

The effect of nutritional ketosis on performance and immune function in endurance athletes

Submitted by

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Abstract

Prolonged (>90 min), strenuous exercise depletes endogenous carbohydrate (CHO) stores and is associated with a transient state of immunodepression and increased illness risk. Commencing exercise with low CHO availability accelerates fatigue and exacerbates perturbations to several *in vitro* immune components, particularly inflammatory T-lymphocyte (T-cell) cytokine production to an immune challenge (i.e. stimulation). Recurrent illness impairs training availability and performance; therefore, CHO fuelling strategies are recommended to support both endurance performance and immune function. Ketone bodies are an additional energetic substrate derived exogenously via ingesting ketone or ketogenic supplements or endogenously via hepatic ketogenesis following conformity to a very low-CHO, ketogenic diet (KD). Ketone supplementation and adaptation to a KD increase blood KB concentrations (minutes vs. days to weeks, respectively) from 0.1-0.2 to >0.5 mmol·L⁻¹ (i.e. nutritional ketosis or hyperketonaemia); albeit, exert disparate effects on CHO and fat metabolism. Therefore, the aim of this thesis was to examine the effect nutritional ketosis – via exogenous and endogenous origin – on endurance performance and immune function.

To identify the optimal dose of the racemic ketogenic supplement, R,S-1,3-butanediol (BD), the first experiment (Chapter 4) explored dose response effects (0 + 0 g·kg⁻¹; 0.5 + 0 g·kg⁻¹; 0.7 + 0 g·kg⁻¹; 0.35 + 0.35 g·kg⁻¹ BD; boluses separated by 1.5 h) on blood D-β-hydroxybutyrate (D-βHB) concentration and T-cell related interleukin (IL)-4, IL-10 and interferon (IFN)-γ gene expression within Staphylococcal Enterotoxin B (SEB)-stimulated peripheral blood mononuclear cells (PBMC) at rest. BD ingestion increased blood D-βHB concentration up to ~1 mmol·L⁻¹; however, there was no effect on T-cell related cytokine gene expression within SEB-stimulated PBMCs compared to placebo (PLA). The second (Chapter 5) and third (Chapter 6) experiments examined the effect of BD ingestion compared to PLA on a preloaded (85 min at 85% second ventilatory threshold) ~30 min cycling time-trial (TT) performance and T-cell related IL-4, IL-10 and IFN-γ gene expression within SEB-stimulated PBMCs, respectively. BD ingestion increased blood D-βHB concentration to 0.4-0.8 mmol·L⁻¹ during exercise and 1.38 ± 0.35 mmol·L⁻¹ at 1-h post-TT; however, blood glucose and lactate concentrations, exercise efficiency (i.e. oxygen uptake) and TT performance were unaltered (PLA, 28.50 ± 3.59; BD, 28.72 ± 3.23 min), with participants reporting gastrointestinal distress and

minor symptoms of nausea, euphoria and dizziness. BD ingestion increased T-cell related gene expression throughout the trial; whereas, IL-4 and IL-10 gene expression were unaltered. These findings suggest blood D- β HB concentrations up to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$ do not benefit high-intensity endurance performance, but may transiently amplify the initiation of a pro-inflammatory type-1 T-cell cytokine response to an immune challenge during and 1-h following exercise.

The fourth (Chapter 7) and fifth (Chapter 8) experiments examined the effect of a 31-day KD (13.7 MJ, 4% [$0.5 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 78% [$4 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) compared to the participants habitual, mixed diet (13.1 MJ, 43% [$4.6 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 38% [$1.8 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) on moderate-intensity (70% maximal oxygen uptake [$\text{VO}_{2\text{max}}$]) running capacity and T-cell related IL-4, IL-10 and IFN- γ gene expression within multi-antigen-stimulated PBMCs and salivary secretory immunoglobulin A (SIgA), respectively. Adaptation to the KD increased fat oxidation (2- to 3-fold) and blood D- β HB concentration to $0.6\text{-}1.6 \text{ mmol}\cdot\text{L}^{-1}$ during exercise and $2.8 \text{ mmol}\cdot\text{L}^{-1}$ at 1-h post-exhaustion, whilst reducing CHO oxidation. Despite impairing exercise efficiency, as evidenced by increased oxygen uptake and energy expenditure, mean time-to-exhaustion was unaffected following the KD (pre-HD, 237 ± 44 vs. post-HD, 231 ± 35 min; pre-KD, 239 ± 27 vs. post-KD, 219 ± 53 min). T-cell related IFN- γ gene expression transiently increased immediately after exhaustion; whereas, IL-4 and IL-10 gene expression were unaltered following the KD. Diet and exercise had no effect on SIgA concentration and secretion rate. These findings suggest conforming to a KD can preserve submaximal exercise capacity and may transiently amplify the initiation of a pro-inflammatory type-1 T-cell cytokine response to an immune challenge immediately following exercise; however, it does not alter mucosal immunity.

In conclusion, the findings of this thesis demonstrate the unique effects of ketone supplementation and adaptation to a KD on exercise metabolism, endurance performance and immune function. BD ingestion does not improve high-intensity endurance performance and should be avoided due to adverse gastrointestinal and systemic effects. Whereas, adaptation to a KD can preserve submaximal exercise capacity and may be considered a viable dietary option *for select individuals*. Moreover, elevated blood D- β HB concentration – via BD ingestion and adaptation to a KD – can transiently increase pro-inflammatory circulatory type-1 T-cell cytokine gene expression to an immune

challenge following exercise onset and cessation; however, the clinical implications for immune function are uncertain.

Attestation of Authorship

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another performance (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been published for the award of any other degree or diploma of a university or other institution of high learning.”

David Shaw

April 2019

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List of Common Abbreviations and Nomenclature

AcAc	Acetoacetate
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
AUC	Area under the curve
β_2 -MG	β_2 -microglobulin
β HB	β -hydroxybutyrate
BD	1,3-butanediol
BMI	Body mass index
C β	T-cell receptor constant region of β -chain
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
DTE	Distance-to-exhaustion
CHO	Carbohydrate
CV	Coefficient of variation
EE	Energy expenditure
EE _{aero-max}	Maximal aerobic energy expenditure
EI	Energy intake
ES	Effect size
Fat _{max}	Intensity at which maximal fat oxidation rates occur
FBS	Foetal bovine serum
FSD	Full scale of deflection
HD	Habitual diet
HR	Heart rate
IFN	Interferon
IL	Interleukin
IMTG	Intramuscular triglyceride
K ₂ EDTA	Dipotassium ethylenediamine tetra-acetic acid
KB	Ketone body
KD	Ketogenic diet
MFO	Maximal fat oxidation
mRNA	Messenger ribonucleic acid

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDHc	Pyruvate dehydrogenase complex
PLA	Placebo
RD	Registered dietitian
RER	Respiratory exchange ratio
RPE	Ratings of perceived exertion
RTE	Run-to-exhaustion
RT-PCR	Reverse transcription polymerase chain reaction
SEB	Staphylococcal Enterotoxin B
SIgA	Salivary secretory Immunoglobulin A
SS	Steady-state
T-cell	T lymphocyte
TCA	Tricarboxylic acid
TReg cell	Regulatory T lymphocyte
TRIMP	Training impulse
TT	Time-trial
TTE	Time-to-exhaustion
URS	Upper respiratory symptom
URTI	Upper respiratory tract infection
USG	Urine specific gravity
VCO ₂	Volume of carbon-dioxide produced
VO ₂	Volume of oxygen consumed
VO _{2max}	Maximal oxygen uptake
VO _{2peak}	Peak oxygen uptake
VT ₂	Second ventilatory threshold
W _{max}	Maximal power output
°C	Degrees celsius

Ethical Approval

Ethical approval for the research carried out in Chapters 4-6 of this thesis was granted by the Health and Disability Ethics Committee (HDEC, Wellington, New Zealand), Auckland University of Technology Ethics Committee (AUTEC, Auckland, New Zealand) and prospectively registered with the Australian New Zealand Clinical Trials Registry (ANZCTR). Ethical approval for the research carried out in Chapters 7 and 8 for this thesis was granted by the AUTEC.

HDEC references

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Publications and Presented Abstracts

Publications

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DM Shaw, F Merien, A Braakhuis, D Plews, P Laursen & D Dulson. The effect of 1,3-Butanediol on Cycling Time-Trial Performance. *Int J Sport Nutr Exerc*. 2019; 29(5): 466-473.

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DM Shaw, F Merien, A Braakhuis, L Keaney & D Dulson. Acute hyperketonaemia alters T-cell-related cytokine gene expression within stimulated peripheral blood mononuclear cells following prolonged exercise. *Eur J Appl Physiol*. (in review).

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Statement of Contribution

Chapter 4. The dose response effect of 1,3-butanediol on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells at rest

David Shaw 85%, Dr Deborah Dulson 5%, Dr Fabrice Merien 5%, Dr Andrea Braakhuis 2.5%, Lauren Keaney 2.5%.

Chapter 5. The effect of 1,3-butanediol on cycling time-trial performance

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Chapter 6. The effect of 1,3-butanediol on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells following prolonged, strenuous exercise

David Shaw 85%, Dr Deborah Dulson 5%, Dr Fabrice Merien 5%, Dr Andrea Braakhuis 2.5%, Lauren Keaney 2.5%.

Chapter 7. The effect of a 31-day ketogenic diet on submaximal-intensity exercise capacity and efficiency

David Shaw 85%, Dr Deborah Dulson 5%, Dr Fabrice Merien 5%, Dr Andrea Braakhuis 2.5%, Ed Maunder 2.5%.

Chapter 8. The effect of a 31-day ketogenic diet on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells and salivary secretory immunoglobulin A following submaximal-intensity, exhaustive exercise

David Shaw 85%, Dr Deborah Dulson 5%, Dr Fabrice Merien 5%, Dr Andrea Braakhuis 2.5%, Lauren Keaney 2.5%.

Introduction

1.1 Theoretical background

Due to their high training loads, endurance athletes may be at a greater risk of illness, particularly upper respiratory symptoms (URS) [1,2]; with recurrent episodes detracting from training availability and performance at pinnacle events [3,4]. Prolonged (>90 min), strenuous exercise perturbs several *in vitro* and *in vivo* immune components, and is associated with a transient state of immunodepression. This period has been classically termed *the open window of opportunistic infection* and may last from several hours to several days following an acute exercise bout [1,2]. Therefore, athletes are perennially searching for strategies to enhance training adaptation and performance, whilst mitigating illness risk. Optimising fuelling strategies by manipulating substrate availability is a key strategy. Carbohydrate (CHO) availability in the form of skeletal muscle and hepatic glycogen and blood glucose appears integral to endurance performance and exercise capacity at moderate- to high-intensities [5,6], as well as reducing exercise-induced immune perturbations [1,7]. The emergence of ketone and ketogenic supplements, and resurgence of ketogenic diets (KD), has complicated our understanding of manipulating substrate availability and utilisation via dietary-training strategies [8]; and subsequently posed questions for performance and immune function. In concordance, understanding the effect of nutritional ketosis – via exogenous and endogenous origin – on both performance and immune function is warranted.

Nutritional ketosis, or hyperketonaemia, is characterised by elevated blood ketone body (KB) concentration $>0.5 \text{ mmol}\cdot\text{L}^{-1}$ [9]. The D- isomer of beta-hydroxybutyrate (D- β HB) is the primary circulating KB, with acetoacetate (AcAc) elevating less markedly [10]. Ketone bodies have been implicated in exercise performance research as they provide substrate for the tricarboxylic acid (TCA) cycle and uniquely regulate CHO and fat metabolism [11,12]. Importantly, strategies to induce hyperketonaemia can also influence substrate availability, as well as circulating metabolites and hormones. Ketone supplement ingestion increases blood D- β HB concentrations to $>0.5 \text{ mmol}\cdot\text{L}^{-1}$ within 30 min without necessitating dietary CHO restriction [13] and, therefore, may be suited for endurance events performed at intensities approaching maximal oxygen uptake ($\text{VO}_{2\text{max}}$). Whereas, KDs require conforming to a very low-CHO diet ($<50 \text{ g CHO}\cdot\text{day}^{-1}$) several days-to-weeks, which may compromise high-intensity performance [14], but preserve endurance capacity at low-to moderate intensities due to the amplification of fat oxidative pathways [15]. Therefore, it seems appropriate to examine the effects of strategies to

induce nutritional ketosis on immune function in conjunction with the event-specific physiological demands with which they are most likely to interact.

T-lymphocytes (T-cells) are sensitive to exercise and nutrition modulation, with shifts in *in vitro* function linked to illness and infection risk *in vivo* [16,17]. Their production of pro- and anti-inflammatory cytokines orchestrate the immune response to a pathogenic challenge and provide valuable mechanistic insight into the reasons why athletes may become susceptible to illness and infection following periods of physical exertion [18]. T-cells can be divided into subsets according to their phenotype and function, in particular, type-1, type-2 and regulatory T-cells [19-21]; albeit, other T-cell subsets do exist. Immune cell cytokine production is measured *in vitro* using a stimulated culture and represents a balance of cytokine protein synthesis and degradation [22]. Cytokine gene transcription is a key determinant during the early stages of the T-cell response to an immune challenge [23]. As such, measuring stimulated T-cell cytokine gene expression can characterise the initiation of cytokine production and the immune activation to exercise and dietary interventions.

The majority of studies examining the stimulated T-cell cytokine response to exercise and diet have measured cytokine protein production. Circulating type-1 T-cells appear to have a lower capacity to produce the pro-inflammatory cytokine, interferon (IFN)- γ , following prolonged, strenuous exercise [24,25]; therefore, protection against intracellular pathogens (e.g. viruses) may be compromised. However, the effect of periods of heavy training on T-cell IFN- γ production is unclear [16,24,26,27]. Conversely, production of anti-inflammatory cytokines, interleukin (IL)-4 by type-2 T-cells and IL-10 by regulatory T-cells, appear unaltered following acute exercise [24,25,27-29]; whereas, there is typically an increase following periods of heavy training [17,26,30]. In turn, there is a shift towards type-2 T-cell predominance at the protein level; meaning the anti-inflammatory response to an immune challenge is heightened and could compromise protection against invading and incumbent viral pathogens.

Carbohydrate exerts an immunomodulating effect directly by providing a fuel source for immune cells and indirectly by attenuating the exercise-induced rise in circulating stress hormones, such as cortisol [1]. Previously, CHO ingestion during prolonged, strenuous exercise attenuated the reduction in post-exercise circulatory type-1 T-cell concentration

and their mitogen-stimulated production of IFN- γ ; potentially by increasing blood glucose concentration and attenuating the rise in cortisol [25]. Whereas, circulating type-2 T-cell concentration and their mitogen-stimulated production of IL-4 were unaffected. Regulatory T-cells appear to oppose the exercise-induced trafficking patterns of type-1 T-cells [31]; however, the effect of manipulating CHO availability on their cytokine production has not been investigated. Nonetheless, the single study investigating the influence of exercise and CHO availability on (single-antigen-) stimulated circulatory T-cell IFN- γ and IL-4 gene expression demonstrated no effect of either exercise or CHO ingestion [32]. Therefore, further research is warranted to confirm patterns of stimulated circulatory T-cell IFN- γ , IL-4 and IL-10 gene expression to exercise and substrate availability.

It is possible that nutritional ketosis effects T-cell cytokine production in response to prolonged, strenuous exercise by modulating CHO availability. For example, ketone supplementation can inhibit hepatic glycogenolysis, thus lowering blood glucose concentration [33,34], and conforming to a KD can reduce hepatic and skeletal muscle glycogen stores and blood glucose concentration [15,35]. In turn, the exercise-induced rise in blood cortisol may be exacerbated; thus, favouring IL-4 and IL-10 production, and, potentially, gene expression [36]. However, it remains uncertain if hyperketonaemia compensates for reductions in CHO availability or is influential via alternative mechanisms on the immune response following exercise. As there is only a single study having investigated the effect of nutritional ketosis on immune function in athletes, which demonstrated no effect on mucosal immunity [37], further research is paramount.

1.2 Purpose statement and significance of research

Athletes are at risk of adopting dietary strategies that may compromise performance and immune function due to a deluge of unsubstantiated claims [38]. Indeed, numerous anecdotes regarding ketone supplements and KDs have been published within non-scientific media outlets (e.g. social media, magazines, blogs). This is concerning as the physiological demands of optimising training adaptation, performance and health that underpin nutritional requirements, particularly for competitive athletes, are often neglected [39]. Nevertheless, it is possible that nutritional ketosis, either via ketone supplementation or adaptation to a KD, may support endurance performance and/or immune function for *some* individuals. Therefore, the purpose of this thesis was to

investigate the efficacy of nutritional ketosis for endurance performance, whilst simultaneously examining effects on immune function, to discern potential benefits and risks for trained endurance athletes. The subsequent findings are expected to contribute to best practice dietary fuelling guidelines for athletes, dietitians, nutritionists, sport scientists and coaches.

The specific aims of this thesis were:

- Determine the effect of acute hyperketonaemia via R,S-1,3-butanediol (BD) ingestion on T-cell IL-4, IL-10 and IFN- γ gene expression within stimulated peripheral blood mononuclear cells (PBMC) at rest.
- Determine the effect of acute hyperketonaemia via BD ingestion on T-cell IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs following prolonged, strenuous exercise.
- Determine the effect of acute hyperketonaemia via BD ingestion on prolonged, high-intensity endurance performance.
- Determine the effect of adaptation to a KD on submaximal-intensity endurance capacity.
- Determine the effect of adaptation to a KD on T-cell IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs and salivary secretory immunoglobulin A (SIgA) following prolonged, exhaustive exercise.

1.3 Thesis organisation

This thesis comprises 9 chapters within four primary sections (Figure 1.1, p. 7). Chapters 4-8 are written as stand-alone chapters that incorporate standard paper format (abstract, introduction, methodology, results, discussion) and are specific to the aims of that chapter.

The first section includes two literature reviews (Chapters 2 and 3) due to the two distinct and complex areas of research within this thesis. Chapter 2 reviews the literature on the effect of ketone supplementation and adaptation to a KD on substrate metabolism during exercise and endurance performance. Chapter 3 reviews the literature on the effect of

prolonged, strenuous exercise on stimulated circulating T-cell cytokine production and potential mechanisms of influence derived from nutritional ketosis.

The second section (Chapters 4-6) investigated the effect of the ketogenic supplement, BD, on prolonged, high-intensity exercise performance and immune function. Chapter 4 was an important precursor to the proceeding chapters (Chapters 5 and 6) and examined the dose response effect of BD on blood D- β HB concentration and T-cell related IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs at rest. Chapter 5 examined the effect of BD ingestion compared to placebo on a cycling time-trial (TT) performance preceded by 85 min of steady-state (SS), glycogen-lowering exercise in trained male cyclists. Chapter 6 examined the effect of BD ingestion compared to placebo on T-cell related IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs following the SS and TT phases, and 1 h into recovery.

The third section (Chapters 7 and 8) investigated the effect of a 31-day KD on submaximal intensity exercise capacity and immune function. Chapter 7 examined the effect of the KD compared to a higher-CHO diet on whole body substrate oxidation, efficiency and exercise capacity by running to exhaustion at 70% $\text{VO}_{2\text{max}}$. Chapter 8 examined the effect of the KD compared to a higher-CHO diet on T-cell related IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs and SIgA following running to exhaustion at 70% $\text{VO}_{2\text{max}}$ compared to a higher-CHO diet.

The fourth and final section, Chapter 9, provides a general summary of the main findings and discusses the results of the thesis as a whole, followed by limitations of the studies within the thesis, directions for future research and concluding remarks on the utility of ketone (and ketogenic) supplements and KDs on performance and immune function.

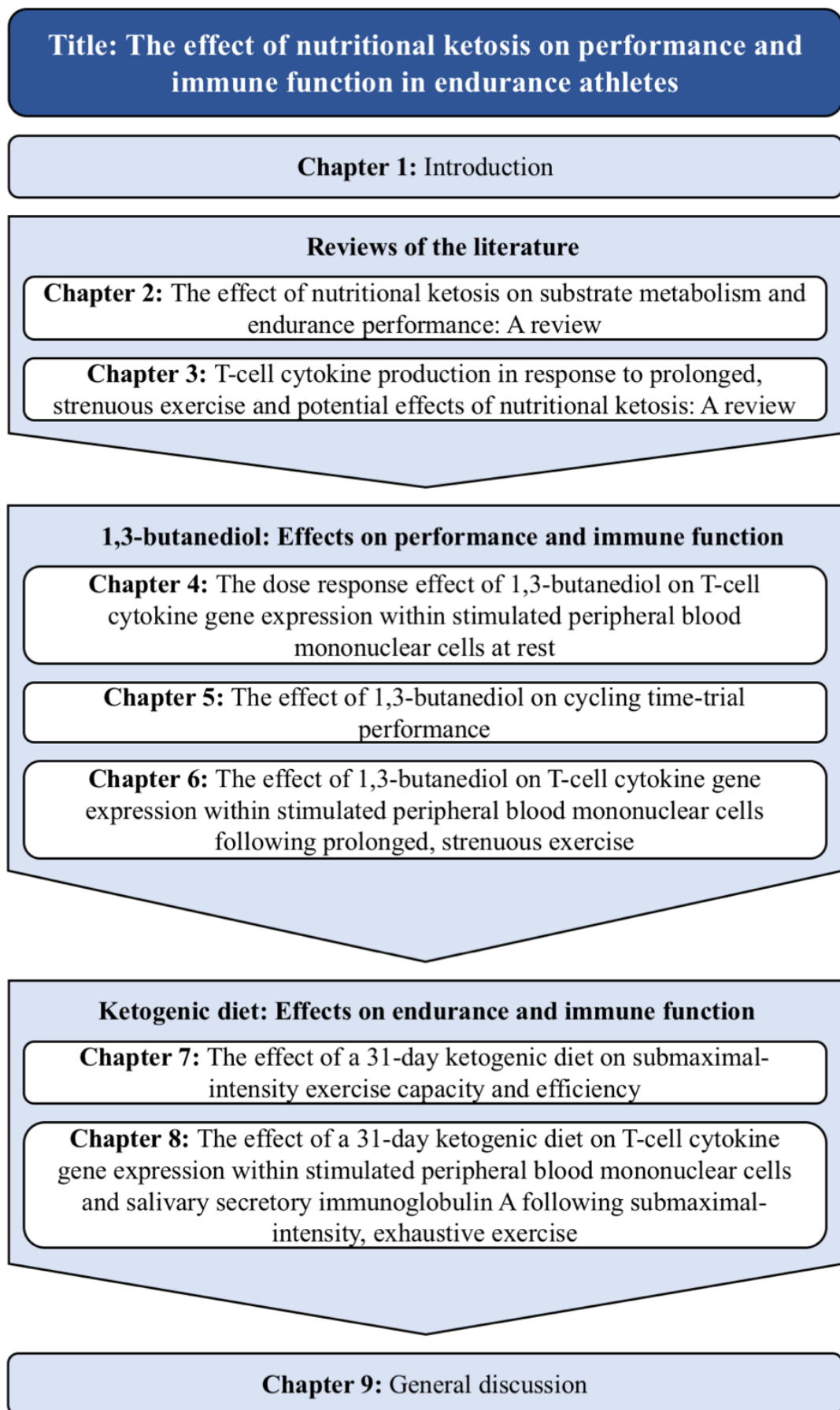


Figure 1.1. Organisation of the thesis.

The effect of nutritional ketosis on substrate metabolism and endurance performance: A review

2.1 Ketone bodies and nutritional ketosis

Ketone bodies refers to AcAc, acetone and β HB; however, only AcAc and acetone contain a carboxyl group with two hydrocarbons, thus classifying them as ketones. Whereas, β HB is technically a KB as the hydrocarbon atom is replaced by a hydroxyl group on the third carbon (Figure 2.1). Compared to AcAc and β HB, acetone is largely excreted in the urine and breath and, therefore, it is considered to be of negligible physiological importance [10]. Postprandial blood KB concentration is typically 0.1-0.2 mmol·L⁻¹ and varies depending on CHO availability [10]. Nutritional ketosis, or hyperketonaemia, is defined as total blood KB (AcAc and D- β HB) concentration >0.5 mmol·L⁻¹ [9]; however, concentrations >0.2 mmol·L⁻¹ have also been proposed [10]. Ketone bodies can bypass the blood-brain barrier via passive diffusion [40] and enter extra-hepatic tissues (e.g. brain, heart and skeletal muscle) via monocarboxylate transporters [41] to provide an alternative oxidative fuel source during periods of low CHO-availability, such as starvation, fasting, prolonged exercise or conformity to a KD [10]. They also possess multidimensional roles in metabolism, homeostasis, cell signalling and transcription under a variety of physiological and pathological states [10,42,43]. However, it is their role in substrate provision and metabolic regulation that has renewed interest in nutritional ketosis for endurance performance.

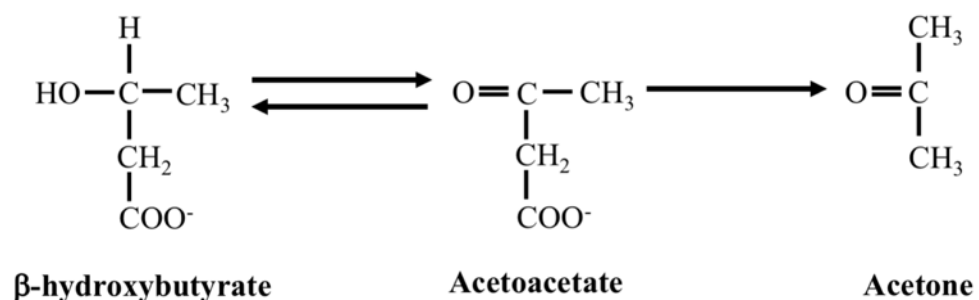


Figure 2.1. Structure of ketone bodies.

2.1.1 Strategies to increase endogenous ketone production

Endogenous ketogenesis occurs predominantly in the liver from free fatty acids (FFA) and specifically increases the production of AcAc, which is the central KB involved in energy metabolism [10]. However, the majority of AcAc is reduced to the D- isomer of β HB, which is the primary circulating KB [10]. The ingestion of a KD can increase blood

KB concentrations to $>0.5 \text{ mmol}\cdot\text{L}^{-1}$ within days [10]. After a prolonged fast (≥ 5 days), blood KB concentrations are $\sim 7\text{-}10 \text{ mmol}\cdot\text{L}^{-1}$, but remain below $10 \text{ mmol}\cdot\text{L}^{-1}$ due to metabolic regulation; thus preventing pathophysiological increases in concentration observed during diabetic ketoacidosis [44]. The ratio of circulating D- β HB to AcAc is 1:1 in a typical postprandial state and can increase to between 4:1 and 6:1 during starvation [44]. The ability for a fat-derived fuel to be used by the brain during periods of CHO-insufficiency is critical to meet the brain's energy requirements, thus negating the requirement for gluconeogenic protein catabolism [45,46]

Ketogenic diets are a popular strategy to increase hepatic ketogenesis. A KD is typically defined as $\leq 50 \text{ g CHO}\cdot\text{day}^{-1}$ or $<5\%$ of energy intake (EI) from CHO, 15-20% of EI from protein and 75-80% of EI from fat [47]. However, the defining feature demarcating a KD from low(er)-CHO, high(er)-fat (LCHF) diets ($\sim 2.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ or $<25\%$ of EI) is hyperketonaemia [9]. In athletes, blood KB concentration can increase to $>1\text{-}2 \text{ mmol}\cdot\text{L}^{-1}$ following the ingestion of a 3-4 week KD [14,15] and remain elevated following several months of dietary adherence [35,48]. Prolonged, glycogen-depleting exercise can also increase blood KB concentration to $1\text{-}2 \text{ mmol}\cdot\text{L}^{-1}$, which is termed *post-exercise ketosis* [49,50]. However, this response is reduced by increased CHO availability [49-53] and a higher training status [54-56], with the latter potentially due to the reduction of circulating FFA [54-56] and upregulation of enzymes pertinent to ketolysis [57,58].

For the athlete, KDs are a preferred strategy to increase hepatic ketogenesis, compared with fasting and starvation, as energy availability can be maintained. Further, endurance performance following several days of fasting does not coincide with contemporary fuelling strategies [8] and should be avoided. As such, dietary standardisation protocols will be implemented within this thesis to create appropriate physiological conditions to examine strategies inducing nutritional ketosis on endurance performance (and immune function).

2.1.2 Hormonal and enzymatic regulation of endogenous ketone production

Fat is the primary substrate for hepatic ketogenesis; however, protein catabolism (specifically leucine) contributes $\sim 4\%$ during the early stages ($\sim 3 \text{ h}$) of fasting [59]. A healthy human liver can produce up to $185 \text{ g KB}\cdot\text{day}^{-1}$, with the rate of ketogenesis

proportionate to the rate of fat oxidation in hepatic tissues [60]. This is likely to occur due to a spillover effect of acetyl-CoA via β -oxidation of acyl-CoA, which saturates citrate synthase activity and/or oxaloacetate availability for condensation to form citrate, thus preventing complete oxidation via the TCA cycle. In turn, acetoacetyl-CoA (AcAc-CoA) is formed, which is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and subsequently to AcAc, which can be reduced to D- β HB [60]. Hormonal and enzymatic factors govern the rate of ketogenesis. Insulin suppresses hormone sensitive lipase and activates acetyl-CoA carboxylase; this inhibits the catabolism of triglycerides to FFAs and increases the production of malonyl-CoA, which can suppress the action of carnitine palmitoyltransferase-1 and the flux of FFAs into the mitochondria [60]. Alternatively, adrenaline and glucagon increase ketogenesis by elevating fatty acid release from adipocytes. In the liver, mitochondrial HMG-CoA synthase is downregulated by insulin and upregulated by starvation and the ingestion of a KD [60]. High blood KB concentrations are anti-lipolytic and self-inhibit, or regulate, their own production. This may be due to either a direct effect or via the action of insulin to maintain blood KB concentration within specific ranges (i.e. $<10 \text{ mmol}\cdot\text{L}^{-1}$) [60].

2.2 Ketone supplementation

Ketone and ketogenic supplements provide an alternative strategy to induce nutritional ketosis without necessitating CHO restriction. To date, studies have investigated the intravenous and oral administration of synthetic KBs on metabolism and athletic performance [11,12]. Elevated KB concentrations are not prohibited by the World Anti-Doping Agency; however, the intravenous administration of fluid $>50 \text{ ml}$ is and, therefore, this review will focus on the oral ingestion of ketone supplements.

Ketone bodies ingested in their free form fail to significantly elevate blood KB concentration [11]. As such, synthetic KBs are combined with a salt (sodium, potassium, calcium or magnesium) to promote absorption. Ketone salts, primarily sold as a racemate (i.e. D,L- β HB), can increase blood D- β HB up to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$ [13,61-64]; however, they provide an undesirable salt load that may result in cation overload and gastrointestinal distress [65]. Alternatively, synthetic KBs can be esterified to an alcohol, for example, R,S-BD AcAc mono- and diesters [66,67] and R-BD D- β HB monoester [33,44]. In humans, R-BD D- β HB monoester ingestion has attracted the most attention, with studies

investigating its safety and tolerability [68,69], pharmacokinetics [13,68,70], blood glucose regulation [34], appetite regulation [71], inflammation [72], endurance performance [73,74], recovery [75,76] and overreaching [77], ratings of perceived exertion (RPE) [78] and acid-base balance [79].

Following ingestion, both the R,S-BD AcAc esters and R-BD D- β HB monoester are catabolised by carboxylesterases and esterases predominantly situated in the gastrointestinal tract [80]. The liberated (R- and/or S-) BD is metabolised to (D- and/or L-) β HB by the liver (see section 2.2.2). The ingestion of R,S-BD AcAc esters has demonstrated similar elevation in blood D- β HB concentration as racemic ketone salts [67]. Whereas, R-BD D- β HB monoester, at doses of 0.14, 0.357 and 0.714 g·kg⁻¹ (in conjunction with a milk-based drink), increases blood D- β HB concentrations to 0.28, 1.00 and 3.30 mmol·L⁻¹ after 1.5-2.5 h [68]; with concentrations up to ~5 mmol·L⁻¹ occurring ~20 min following the ingestion of 0.573 g·kg⁻¹ of R-BD D- β HB monoester (without the combination of a milk-based drink) [73]. As such, the rise in blood D- β HB concentration following ketone supplementation is attenuated by the accompaniment of food [13], indicating that the contents of the gut may affect the digestion and absorption of ketone supplements.

Currently, limited access and the expense of R,S-BD AcAc mono- and diesters and R-BD D- β HB monoester has prevented widespread application and investigation, thus driving research into alternative ketone or ketogenic supplements. One option is the racemic BD, which is a component part of the R,S-BD AcAc mono- and diesters.

2.2.1 1,3-butanediol

1,3-butanediol, also known as 1,3-butylene glycol, is a nontoxic dialcohol. Following ingestion, the racemic R,S-BD increases blood concentrations of the R- and S- moieties of BD and metabolised to the isotopic enantiomers, D- and L- β HB, within minutes by alcohol dehydrogenase and aldehyde dehydrogenase primarily in the liver [81-85] (Figure 2.2, p. 14). These steps are rate limiting to BD metabolism and influenced by previous ethanol exposure [86]. Additionally, gastric alcohol dehydrogenase is involved in first pass metabolism of BD and, conversely to hepatic alcohol dehydrogenase, its enzymatic activity may be negatively associated with previous ethanol exposure [87]. In turn, when

BD is administered in amounts beyond enzymatic saturation, there is an accumulation of blood BD [88]. In rats, intragastric administration of 5 g·kg⁻¹ [89] and 10 g·kg⁻¹ [66] of BD raised blood D-βHB concentration to ~1 mmol·L⁻¹ and ~5 mmol·L⁻¹, respectively, with peak concentrations occurring within ~30-120 min. Whereas, in humans, 0.5 g·kg⁻¹ raised blood D-βHB concentrations to 0.8 mmol·L⁻¹ within 30 min at rest [90]. Collectively, however, there is currently a lack of published literature investigating the pharmacokinetics and pharmacodynamics of BD's dose response effect in humans.

2.2.2 Chirality

Multiple pathways exist for the absorption and metabolism of ketone supplements. Figure 2.2 (p. 14) provides an overview of the cleavage and major metabolic pathway of ketone and ketogenic supplements prior to oxidation in extrahepatic tissues. The D- isomer of βHB derived from R-BD metabolism is identical to D-βHB produced via endogenous ketogenesis; whereas, the L-βHB derived from S-BD is typically a by-product of fat metabolism present in trace and low amounts in the blood [91] and extrahepatic tissue [92], respectively. In rats, the administration of R-BD contributed to 86-98% of total ketogenesis (i.e. D-βHB and AcAc); whereas, S-BD contributed to 47-75% of total ketogenesis [82]; indicating S-BD has significantly lower rate of conversion to D-βHB compared to R-BD. D-βHB is rapidly catabolised to acetyl-CoA and adenosine triphosphate (ATP) via the TCA cycle in extrahepatic tissues; whereas, L-βHB does not enter the TCA cycle and is preferentially converted by the liver to FFAs and sterols or acetyl-CoA prior to D-βHB or CO₂ [93,94]. The slow conversion of L-βHB means it can remain present in the blood for up to 24 h; increasing the potential of blood L-βHB accumulation [13] and lowering of the D-βHB/L-βHB ratio, which may interfere with the D-βHB-induced suppression of glucose metabolism [92]. This may have implications for substrate metabolism during exercise in humans and could offer a metabolic benefit in some endurance performance contexts.

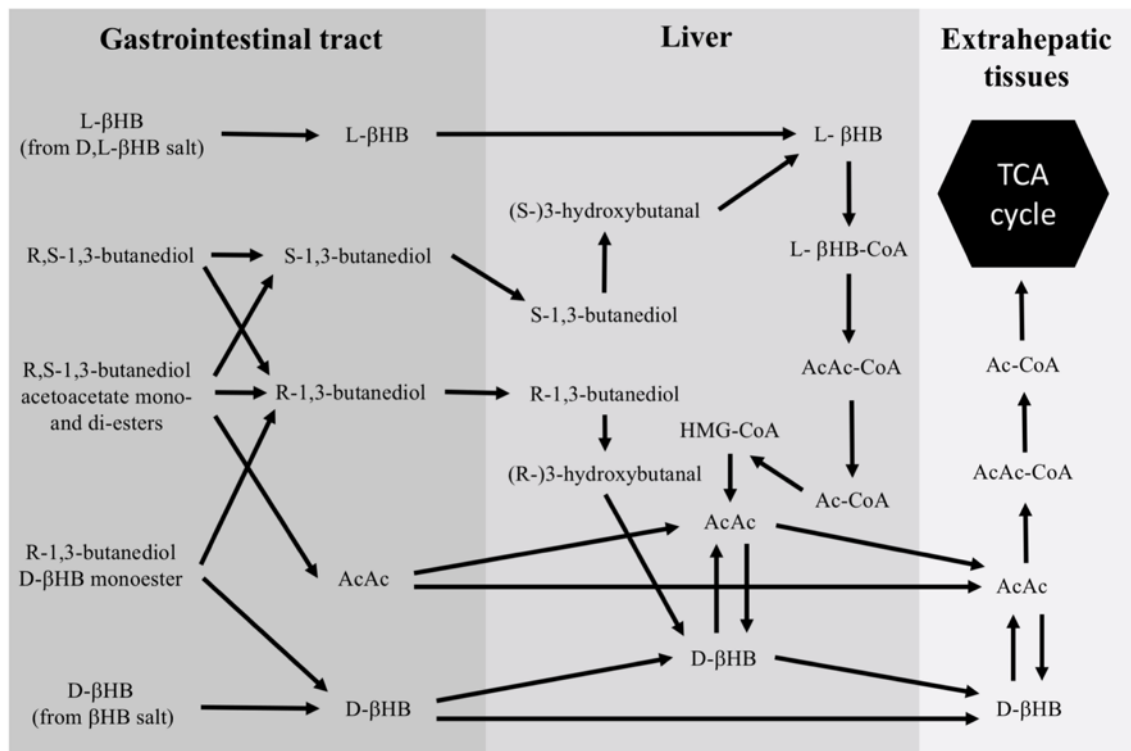


Figure 2.2. A simplified schematic of the cleavage and major metabolic pathways of 1,3-butanediol-based ketone esters and ketone salts prior to oxidation in the tricarboxylic acid cycle within extrahepatic tissues. β HB, β -hydroxybutyrate; AcAc, acetoacetate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; AcAc-CoA, acetoacetyl-CoA; Ac-CoA, acetyl-CoA; TCA, tricarboxylic acid. Following oral ingestion, ketone esters and salts are cleaved and absorbed in the gut. R,S-1,3-butanediol is initially converted to R,S-3-hydroxybutanal prior to the ketone bodies, acetoacetate, β HB and acetone. Acetone is formed by the decarboxylation of AcAc and is not shown in the schematic as it does not contribute to energy production. Circulating ketone bodies are transported to extrahepatic tissues (e.g. skeletal muscle, kidney, brain and heart) and enter primarily via monocarboxylate transporters to be oxidised in the TCA cycle for energy production. Excretion of nonmetabolised ketone bodies occurs via the faeces, exhalation by the lungs as acetone or kidneys as AcAc and β HB.

2.3 Quantifying ketosis

Various methods measure biological KB concentrations [95]. The cheapest and simplest technique involves dipsticks to semi-quantitatively measure the presence of urinary AcAc. Despite not detecting D- β HB, and having a low sensitivity to acute shifts in blood KB concentration [95], urinary dipsticks can be a suitable option to confirm daily adherence to a KD [96]. To measure capillary blood KBs, several point-of-care, handheld devices are currently available and are widely used in healthcare settings for individuals with diabetes [95]; however, these specifically measure D- β HB, not L- β HB, by using D- β HB dehydrogenase coupled with electrochemical detection. Handheld devices also

appear to overestimate D- β HB concentration by 2- to 3-fold, compared to laboratory measures using serum and plasma samples [67,97]. This measurement variability makes it difficult to identify the optimal range of hyperketonaemia for performance and health.

2.4 Ketone body utilisation during exercise

The uptake and oxidation of KBs within skeletal muscle during exercise has been comprehensively reviewed elsewhere [10-12]. Briefly, the entry of KBs from the blood into skeletal muscle occurs via monocarboxylate transporters [41]. Monocarboxylate transporters are highly expressed in type I muscle fibres [98] and upregulated by exercise training in an intensity-dependant manner [99]. Ketone bodies are metabolised by the ketolytic enzymes 3-hydroxybutyrate dehydrogenase, succinyl-CoA:3-oxoacid CoA transferase and acetyl-CoA acetyltransferase [60]. The activity of these enzymes are typically highest in type I muscle fibres, compared to type IIa and type IIb, with exercise training also increasing their activity [57]; however, this appears to be isolated to skeletal muscle, with brain, heart and kidney ketolytic activity remaining stable [57,100]. Therefore, highly-trained individuals, with a high proportion of type I muscle fibres and greater oxidative capacity, are metabolically endowed to utilise KBs for energy production.

The utilisation of KBs appears positively associated with blood KB concentrations at low concentrations and tends to plateau at higher concentrations [11]. At rest, the contribution of KBs to energy production accounts for ~10% after an overnight fast and ~20-50% after 72 h of starvation, which declines to ~15% after 24 days of starvation [11]. It has been suggested that skeletal muscle exhibits saturation kinetics when blood KB concentrations are 1-2 mmol·L⁻¹ [33,44]. Following the onset of exercise, the uptake of KBs, when concentrations are <1 mmol·L⁻¹ (achieved after an overnight fast), into skeletal muscle increases by 50-70% [44]. The exercise-induced muscle uptake of blood KB may result in a decline in blood KB concentration. However, when blood KB concentration is >2.5 mmol·L⁻¹, such as during a prolonged fast, the exercise stimulated uptake of KBs from the blood is attenuated [44]. Therefore, blood KB concentration does not appear to be the limiting factor for KB utilisation when above >2.5 mmol·L⁻¹. Additionally, in isolated rodent tissue models, the interaction between blood KB concentration and CHO availability has been shown to mediate KB metabolism [101] and high rates of KB oxidation appeared to require high CHO availability to maintain the anaplerotic flux in

the TCA cycle [102,103]; therefore, it is possible that KB oxidation in human skeletal muscle is also altered by blood KB concentration and carbohydrate availability during exercise, but this remains speculative.

Measuring KB utilisation is difficult, particularly as stoichiometric equations have only been validated for CHO and fat [104]. Indirect methods require estimates of volume distribution values (i.e. total amount of KBs in the body divided by KB plasma concentration) and KB uptake into skeletal muscle [105]; with methods proposed for ketone supplementation using incremental area-under-the-curve (iAUC) of blood D- β HB concentration between resting and exercising conditions [73,105]. However, these cannot be implemented for hepatic ketogenesis as rates of endogenous ketogenesis must be known, which can only be ascertained using tracer methodology. Furthermore, not all KBs extracted from the blood are oxidised and can be stored in the form of D-3-hydroxybutyrylcarnitine (i.e. keto-carnitine), for which its role in energy production is uncertain [73,106]. Insensible losses also occur via the breath and urine [10]. Therefore, future studies are warranted to confirm the contribution of KBs to energy expenditure (EE) during a range of exercise intensities in a variety of nutritional contexts (e.g. ketone supplementation and hepatic ketogenesis induced via KD ingestion).

Despite the limitations of indirect calorimetry, a recent study estimated the contribution of KBs to EE following R-BD D- β HB monoester ingestion. In this study, highly-trained cyclists consumed 0.573 g·kg⁻¹ of R-BD D- β HB monoester 10 min prior to 45 min of SS cycling at either 45 or 75% W_{\max} [73]. Blood D- β HB concentrations in the high-intensity trial were ~3 and 1 mmol·L⁻¹ lower than the resting and low-intensity trials, respectively [73]. This was the first study to show an exercise intensity-dependent effect on KB utilisation. Using iAUC between exercise and resting states and stoichiometric equations [105], the estimated contribution of D- β HB to total oxygen consumption at 45 and 75% W_{\max} was ~16 and 18%, respectively (i.e. ~0.35 and 0.5 g·min⁻¹, respectively) [105]. Importantly, the contribution to total EE was grossly higher than previous estimates using direct calorimetry following intravenous infusion of sodium AcAc after an overnight fast whilst exercising at ~52% $VO_{2\max}$ (blood D- β HB and AcAc concentrations of ~2.5 mmol·L⁻¹ and ~3.5 mmol·L⁻¹, respectively) [107]. Therefore, indirect methods may overestimate KB oxidation during exercise and should be interpreted cautiously until validated.

2.5 Substrate utilisation during exercise

Since the early 1900s, physiologists have been intrigued by the catabolism of energetic substrate to power muscular contraction [108]. The energy currency of the cell, ATP, is resynthesised via a series of enzymatically-linked reactions largely via the metabolic conversion of CHO and fat [109]. Exercise increases the flow of energetic substrates in order to maintain the concentration of ATP. Compared to rest, the rate of ATP turnover can increase 100-fold during maximal exercise [110]. The majority of ATP is resynthesised by oxidative phosphorylation within the electron transport chain in the mitochondria of the cell [111]. Therefore, skeletal muscle is a primary site of CHO and fat metabolism during exercise.

Total power output governs the requirement of ATP and the relative intensity of exercise underpins the contribution of CHO and fat (Figure 2.3, p. 18). At rest, and during low-intensity exercise (25% $\text{VO}_{2\text{max}}$), fat contributes >70% towards energy production. When exercise intensity is increased, CHO utilisation becomes increasingly important [112-114]. For example, an isotopic tracer study in trained cyclists demonstrate a 2-fold increase in energy requirements from low- to moderate-intensity exercise (65% $\text{VO}_{2\text{max}}$), which was met by relative increases in intramuscular glycogen and triglyceride (IMTG) utilisation [113]. During high-intensity exercise (85% $\text{VO}_{2\text{max}}$), absolute fat oxidation did not differ compared to exercising at 25% $\text{VO}_{2\text{max}}$; however, its relative contribution to total energy production declined, meaning that CHO provided ~70% of the total energy demand. At this intensity, muscle glycogen utilisation contributed over half of the energy requirements, with blood glucose utilisation being ~2-fold higher than when exercising at 65% $\text{VO}_{2\text{max}}$ [113].

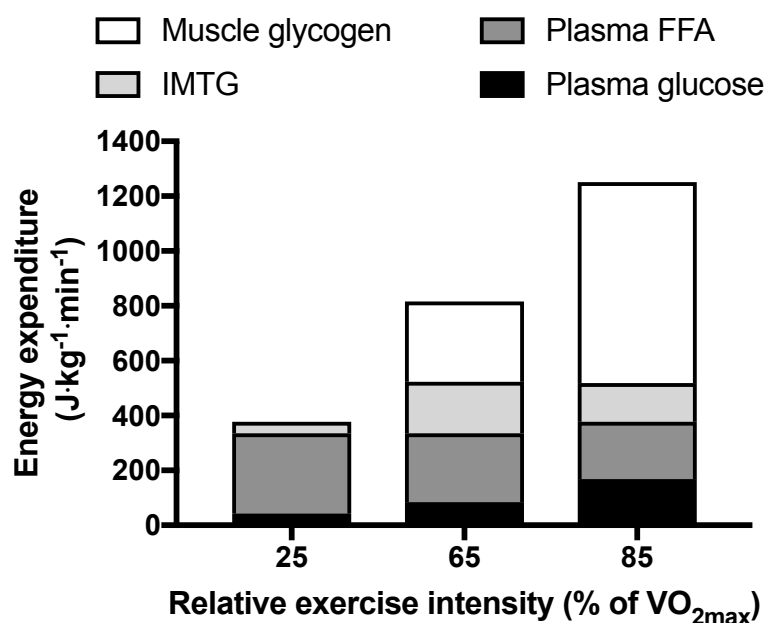


Figure 2.3. Contribution to energy expenditure relative to body mass during steady-state exercise at different relative intensities. Energy derived from plasma does not change relative to exercise intensity. IMTG, *intramuscular triglyceride*; FFA, *free fatty acids*. Adapted from Romijn et al. (1993).

Endurance performance during prolonged (up to ~3 h), high-intensity (>80% VO_{2max}) exercise appears to be CHO-dependent (i.e. skeletal muscle and hepatic glycogen, blood glucose, lactate and exogenous sources) [115]. For example, during a simulated marathon, following an overnight fast without CHO ingestion [116], the fast runners (2 h 43 min) achieved higher rates of CHO oxidation than the slow runners (3 h 30 min) (~3.5 g·min⁻¹ vs. ~3.3 g·min⁻¹); coinciding with greater fractional utilisation of VO_{2max} (73 vs. 65%) and elevated respiratory exchange ratio (RER) (0.99 vs. 0.90) [116]. Moreover, the contribution of CHO to EE during cycling TT protocols lasting 60, 90 and 120 min (i.e. 82-83% VO_{2max}) in trained cyclists following a standardised breakfast meal (i.e. 2 g·kg⁻¹ CHO), ranged between 83-92% (RER, 0.94-0.97) [117]; further emphasising the importance of CHO at these intensities and durations. Considering the energy liberated per L of oxygen is 5.2% higher from CHO than fat (21.14 vs. 20.04 kJ) [108] and VO_2 is linearly related to velocity, the capacity to sustain high rates of CHO oxidation is important to endurance performance.

In contrast, exercise duration exerts less effect on substrate preference compared to exercise intensity. During exercise, up to ~90 min, the ratio between CHO and fat oxidation is relatively stable [113]. However, when exercising at moderate-intensities

(65-75% $\text{VO}_{2\text{max}}$) for 2-4 h in a fasted state, the contribution of fat and CHO to EE shifts [118] (Figure 2.4A, p. 20). At the onset of exercise, fat oxidation contributes to ~50% of EE within contracting muscle, which is equally shared between circulating FFAs and IMTG [118]. At this stage, muscle glycogen provides almost the entire CHO source, with a slight contribution from blood glucose. As exercise continues, the contribution of fat increases and CHO decreases. After 4 h, fat provides ~60% of the fuel source, with a higher proportion derived from circulating FFAs. Coinciding with the decline in muscle glycogen content, the reliance on blood glucose oxidation rises [118]. As such, exhaustion at moderate exercise intensities appears to be associated with depleted endogenous CHO stores (i.e. skeletal muscle [119] and hepatic [120] glycogen), and manifests as the inability to maintain the CHO oxidation rates exhibited during the early stages of exercise (Figures 2.4B and 2.4C, p. 20) [119,121,122].

Obviously, maintaining CHO availability is important for endurance exercise performance and capacity at moderate- to high-intensities. Ingesting a high-CHO diet before and supplementing CHO during competition appear to be the most efficacious dietary strategies for maintaining power output during prolonged, high-intensity exercise and delaying fatigue at submaximal intensities [5,118]. For example, Figure 2.4C demonstrates the ergogenic effect of CHO supplementation for maintaining CHO oxidation rates and prolonging exercise capacity at ~70% $\text{VO}_{2\text{max}}$, which occurred despite a similar reduction in muscle glycogen content (as shown in Figure 2.4B). Alternatively, dietary-training strategies to reduce the reliance of CHO in order to maintain CHO availability for use during critical stages of an event, such as breakaways and surges to the finish line, are also of interest [8]. Of these, strategies to induce nutritional ketosis have been proposed, such as ketone supplementation and adaptation to a KD [8].

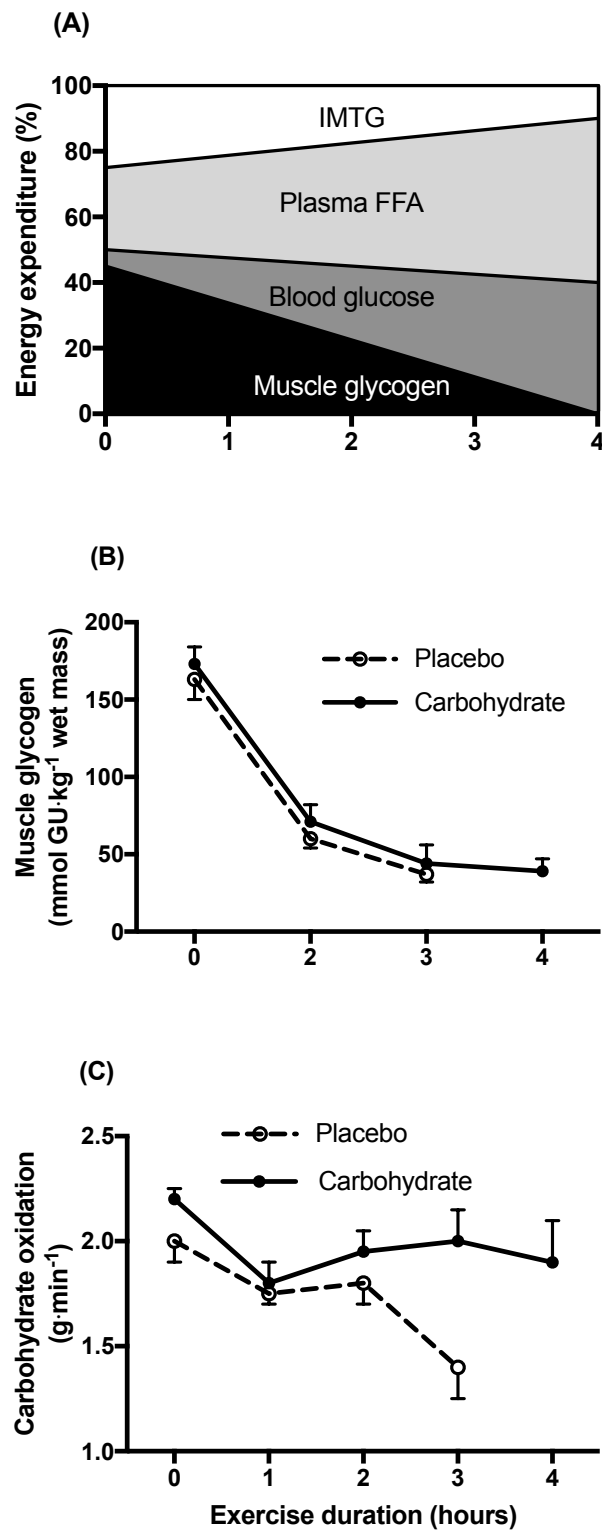


Figure 2.4. Substrate utilisation during prolonged exercise at moderate intensities (65-75% $\text{VO}_{2\text{max}}$) presented as (A) relative contribution to energy expenditure, (B) time-to-exhaustion and glycogen utilisation when ingesting placebo or carbohydrate during exercise and (C) time-to-exhaustion and rate of carbohydrate oxidation when ingesting placebo or carbohydrate during exercise. GU, *glycosyl units*. Adapted from Coyle (1995).

2.5.1 Maximal fat oxidation

The relationship between exercise intensity and fat oxidation is typically measured using an incremental exercise protocol [123]. Each stage (or exercise intensity) comprises of 3-6 min in combination with indirect calorimetry and demonstrates fat oxidation patterns follow an inverted hyperbola with increasing exercise intensity [123]. However, the short stages may not ascertain a SS flux of respiratory gases between tissue and fluid compartments and the alveoli, which means there is an increased excretion of non-respiratory CO₂ and underestimation of whole body fat oxidation [124], particularly in less-trained individuals [125]. Nonetheless, these tests are commonly used with the field of exercise physiology and provide estimates of the following: maximal fat oxidation (MFO); the exercise intensity at which MFO occurs (FAT_{max}), which is expressed as a percentage of VO_{2max}; the exercise intensity whereby fat oxidation rates are negligible or can no longer be estimated (i.e. RER >1.0; FAT_{min}). Due to the potential role of fat oxidation for metabolic health [126,127] and (ultra-) endurance performance [128], this area has been extensively reviewed [123,129,130].

Briefly, in a cross-sectional study involving 300 healthy men and women performing an incremental cycling protocol [131], MFO was $0.46 \pm 0.01 \text{ g}\cdot\text{min}^{-1}$ at a FAT_{max} of $48 \pm 1\% \text{ VO}_{2\text{max}}$; ranging between $0.18\text{-}1.01 \text{ g}\cdot\text{min}^{-1}$ at a FAT_{max} of $27\text{-}77\% \text{ VO}_{2\text{max}}$. As such, there is a large inter-individual variability in MFO, with 34% of the variation attributed to differences in training status, fat free mass, fat mass, physical activity levels and sex; leaving 66% of the variance due to other factors such as genetics and nutrition [131,132]. More recently, a study involving 281 athletes (47 female) demonstrated MFO of $0.59 \pm 0.17 \text{ g}\cdot\text{min}^{-1}$ whilst running at a FAT_{max} of $53 \pm 15\% \text{ VO}_{2\text{max}}$ [133]. Similarly, body composition, VO_{2max}, sex and age accounted for 33% of the variance in MFO. Exercise modality also influences MFO, with running protocols showing higher values compared to cycling over a range of intensities [134]. As such, shifts in MFO and FAT_{max} can infer metabolic adaptations pertinent to fat metabolism. It would, therefore, be expected that adaptation to a low-CHO or KD increases MFO and FAT_{max} (see section 2.7.1) to provide a surrogate for short-term dietary adherence.

2.6 Ketone supplementation: Substrate utilisation and exercise efficiency

Ketone supplementation provides an opportunity to isolate the effects of hyperketonaemia on substrate metabolism from the interference derived from drastic dietary change. A prominent feature of acute hyperketonaemia is the attenuation of CHO metabolism. An initial investigation into the effects of acute hyperketonaemia on exercise metabolism demonstrated that blood lactate concentration did not change following 120 min of SS exercise at 52% $\text{VO}_{2\text{max}}$, which commenced 90 min after the continuous infusion of sodium AcAc (blood concentrations of D- β HB and AcAc were $\sim 3.5 \text{ mmol}\cdot\text{L}^{-1}$ and $\sim 2.5 \text{ mmol}\cdot\text{L}^{-1}$, respectively) compared to a 3-5 day fast, which increased blood lactate concentration to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$ [107]. In two recent studies, using highly-trained cyclists, blood lactate accumulation was reduced by $\sim 3 \text{ mmol}\cdot\text{L}^{-1}$ following R-BD D- β HB monoester ingestion after 60 min of SS cycling at 75% W_{max} (plasma D- β HB concentration $\sim 4 \text{ mmol}\cdot\text{L}^{-1}$) [73] and by $\sim 4.5 \text{ mmol}\cdot\text{L}^{-1}$ following the ingestion of the racemic R,S-BD AcAc diester after a ~ 50 min TT (serum D- β HB concentration $\sim 0.4 \text{ mmol}\cdot\text{L}^{-1}$ and AcAc $\sim 0.4 \text{ mmol}\cdot\text{L}^{-1}$) [67]. However, the ingestion of racemic ketone salts [61-63] and BD [90], which elicit blood D- β HB concentrations up to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$, do not appear to effect blood lactate accumulation during exercise.

The ingestion of R-BD D- β HB monoester was also found to reduce skeletal muscle glycogen utilisation and increase IMTG breakdown [73]. The authors made parallels with a *Randle cycle-like* effect whereby D- β HB may provide additional acetyl-CoA groups and increase the acetyl-CoA/CoA ratio, the ratio for reduced and oxidised nicotinamide adenine dinucleotide (NADH/NAD⁺), and/or citrate leading to a feedback inhibition of glycolysis [135]; the reduction in glycogenolysis/glycolysis may also be due to inhibition of phosphofructokinase and pyruvate dehydrogenase [73,136]. Moreover, the R-BD D- β HB monoester [73] and racemic R,S-BD AcAc diester [67] have been shown to lower FFA and blood glucose concentrations during exercise; with the latter potentially due to the limiting effect of KBs on hepatic glycogenolysis and increase in peripheral tissue glucose uptake [33,34]. It is difficult to accurately estimate shifts in whole blood substrate utilisation using indirect calorimetry as the stoichiometry of AcAc and D- β HB (1.00 and 0.89, respectively) may disrupt RER values [105], and adjustments made to stoichiometric calculations have not yet been validated.

In mice livers, total combustion of D- β HB liberates $1021 \text{ kJ}\cdot\text{mol}^{-1}$ ($13 \text{ ATP}\cdot\text{mol}^{-1}$) per C_2 unit, compared to $933 \text{ kJ}\cdot\text{mol}^{-1}$ ($12.67 \text{ ATP}\cdot\text{mol}^{-1}$) and $778 \text{ kJ}\cdot\text{mol}^{-1}$ ($10 \text{ ATP}\cdot\text{mol}^{-1}$) per C_2 unit for glucose and pyruvate, respectively [137]. It has also been shown that isolated working perfused rat heart, there is 24% increase in the hydraulic efficiency when administered a combination of glucose ($10 \text{ mmol}\cdot\text{L}^{-1}$), with β HB ($4 \text{ mmol}\cdot\text{L}^{-1}$) and AcAc ($1 \text{ mmol}\cdot\text{L}^{-1}$) compared to glucose alone [138,139]. This was reasoned to be due to reducing the mitochondrial NAD couple, oxidation of the co-enzyme C couple and increasing the Gibbs free energy (ΔG) of ATP hydrolysis [65]. Thus, a greater mitochondrial electrochemical gradient is produced per ATP derived from β HB compared to CHO. This suggests the addition of KBs to substrate provision is energetically superior than relying on the end product of glycolysis. Nevertheless, there have been no observable differences in oxygen utilisation using racemic β HB salts [61-63], BD [90] or ketone esters [67,73], suggesting the enhanced metabolic efficiency of KBs demonstrated in isolated rodent tissue may not translate to whole body exercise in humans.

2.6.1 Endurance performance

Appendix A provides a tabulated summary of the effects of ketone and ketogenic supplementation on endurance performance. Initially, a theoretical performance advantage of ketone supplementation was based on an evolutionary perspective [140]; with KBs providing an additional energetic substrate for EE. In turn, a group of eight highly trained cyclists ingested CHO plus R-BD D- β HB monoester ($0.573 \text{ g}\cdot\text{kg}^{-1}$) or an isoenergetic CHO only solution (after an overnight fast) 20 min before and at the end of 60 min cycling at $75\% W_{\max}$, which preceded a 30 min TT [73]. Blood D- β HB concentration rose from 0.1 to $\sim 2 \text{ mmol}\cdot\text{L}^{-1}$ by the onset of exercise and ranged up to $2.5 \text{ mmol}\cdot\text{L}^{-1}$ by the end of SS cycling. Interestingly, TT performance improved by $411 \pm 162 \text{ m}$ ($\sim 2\%$) when CHO was ingested with the ketone ester, compared to CHO alone, despite lower plasma glucose, lactate and FFA concentration following the TT [73]. However, these performance outcomes are yet to be replicated and should be interpreted with caution as various aspects of the study design do not reciprocate real world performance nutrition strategies.

To investigate ketone supplementation, in conjunction with recommended performance nutrition strategies, a group of 11 internationally competitive cyclists undertook a dietary standardisation protocol [67]. The night prior to the trial, participants ingested an evening meal (2 g CHO·kg⁻¹) and snack (1 g CHO·kg⁻¹), followed by a breakfast (2 g CHO·kg⁻¹ with 200 mg of caffeine) on the day of the trial. Participants further ingested 0.25 g·kg⁻¹ of the racemic R,S-BD AcAc diester or placebo 70 and 90 min later (i.e. total of 0.5 g·kg⁻¹), then undertook a 20 min warm up. An additional 50 mg of caffeine and 27 g of CHO was ingested, then participants performed a ~31 km simulated TT replicating the world championship course; with an additional 250 ml of a 6% CHO solution ingested at the halfway point. Blood glucose, FFA and lactate concentration were lower in the ketone supplemented trial and performance time was impaired by ~2%, which corresponded to a ~3.7% reduction in average power output (~339 vs. ~335 W) [67]. Noteworthy, D-βHB concentrations were markedly lower compared to the earlier study, as determined by a capillary whole blood point-of-care device (0.8-1.2 mmol·L⁻¹) and laboratory test (serum ~0.4 mmol·L⁻¹); thus, emphasising metabolic differences between ketone esters. Additionally, participants exhibited severe levels of gastrointestinal distress, which appeared to increase RPE, thus compromising performance and highlighting a major concern of ketone supplementation.

The effects of the R-BD D-βHB monoester in conjunction with recommended performance nutrition strategies was later investigated [74]. A group of 11 team sport athletes performed a run-to-exhaustion (RTE) characterised by intermittent jogging (20 m at 50% estimated VO_{2max}) and running (20 m at 95% estimated VO_{2max}), preceded by 5 x 15 min high-intensity intermittent running (3 x 20 m at walking speed, 1 x maximal 15 m sprint, 4 sec recovery, 3 x 20 m jogging at 55% VO_{2max} and 3 x 20 m running at 95% VO_{2max}). Participants ingested a standardised high-CHO diet for the day prior (~6 g·kg⁻¹) and in the two meals on the day before the trial (3 g·kg⁻¹), with a 6.4% CHO solution provided at a rate of 1.2 g·min⁻¹ during the trial. The R-BD D-βHB monoester (0.75 g·kg⁻¹) was split into three boluses at 20 min pre-exercise, then after 30 and 60 min of exercise to elicit a plasma D-βHB concentration of ~1 mmol·L⁻¹ by the onset of exercise, which steadily rose to ~2.5 mmol·L⁻¹ by exhaustion. The ketone ester reduced blood glucose and lactate concentration, and had no effect on time-to-exhaustion (TTE) (~229 vs. ~267 sec; *p* = 0.13); with participants experiencing moderate-to-severe gastrointestinal distress.

Studies investigating racemic ketone salts and BD have elicited trivial [63,90] and detrimental effects on endurance performance [62]. It is possible these exercise protocols failed to sufficiently reduce skeletal muscle glycogen stores, similar to studies that investigated ketone esters, to elucidate an ergogenic effect of acute hyperketonaemia. For example, the longest trial investigating ketone supplements (i.e. racemic ketone salt) consisted of a 4 min cycling TT preceded by 90 min of cycling at 80% of the power eliciting secondary ventilatory threshold (VT₂) [63]. Despite unfavourable results to date, ketone supplements increasing blood D-βHB concentration up to 1 mmol·L⁻¹ could possess ergogenic effects, particularly if the concomitant rise in L-βHB negates the suppressive action of D-βHB on CHO metabolism [92]; however, ketone salts are not a preferred option because they markedly increase the risk of gastrointestinal distress. As an alternative, the racemic BD was investigated on a 5 km treadmill TT following 60 min at 75% VO_{2max} in trained runners [90]. Participants ingested 50% and 25% 30 min and immediately before exercise, respectively, of a ~650 ml BD + CHO solution consisting of 0.5 g·kg⁻¹ BD and 60 g CHO or an isoenergetic CHO only solution (~110 g CHO), with the remaining 25% ingested immediately prior to the TT. Although no effect on TT performance was observed, it is possible that BD ingested at higher doses without high-CHO intakes could exert a greater effect on blood D-βHB concentration, potentially by reducing competition for absorption in the gastrointestinal tract, and substrate metabolism.

Clearly, the optimal physiological conditions to elucidate an ergogenic effect of ketone supplements are difficult to identify. This is exacerbated by differences in componentry of ketone and ketogenic supplements and their varying rates of conversion to D-βHB and AcAc. As such, further trials specifically investigating their effect on metabolism and performance during protocols with reduced glycogen content without the interference of CHO ingestion seem warranted.

2.7 Ketogenic diets: Substrate utilisation and exercise efficiency

Conformity to a ketogenic diets produce an array of metabolic adaptations, collectively termed *keto-adaptation*. There is no clear definition of what constitutes *keto-adaptation* or strategies for its optimisation, except adherence for at least 3-4 weeks and supplementation with sodium and potassium [141]. However, it is likely to be population-

specific and exist on a continuum depending on the duration of dietary adherence. Typical adaptation observed during exercise include: 1) increased in MFO [14,15,35,48]; 2) reduced blood glucose utilisation [15] and; 3) reduced muscle [15,35] and hepatic [35] glycogen utilisation. The importance of KBs to EE is uncertain; however, it is postulated as the defining feature differentiating adaptation to keto- versus nonketogenic, lower-CHO ($\sim 2.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), higher-fat (LCHF) diets (i.e. fat-adaptation) [9].

2.7.1 Maximal fat oxidation

Keto- and fat-adaptation exert large increases in MFO and FAT_{max} to consistently $>1 \text{ g}\cdot\text{min}^{-1}$ and $>65\% \text{ VO}_{2\text{max}}$, respectively [14,15,35,48]. In a group of 10 chronically keto-adapted (~ 20 months), well-trained, ultra-endurance runners ($\text{VO}_{2\text{max}}$, $\sim 64.3 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), MFO rates were $\sim 1.54 \text{ g}\cdot\text{min}^{-1}$ (range of $1.15\text{--}1.74 \text{ g}\cdot\text{min}^{-1}$) at a FAT_{max} of $\sim 70\% \text{ VO}_{2\text{max}}$ [48]. Similar rates of $\sim 1.57 \text{ g}\cdot\text{min}^{-1}$ have been reported following a 3-week KD intervention in elite race-walkers ($\text{VO}_{2\text{max}}$, $\sim 66.3 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), which ranged up to $\sim 1.9 \text{ g}\cdot\text{min}^{-1}$ during 2 h of walking at $80\% \text{ VO}_{2\text{peak}}$ [14]. This increase in fat oxidation appears to be underpinned by a variety of metabolic responses, including; 1) increased FFA delivery and transport across cell membrane transporters into skeletal muscle; 2) elevated IMTG stores and breakdown; 3) increased FFA transport through the carnitine-palmitoyl transferase complex into the mitochondria and; 4) increased β -oxidation [142]. Although MFO and FAT_{max} values can infer metabolic shifts towards keto- and fat-adaptation, these are not necessarily reliable surrogates for *optimal* adaptation as fat oxidation rates can increase to $>1 \text{ g}\cdot\text{min}^{-1}$ within three days of ingesting a LCHF diet [143]; with this duration of dietary adaptation typically associated with performance impairments [144].

2.7.2 Metabolic flexibility

The ability to rapidly and efficiently switch between CHO and fat stores for energy production relative to energy demand is characteristic of metabolic flexibility [145]. Metabolic flexibility is a necessary adaptation for maximal-intensity exercise, such as tactile surges, sprints and hill work, whilst preserving CHO stores during the submaximal phases of endurance competition [146].

Despite large increases in fat oxidation over a range of intensities, low-CHO diets negate CHO metabolism during high-intensity exercise. In the earliest study investigating keto-adaptation on submaximal endurance performance, this was described as a *throttling* effect of whole body CHO oxidation at near maximal intensities (i.e. RER <1.0 at $\text{VO}_{2\text{max}}$) [15] and may be explained by an attenuation of glycogenolysis and pyruvate dehydrogenase complex (PDHc) activity [147]. The PDHc catalyses the conversion of pyruvate, the end product of glycolysis, into acetyl-CoA in a nonreversible reaction and is a key enzyme determining the rate of CHO oxidation during exercise [148]. When pyruvate production exceeds its rate of oxidation by the PDHc, such as during high-intensity exercise, lactate is produced [149]. Elevated blood lactate concentrations during submaximal intensity exercise appear to occur in chronically keto-adapted (>8 months) individuals [35,48]. Prior studies have suggested lactate oxidation [150] and gluconeogenesis via lactate [35] may be reduced following conformity to a LCHF diet and, therefore, this could increase blood lactate concentrations. Whether this effects submaximal intensity exercise performance or capacity is uncertain.

To optimise adaptation to a KD, periods of several months-to-years have been proposed. This premise is largely underpinned by a study comparing metabolic characteristics between a group of trained, ultra-endurance runners, chronically adapted (>9 months) to a KD to athletes habitually ingesting a high-CHO diet [48]. Unexpectedly, the keto-adapted athletes demonstrated equivalent pre-exercise skeletal muscle glycogen stores and rates of utilisation (during 3 h of running at 70% $\text{VO}_{2\text{max}}$) and glycogen restoration in the absence of dietary CHO intake during recovery. However, these results have not been replicated and conflict with other studies demonstrating lower starting skeletal muscle glycogen content and utilisation during exercise following keto-adaptation periods of 4 weeks [15] and >8 months [35]. Further, it should be noted that KBs do not provide the major fuel source for exercising muscles following keto-adaptation; rather, fat is the primary substrate with the stoichiometry of indirect calorimetry unlikely to be altered when calculating whole body oxidation rates, assuming negligible insensible losses [151]. As such, keto-adaptation appears better suited towards ultra-endurance events as the rate of EE can be met predominantly by oxidising fat.

2.7.3 Exercise efficiency

The proportionate increase in fat oxidation following keto- and fat- adaptation strategies increases oxygen uptake for a given exercise intensity [108]. As such, efficiency is impaired when exercising at higher fractional rates of $\text{VO}_{2\text{max}}$ requiring high EE [14]. Therefore, a high-CHO diet in the days preceding competition can augment exercise efficiency [152]. Nevertheless, the increase in oxygen uptake at low- to moderate-intensities does not necessarily persist following keto-adaptation. For example, after 4 weeks of adaptation to a KD, it was proposed that exercise efficiency improved due to a similar oxygen uptake whilst cycling at the same absolute submaximal workload (i.e. 62-64% $\text{VO}_{2\text{max}}$) [15]. In this study, it should be noted that if exercise efficiency was maintained, oxygen uptake would have been expected to increase with the increase in fat oxidation. It is, therefore, possible that KB oxidation compensated for the increase in fat oxidation; however, this is only speculative. Furthermore, at submaximal intensities, the shift in oxygen uptake may simply be a reflection of substrate preference, not exercise efficiency [153,154]; with a more appropriate measure of exercise efficiency being the energy cost of exercise [153,154].

The mechanisms impairing exercise efficiency may be related to the mitochondrial electrochemical gradient. Due to the yield from oxidative phosphorylation being greater when NADH is the primary electron transporter (three coupling sites; complex 1, 3 and 4), compared to flavin adenine dinucleotide (FADH_2) (two coupling sites; complex 3 and 4), the greater NADH/ FADH_2 ratio from glycolysis compared to β -oxidation means there is a higher energy yield per unit of oxygen [155]. This equates to a loss of synthesis of about one of the six possible ATP molecules per mole of oxygen, resulting in a ~5% reduction in efficiency [65]. Furthermore, elevated fatty-acid activated transcription factor peroxisome proliferator-activated receptor α (PPAR α) upregulates both fat oxidative genes and the expression of mitochondrial uncoupling proteins [156]. Mitochondrial inefficiency may result in a discrepancy between measured and predicted oxygen uptake based on shifts in RER, whilst assuming no change in efficiency; this has been shown to increase in sedentary [157], but not trained individuals [158]. Currently, the effect of keto- or fat-adaptation on mitochondrial uncoupling in human skeletal muscle is unclear [157-159] and requires further research.

2.7.4 Endurance performance and capacity

Appendix B provides a tabulated summary of the effects of adaptation to a KD on endurance performance. Recent reviews and commentaries have discussed the benefits of low-CHO diets on performance [9,160-162], which have been refuted by others [146,163,164]. To date, the majority of published studies investigating the ergogenic effects of keto- and fat-adaptation have employed high-intensity ($\geq 80\%$ $\text{VO}_{2\text{max}}$) performance protocols (i.e. <120 min) [14,144], which favour high rates of CHO utilisation. Coinciding with the loss in exercise efficiency when approaching $\text{VO}_{2\text{max}}$, there appears to be an impairment in high-intensity endurance performance [14,165,166]. However, enhanced performance in select individuals during preloaded cycling TTs (total protocol duration of ~ 5 h) have been demonstrated following short-term fat-adaptation with CHO restoration protocols [167,168]. Nevertheless, these early findings have failed to impact contemporary fuelling recommendations, particularly for endurance events up to ~ 3 h in duration [5,169].

It is arguable that high rates of fat oxidation may be preferential for events performed at low- to moderate-intensities. As previously discussed, fatigue at these intensities is associated with depleted muscle glycogen content and an inability to sustain rates of CHO oxidation exhibited during the early states of exercise. If fat oxidation cannot adequately compensate for reduced CHO availability (or be met by exogenous CHO ingestion), then exhaustion is accelerated [170,171]. Therefore, a shift towards fat oxidation may be desirable due to the effectively unlimited supply of stored energy (i.e. ~ 259 MJ in a 70 kg individual with 10% body fat). Additionally, compared to a nonketogenic LCHF diet, a KD may possess a metabolic advantage due to hyperketonaemia, which can provide a fuel source for skeletal muscle and the brain, as well as exhibit a variety of pleiotropic roles in metabolism, homeostasis, cell signalling and transcription [10,42,65,172]. Collectively, these adaptations may be sufficient to maintain the energy costs for an amateur athlete without necessitating CHO supplementation. However, it is unlikely to benefit elite endurance athletes who require additional CHO intake to maintain higher energy outputs (Figure 2.5, p. 30) [6].

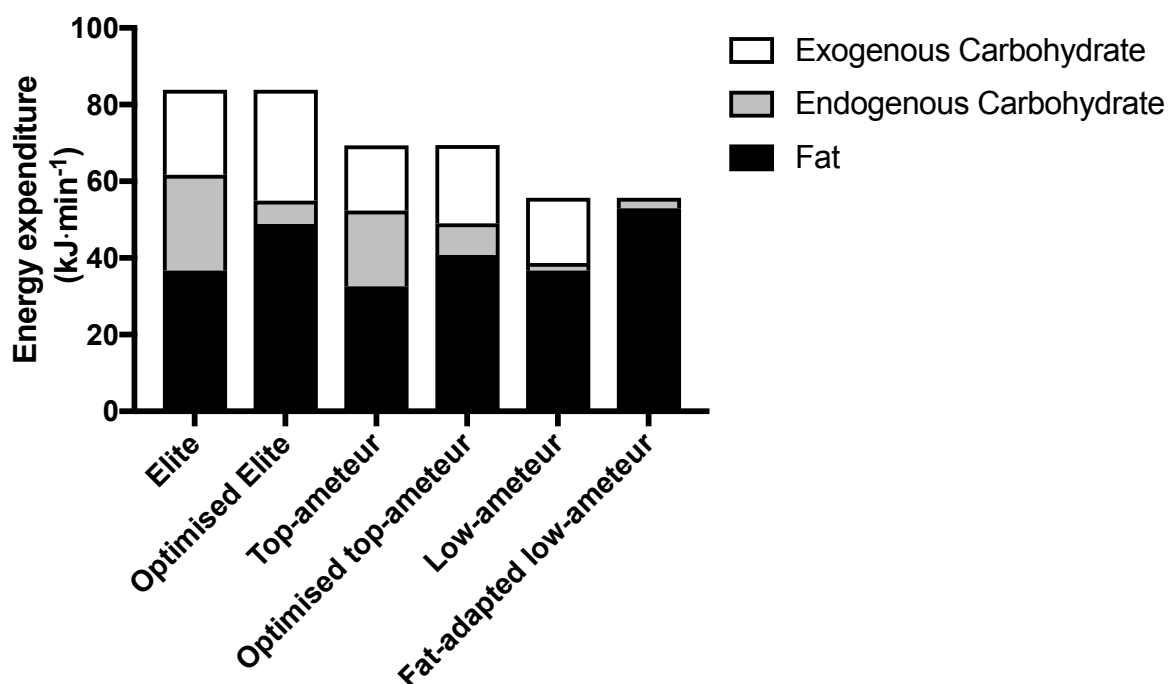


Figure 2.5. Contribution of carbohydrate and fat to energy expenditure during an Ironman triathlon depending on athletic calibre and dietary intake. *Optimised* refers to adaptations elicited by training, specifically increases in fat utilisation, and the ingestion of multiple-transportable carbohydrates. Adapted from Maunder et al. (2018).

A single study has investigated the effect of a KD on submaximal intensity exercise capacity (62-64% $\text{VO}_{2\text{max}}$). The researchers employed a single-arm design with the pre-test acting as the CHO-diet trial (66% of EI from CHO, 33% of EI from fat) and the post-test following 28 days of ingesting an isoenergetic and isoprotein KD (<20 g CHO·day⁻¹, 1.75 g protein·kg⁻¹·day⁻¹) [15]. Of the five trained cyclists ($\text{VO}_{2\text{max}}$ 69.0 ml·kg⁻¹·min⁻¹), three improved and two reduced their TTE, resulting in no overall difference between dietary conditions (CHO trial, 147 ± 13 vs. KD trial, 151 ± 25 min) (Figure 2.6, p. 31). During the intervention period, participants were requested to maintain their current training load; however, training variables were not reported. Thus, there was the potential of an order- or training-effect and this is highlighted in the results, as they appear to be heavily skewed by the improvement of a single participant from 148 to 232 min (Figure 2.6, indicated with an X). Additionally, for the CHO-diet trial, participants commenced exercise after an overnight fast and did not ingest CHO during exercise, which is incongruent with recommended performance nutrition strategies [5,173]. Therefore, the study design favoured the keto-adapted (i.e. post) trial.

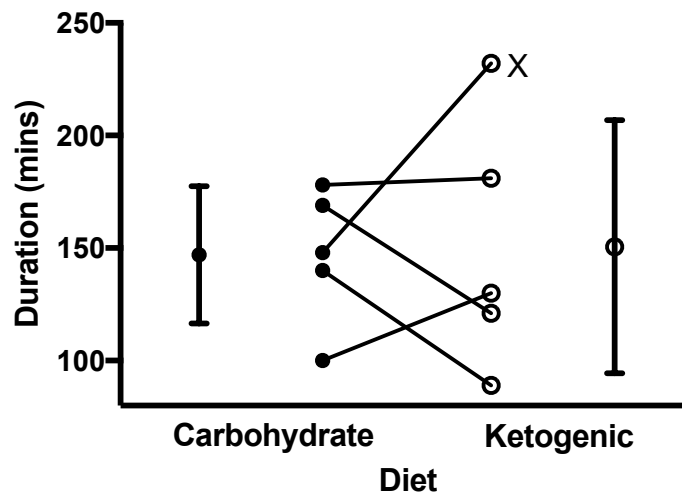


Figure 2.6. Time-to-exhaustion at 62-64% maximal oxygen uptake in five trained male cyclists following adaptation to a 28-day ketogenic diet. Presented as mean \pm SD and individual responses. The X refers to the participant skewing the group mean. Adapted from Phinney et al. (1983).

It appears a minimum of 3-4 weeks is required to restore performance and/or endurance to near normal levels following the initial decrement associated with a KD [14,15]. Despite the suggestion that several months are required to optimise keto-adaptation, the only studies having investigated athletes ingesting a KD for this duration have failed to either: 1) measure performance [35,48] or 2) control and monitor dietary intake and training volume [166,174]. The importance of dietary control in performance studies is well known [175], with long-term dietary interventions being complex and requiring the simultaneous management of various nutrition and lifestyle challenges [176]. Therefore, in light of the equivocal outcomes in case studies of ultra-distance athletes [177,178], and the absence of rigorously controlled studies, the recommendation of ketogenic dietary strategies for (ultra-) endurance performance remains unsubstantiated [8,179].

2.8 Conclusion and thesis hypotheses

The body of knowledge on nutritional ketosis does not substantiate any conclusive recommendations for: 1) ketone or ketogenic supplementation for prolonged, high-intensity endurance performance and 2) keto-adaptation for submaximal-intensity endurance performance/capacity. Several types of ketone supplements exist; however, many induce gastrointestinal distress and are unlikely to benefit endurance performance in their current form. Due to the limited research investigating BD on endurance performance and no clear evidence for adverse gastrointestinal or systemic effects in

humans, further research is warranted to examine the effects of BD on high-intensity endurance performance. Alternatively, adaptation to a KD appears to require at least 3-4 weeks to reconcile metabolic pathways with shifts in substrate availability. The increase in fat oxidation could augment exercise capacity at submaximal exercise intensities; however, future studies must investigate the effects of adaptation to a KD compared to recommended CHO fuelling strategies.

It is, therefore, hypothesised:

- BD ingestion, without coingesting CHO, would downregulate CHO utilisation and hepatic glycogenolysis and, therefore, impair prolonged, high-intensity endurance performance compared to placebo.
- Keto-adaptation would preserve submaximal exercise capacity without necessitating acute CHO fuelling strategies compared to recommended CHO fuelling strategies.

Dietary recommendations for athletic populations must also consider health effects. Since manipulating CHO availability appears the most efficacious nutritional strategy modulating exercise-induced immune perturbations *in vitro* [1,7], it is possible the induction of nutritional ketosis – either via endogenous or exogenous origin – may alter the immune response to prolonged, strenuous exercise and subsequent infection risk. This is discussed further in Chapter 3.

T-cell cytokine production in response to prolonged, strenuous exercise and potential effects of nutritional ketosis: A review

3.1 Are athletes at risk of illness?

Exercise has been known to modulate immune function for over a century [180]. However, it was only ~40 years ago that it was suggested physical exertion had a detrimental effect on illness prevention. The epidemiological research demonstrated marathon and ultra-marathon runners self-reported more illness-related symptoms following competition compared to a resting population [181,182]. It is now recognised that individuals engaging in regular moderate training are at a decreased risk of illness compared to the sedentary population. However, acute bouts of prolonged (>90 min), exhaustive exercise and periods of heavy training result in a transient state of immunodepression [1,2,183-185]. This period may last from several hours to several days and is associated with an increased risk of opportunistic infection by viruses and bacteria, viral reactivation, and the development of noninfectious illness-related symptoms [1,183].

Upper respiratory symptoms are the most commonly reported illness in athletes (35-65% of illness presentations) [183]; with recurrent episodes compromising training availability [3], achievement of athletic potential [186] and performance at pinnacle events [3,4,187]. The aetiology of URS is multifaceted and not all presentations are of infectious origin (30-40%), such as exercise-induced asthma, allergy and laryngeal dysfunction [2,183,188-192]. Nevertheless, viral and, to a lesser extent, bacterial pathogens underpin many URS presentations [189,192], which are classified as upper respiratory tract infections (URTI). Nieman (1994) proposed the J-shape curve to characterise the association between elevations in training load and increased illness/infection risk in sub-elite athletes [193]. The model was later extended by Malm (2006) to a S-shaped curve due to the notion that high-training loads are incompatible with recurrent illness, such as in elite-athletes (Figure 3.1, p. 35) [194,195]. These differences may be due to modifiable risk factors other than training load, such as full- or part-time employment, sleep, diet, stress management, pathogen avoidance and hygiene [196].

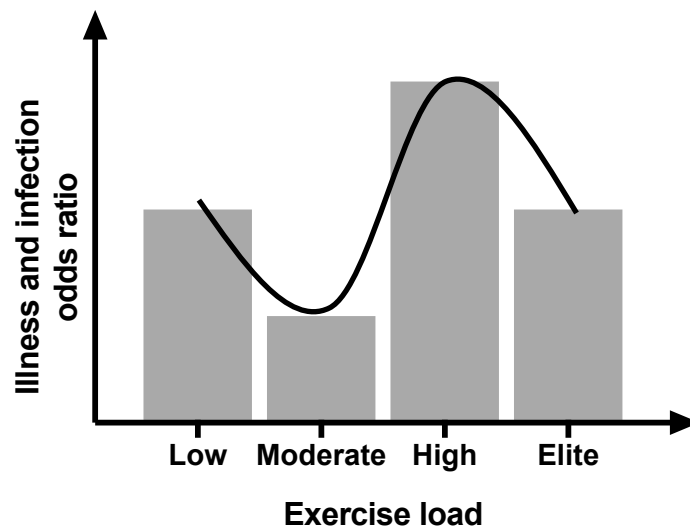


Figure 3.1. Proposed S-shaped relationship between training load and illness / infection risk. Adapted from Malm (2006).

Exercise-induced immunodepression is characterised by changes to several innate and acquired immune components [1]. Depending on the immune component and exercise (duration and intensity), a ~15-25% reduction in immune competency has been suggested [196]. Whether this directly relates to an increased illness and infection risk is uncertain, as there is currently a lack of research investigating the association of *in vitro* and *in vivo* immune markers with URS and URTI incidence. Furthermore, *in vitro* models examining immune function do not necessarily reflect physiological conditions *in vivo* [22] and alterations to single immune components does not imply changes exist to others; rather, findings should be interpreted with the assumption that there is a large degree of cooperation, complementation and compensation within the immune system [22] (Figure 3.2, p. 36). As such, *in vivo immune* models have been preferred (i.e. skin contact delayed-type hypersensitivity test) as they are considered to possess greater clinical implications [197]. Nevertheless, *in vitro* models provide valuable insight into the mechanisms regulating the immune system and underpinning changes to the *in vivo* immune response [22].

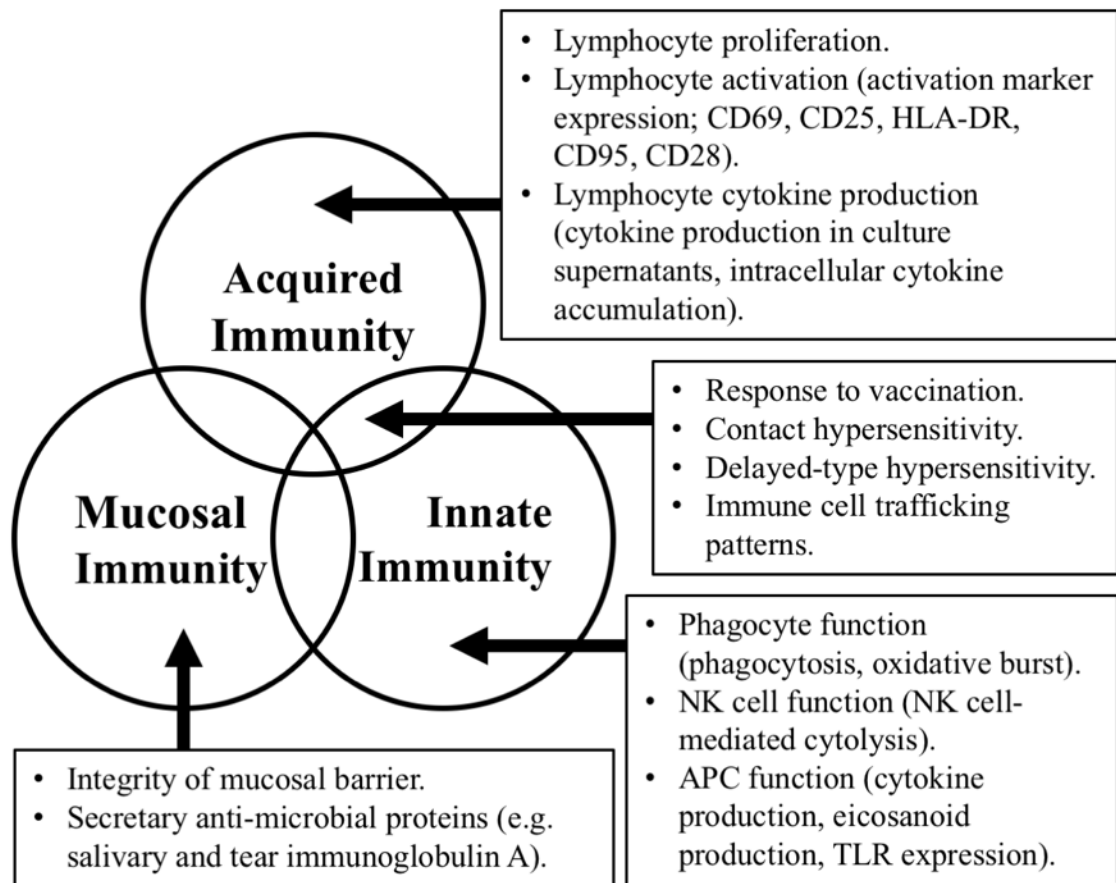


Figure 3.2. An overview of the acquired, innate and mucosal immune systems with examples of immune markers most commonly used in immunonutrition and exercise investigations. CD *cluster of differentiation*, HLA-DR *human leukocyte antigen – DR isotype*, NK cell *natural killer cell*, APC *antigen-presenting cell*, TLR *toll-like receptor*.

3.2 T lymphocytes

Lymphocytes comprise ~20-25% of the peripheral blood leukocyte population, of which, ~60-80% are T lymphocytes, commonly termed T-cells. T-cells, of the acquired immune system, play a pivotal role in the orchestration of the immune response to invading and incumbent pathogens. T-cells differentiate into various effector and regulatory cell subsets with divergent functional capacities to eliminate or neutralise pathogens, whilst preventing an overshooting of the immune response to harmless microbes [19,198-204]. These capabilities of T-cells are generated in the thymus – hence for name – then within secondary lymphoid tissue (spleen, lymph nodes and mucosal-associated lymphoid tissue). After maturation in the thymus (hence their name), each naïve T-cell migrates to secondary lymphoid tissue (spleen, lymph nodes and mucosal tissue) via lymphatic vessels to become an effector, regulatory or memory T-cell depending on their interaction with an antigen-presenting cell (APC) [19,21]. T-cell specificity is due to the T-cell

receptor (TCR), which responds to a single antigen for its lifespan [19]; the major histocompatibility complex molecule on APCs presents fragments of foreign antigens to the T-cell, which is recognised by the TCR and stimulates a T-cell immune response [19]. They mediate other cells of the innate and acquired immune systems by producing specific pro- and anti-inflammatory cytokines acting via autocrine, paracrine and endocrine mechanisms. Acute and chronic exercise influences the concentration of circulatory T-cell subsets and their capacity to produce cytokines [18,205]. This perturbed T-cell status may influence illness and infection risk and have implications for allergy and atopy.

3.3 T-cell subsets

3.3.1 Helper and cytotoxic T-cells

T-cells are divided into helper T-cell (~70%) and cytotoxic T-cell subsets during maturation in the thymus, and are identified by their cluster of differentiation (CD) membrane co-receptors $CD4^+$ and $CD8^+$, respectively. $CD4^+$ T-cells have a role in regulating both the cell-mediated and humoral arm of the immune response through cytokine signalling [19]. Their TCR only interacts with MHC class II molecules, which are present on APCs. Alternatively, $CD8^+$ T-cells are predominantly responsible for destroying virally infected cells and some tumour cells via an armoury of cytolytic proteins including perforins and granzymes stored within intracellular lytic vesicles. $CD8^+$ T-cells are not recognised for their regulatory function and their TCR only interacts with MHC class I molecules, which are present on *all nucleated cells* within the human body [19]; however, similar to $CD4^+$ T-cells, $CD8^+$ T-cells also produce cytokines [19,198,199].

3.3.2 Type-1 and Type-2 T-cells

T-helper and cytotoxic T-cells can polarise into type-1 or type-2 subsets; however, it is the cytokine production by the $CD4^+$ T-cell population that is most responsible for immune regulation [206]. Type-1 T-cells are predominantly involved in cell-mediated immunity to combat intracellular pathogens, such as viruses. They are characterised by their production of the pro-inflammatory cytokine, interferon (IFN)- γ , and can activate

CD8⁺ T-cells, natural killer (NK) cells and phagocytes [20]. Production of IL-12 by APCs of the innate immune system, in conjunction with IFN- γ by NK cells and T-cells, polarise T-cells towards a type-1 phenotype in a feed-forward fashion [20]. T-box transcription factor (T-bet) programs the polarisation of the cell towards a type-1 phenotype by acting as a promoter of IFN- γ expression [207]. A reduction in the capacity to produce IFN- γ has been hypothesised to increase infection risk [208]. Conversely, type-2 T-cells are predominantly involved in humoral immunity, which provides protection against extracellular pathogens, particularly of bacterial and fungal origin [20]. They are characterised by their production of the anti-inflammatory cytokine, IL-4, and can assist B-cells in upregulating the production of some immunoglobulins (e.g. IgE) and recruit eosinophils. IL-4 is antagonistic to IFN- γ and polarises T-cells towards a type-2 phenotype via the action of the transcription factor, GATA-3 [209]. The type-1/type-2 T-cell balance is linked with infection risk [20], mucosal immunity [210], allergy and asthma [20].

3.3.3 Regulatory T-cells

Regulatory T (TReg)-cells represent 5-10% of the peripheral blood CD4⁺ T-cell population. They play a central role in modulating various immune responses and silencing self-reactive T-cells, including autoimmune responses and immunity to transplants, allergens, tumours and pathogens [211,212]. TReg cells express the cell surface marker CD25⁺; however, more specific markers are the intracellular expression of Forkhead transcription factor (FoxP3) and cell surface marker CD127^{low/-} [212,213]. Naturally occurring CD4⁺CD25⁺ TReg (nTReg) cells (produced in the thymus) and transforming growth factor (TGF)- β induced TReg (iTReg) cells (produced in the periphery) are the two major types of TReg cells. TReg cells do not produce pro-inflammatory cytokines upon antigenic stimulation; rather, they suppress the activation, proliferation and effector functions of CD4⁺ (type-1, type-2 and T-helper 17 effector cells), CD8⁺ cells, NK cells, dendritic cells, and B-cells primarily through the release of IL-10, an anti-inflammatory cytokine [212]. The differentiation to T helper 17 (Th17) cells or iTReg cells is interlinked as both require TGF- β , with the cytokine micro-environment being decisive for polarisation. iTReg polarisation is largely dependent upon the ability of nTRegs to produce TGF- β ; whereas, in the presence of pro-inflammatory

cytokines (e.g. IL-6), this may shift to favour a Th17 phenotype by abolishing the induction of FoxP3 [214].

3.4 Effect of strenuous exercise on circulating T-cell concentration

Circulating lymphocytes elicit a biphasic response to exercise proportionate to the intensity and, to a lesser extent, duration [18]. During and immediately following exercise, the number of lymphocytes rises (lymphocytosis), followed within ~30-60 min by a decline to below pre-exercise levels (lymphopenia), which may persist for up to 24 h in some populations [215]. These cell trafficking patterns are rapid, with the egress of effector, pro-inflammatory lymphocytes demonstrating marked differences in the blood compartment within 3 min of exercise cessation [216]. Originally, researchers speculated that an exercise-induced reduction in circulating immune cell counts indicated immunodepression. More recently, immune cell redistribution is suggested to contribute to immune surveillance and cytotoxicity in peripheral tissues [185,217]. These cell trafficking patterns originate in the margined leukocyte pool, lymph nodes, spleen and bone marrow, and move via the blood stream to peripheral tissues (e.g. urogenital tract, skin, gastrointestinal tract, mucosal linings and respiratory tract) where contact with a pathogen is more likely to occur [217]. However, recent research has demonstrated that the skin is not a favoured site of relocation for T-cells [218].

3.4.1 Helper and cytotoxic T-cells

3.4.1.1 Acute exercise

The concentration of circulating CD4⁺ and CD8⁺ T-cells is proportionate to exercise intensity [219]; a greater number of CD4⁺ T-cells mobilise compared to CD8⁺ T-cells secondary to their dominance of the T-cell population. Nevertheless, there is a larger relative increase in CD8⁺ T-cells due to their higher density of β_2 -adrenoreceptors and, therefore, the CD4⁺/CD8⁺ ratio is reduced. This movement of CD4⁺ and CD8⁺ T-cells into the blood augments when exercise has been performed earlier on the same day [220] and when exercise intensity is above (+15%) compared to below (-5%) lactate threshold [219]. When the recovery period between exercise bouts are shortened (3 vs. 6 h), circulating CD4⁺ and CD8⁺ T-cells may fail to return to pre-exercise concentrations [221].

This is followed by a greater exercise-induced increase in CD8⁺ T-cells; whereas, CD4⁺ T-cells rise in a similar magnitude following both recovery periods [221].

3.4.1.2 Training

Several days of intensified training can alter the concentration of circulating lymphocytes and T-cells at rest and following acute exercise. For example, exercise-induced CD4⁺ [27] and CD8⁺ [222] T-cell mobilisation and redeployment was truncated following 6-7 days of intensified training in trained ($\text{VO}_{2\text{max}} > 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) male cyclists. However, periods of intensified training do not appear to effect exercise-induced lymphocytosis and lymphopenia [25,27]. In contrast, regular moderate training may augment immune cell redistribution. For example, in 18 untrained men ($\text{VO}_{2\text{max}}, \sim 46 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), there was a greater post-exercise reduction of circulating CD4⁺ T-cells and lymphocytes after 3 days of cycling at 65% $\text{VO}_{2\text{max}}$ for 1 h despite no difference in pre-exercise lymphocyte and T-cell concentrations [223]; whereas, exercise-induced CD8⁺ T-cell trafficking remained unchanged [223]. Furthermore, during several months of Ironman triathlon training, there was no difference in the resting CD4⁺ and CD8⁺ proportion of circulating T-cells [224]. Altogether, it seems that sharp increases in training load may attenuate T-cell trafficking patterns, which may impair immune surveillance and cytotoxicity in peripheral tissues.

3.4.2 Type-1 and Type-2 T-cells

3.4.2.1 Acute exercise

Strenuous exercise reduces circulating type-1 CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ T-cells (Figure 3.3, p. 43). For example, immediately following 150 min of running at 75% $\text{VO}_{2\text{max}}$, the percentage of mitogen-stimulated type-1 CD4⁺ (IFN- γ ⁺) and CD8⁺ (IFN- γ ⁺) T-cells (of total T-cells) declined in trained men ($\text{VO}_{2\text{max}} 52\text{-}68 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) [225]. This reduction was sustained at 24 h post exercise. Conversely, there was no change in the percentage of mitogen-stimulated type-2 CD4⁺ (IL-4⁺) and CD8⁺ (IL-4⁺) T-cells [225]. These findings have been corroborated in whole blood mitogen-stimulated cultures showing both a reduction in the concentration and percentage of IFN- γ ⁺ T-cells, without an alteration in IL-4⁺ T-cells, immediately and 1 h following cycling to exhaustion at $\sim 74\%$ $\text{VO}_{2\text{max}}$ ($107 \pm 7 \text{ min}$) [24]. Some studies also report an increase or no change in the concentration of stimulated circulatory IFN- γ ⁺ T-cell concentration immediately

following exercise [25,226,227]; however these typically decline to below pre-exercise levels within 2 h of recovery. Other studies have shown an increase in both unstimulated CD8⁺IFN- γ ⁺ and CD8⁺IL-4⁺ T-cells [219,228] and IFN- γ ⁺ and IL-4⁺ T-cells [229] following shorter exercise bouts (30-60 min). However, exercise-induced increases in circulatory T-cell concentrations expressing these cytokines does not necessarily persist after (mitogen) stimulation [228]. Altogether, the reduction in circulatory type-1 T-cell concentration is likely due to a combination of immune cell redistribution to peripheral tissues and polarisation towards a type-2 T-cell phenotype, which may suppress the pro-inflammatory response to an immune challenge and increase the risk of infection and viral reactivation.

3.4.2.2 Training

Several days of intensified training alters the concentration and trafficking pattern of circulating IFN- γ ⁺ T-cells, but not IL-4⁺ T-cells. For example, 7 days of intensified training reduced the number of IFN- γ ⁺ T-cells at rest [24] with IFN- γ ⁺ T-cells exhibiting no exercise-induced changes following an exhaustive cycling bout at ~74% VO_{2max} (85 \pm 5 min) [24]. This contrasts starkly to when the same participants cycled to exhaustion at the same intensity following a normal training volume [24]. Additionally, lower CD4⁺IFN- γ ⁺ and higher CD4⁺IL-4⁺ T-cell percentage of PBMCs have been demonstrated in marathon-trained individuals [230]. This preferred accumulation of anti-inflammatory, type-2 T-cells and failure of type-1 T-cells to effectively relocate to peripheral tissues in response to an exercise bout suggests periods of heavy training may reduce cytotoxicity in peripheral tissues and increase the risk of infection and viral reactivation.

3.4.3 Regulatory T-cells

3.4.3.1 Acute exercise

Strenuous exercise has demonstrated equivocal effects on circulating TReg cell concentration following exercise (Figure 3.3, p. 43); this may be underpinned by the intensity and duration of the exercise bout. For example, shorter exercise durations requiring near maximal power outputs appear to increase circulatory CD4⁺CD25⁺CD127^{low/-} [27,231] and CD4⁺CD25⁺FoxP3⁺ T-cell concentration [232],

with females showing a higher increase once adjusted for pre-exercise values [232]. However, the relative contribution of TReg cells to the CD4⁺ cell population may actually decline [233]. In contrast, longer duration submaximal exercise may not effect CD4⁺CD25⁺CD127^{low/-} T-cell concentration [28,30] or percentage of total lymphocytes [28,30] and CD4⁺ T-cells [30]. Two studies have demonstrated a reduction in CD4⁺CD25⁺FoxP3⁺ T-cell concentration and percentage of total CD4⁺ T-cells following a half-Ironman triathlon or marathon [31,234], which may be due to apoptosis [231] or redistribution of cells to peripheral tissues. In a recent study, circulatory CD4⁺CD25⁺FoxP3⁺ T-cell concentration and percentage of CD4⁺ cells declined 1 h into recovery, then increased above pre-exercise levels 1 day after a marathon, indicating a biphasic response of these anti-inflammatory and immunosuppressive T-cells [31]. Their increase coincided with elevated HLA-DR⁺ (mature, terminally differentiated) TReg cells, which may be a due to greater expansion of these cell subsets or preferential mobilisation [31], and is possibly a mechanism to prevent excessive cell damage resulting from exercise.

3.4.3.2 Training

More consistent findings in circulating TReg cell concentration have been associated with heavy training loads [26,30,235,236]. In particular, individuals involved in sports where aerobic capacity is an important factor for performance appear to have an elevated blood TReg cell concentration [235]. This effect may be augmented by the stimulatory role of IL-2 on TReg cell proliferation, differentiation and activation [237,238], as IL-2 remains elevated in the blood for several hours after exercise [239]. Nonetheless, one study reported reduced CD4⁺CD25⁺FoxP3⁺ T-cell percentage of PBMCs in marathon-trained individuals; although, this discrepancy was resolved when adjusted for body mass index [230]. In the same study, there was also a higher percentage of CD4⁺IL-10⁺ and CD4⁺TGF-β⁺ T-cell subsets within PBMCs in marathon-trained individuals [230]. Furthermore, another study showed no effect from 8 days of intensified training on circulatory CD4⁺CD25⁺CD127^{low/-} T-cell concentration in trained individuals, which may have been due to this population already possessing an enlarged TReg cell population [27]. Considering TReg cells preferentially produce the anti-inflammatory and immunosuppressive cytokine, IL-10, heavy training loads are likely to promote immunodepression and increase illness risk.

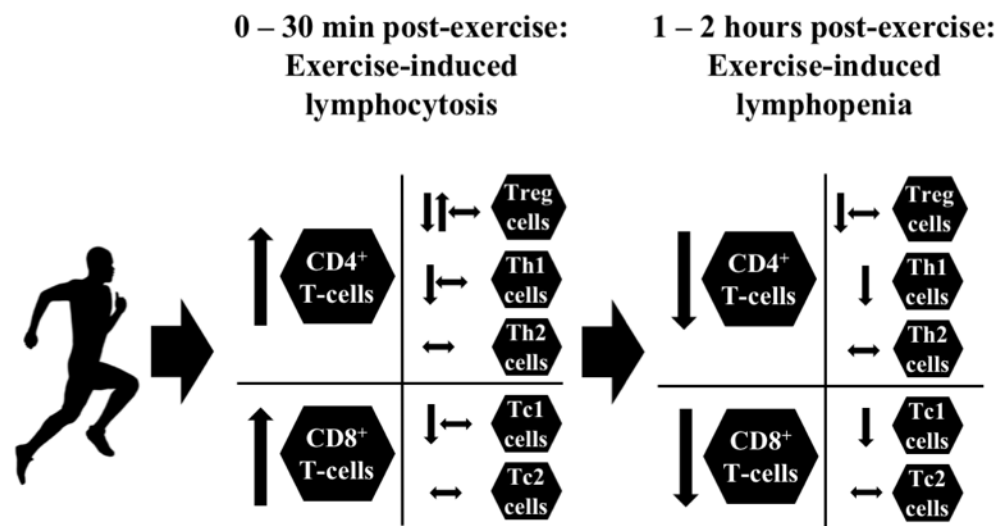


Figure 3.3. Changes in circulatory T-cell subset concentration relative to pre-exercise values following a prolonged, strenuous exercise bout. *CD4⁺ cell* T-helper cell, *CD8⁺ cell* Cytotoxic cell, *TReg cell* Regulatory T-cell, *Th1 cell* Type-1 T-helper cell, *Th2 cell* Type-2 T-helper cell, *Tc1 cell* Type-1 Cytotoxic T-cell, *Tc2 cell* Type-2 Cytotoxic T-cell.

3.5 Measurement of T-cell cytokine production

To measure cytokine production, *in vitro* isolated cell or whole blood cultures are stimulated with an immunogenic agent. This *ex vivo* model is assumed to reciprocate, at least in part, an *in vivo* infection that may possess implications for illness and infection risk [22]. Typically, lymphocytes and T-cells are stimulated with mitogens, superantigens, specific-(single- or multi-) antigens or anti-CD3⁺ antibodies; whereas, monocytes are stimulated with bacterial lipopolysaccharides. Protein concentration of a single cytokine within the culture supernatant is often measured using an enzyme-linked immunosorbent assay (ELISA) or numerous cytokines can be measured simultaneously using a multiplex assay. Changes in cytokine concentration can also be presented on a *per cell basis* presuming the selected cell is the major contributor of the cytokine. Alternatively, if an intracellular protein transport inhibitor is added to the culture, intracellular cytokine production can be measured using flow cytometry. Overall, a large degree of methodological heterogeneity and numerous analytical issues between studies make it difficult to directly compare findings and extrapolate to illness and infection risk [22,240].

As immune cells do not store cytokines, culture stimulation is a dynamic process of protein synthesis and breakdown [22]. Therefore, the duration of the incubation

(stimulation) period can influence the abundance of cytokine protein within the medium. Prior studies have employed stimulation of 1 h (pulse stimulation) to several days, with different time points associated with cytokine-specific changes in concentration resulting from their unique rates of cytokine kinetics [22]. Since the production of proteins are regulated by their gene transcription [23], measuring cytokine specific messenger ribonucleic acid (mRNA) expression via reverse transcription polymerase chain reaction (RT-PCR) and/or quantitative polymerase chain reaction can provide insight into the early stages of activation in the immune response to a pathogenic challenge. Similarly, gene expression for cognate transcription factors governing the differentiation of T-cells can also provide insight into the mechanisms underpinning the production of key cytokines.

The cytokine response is further influenced by the cellular, hormonal, cytokine and substrate make-up within the stimulated culture. Isolated cell cultures are nonphysiological as they insulate the effect from other immune components [22]; whereas, whole blood cultures are more reflective of an *in vivo* immune response. Measuring cytokine concentration in culture supernatants, however, cannot determine cell-specific cytokine production due to the contribution of various immune cells and shifts in the cellular make-up following exercise. Furthermore, each immunogenic agent has unique immunogenicity that elicits a different cytokine response by preferentially initiating the production of some cytokines over others. In particular, mitogens trigger a *nonspecific* and powerful immune response, initiating the production of large amounts of cytokines unreflective of an *in vivo* immune challenge. Whereas, *specific*-single- and *specific*-multi-antigens activate up to 2-5% of the T-cell population [241] to initiate a *recall* T-cell response and more closely model a true *in vivo* immune challenge; henceforth, specific-single- and specific-multi-antigens will be simply termed single- and multi-antigens.

Identifying the most appropriate immunogenic agent for use in culture stimulation protocols is difficult. Although specific-antigen stimulation of resting whole blood cultures may stratify illness risk within prospective cohort studies [17], their utility in acute exercise trials appears limited; the subtler stimulus of specific-antigens may not be sufficient to overcome the noise within the model to identify an effect of the exercise and/or nutrition intervention [27,28,32]. Nevertheless, this may be due to the type of specific-antigen as some exert a more potent stimulus on T-cells than others (e.g.

hepatitis). Superantigens, such as Staphylococcal enterotoxin B (SEB), can be used to investigate T-cell-driven immune response [242,243]. Staphylococcal enterotoxin B binds outside of the MHC class II and *nonspecifically* activates up to 20-25% of the T-cell population displaying the V β T-cell receptor [242,244]. This interaction triggers a pro-inflammatory cytokine response and the proliferation of T-cells bearing specific V β subgroups [242-244]. Therefore, in order to produce measurable differences in the T-cell cytokine response to exercise and diet interventions, a sufficient stimulus is warranted; however, the immunogenic agent does not mirror an *in vivo* infection and immune response and, therefore, extrapolating findings to illness and infection risk should be made with caution.

3.6 Strenuous exercise and circulating T-cell cytokine production

3.6.1 Interferon- γ and interleukin-4

3.6.1.1 Acute exercise

Prolonged, strenuous exercise typically reduces circulatory T-cells IFN- γ production during the early stages of recovery (Figure 3.4, p. 48); this has been demonstrated within numerous studies using stimulated PBMC and whole blood cultures [205]. Conversely, T-cell IL-4 production appears unaffected by acute exercise. Interestingly, the changes in circulatory type-1 or type-2 T-cell concentration do not necessarily parallel their capacity to produce their signature cytokines. Whilst some studies have demonstrated reductions in circulatory IFN- γ^+ T-cell concentration coinciding with reduced mitogen-stimulated IFN- γ production immediately post-exercise [24,25], others have reported an increased circulatory IFN- γ^+ T-cell concentration despite a reduction of mitogen-stimulated IFN- γ production [226]. This may be due to differences in cell trafficking patterns influenced by exercise intensity and duration; with exercise priming the mobilisation of T-cells that are known to preferentially produce IFN- γ , but have a lower capacity to produce IFN- γ . Nevertheless, it appears conclusive that prolonged, exhaustive exercise elicits a reduction in circulatory T-cell IFN- γ production during the initial hours of recovery.

The transcriptional factors T-bet and GATA-3 may help to explain changes in cytokine production. For example, following 1 h of cycling at 70% VO_{2max} in trained males

($\text{VO}_{2\text{max}} \sim 62 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), Tbet and GATA-3 mRNA expression increased in unstimulated PBMCs [245], suggesting an enhanced type-1 and type-2 immune response. Nevertheless, IFN- γ mRNA expression within PBMCs was not altered, meaning that Tbet had no effect on cytokine regulation (IL-4 mRNA expression was not measured) [245]. Another study showed unstimulated mRNA expression of Tbet was unaltered; whereas, GATA-3 mRNA expression was increased 10 days after completion of a marathon ($249 \pm 47 \text{ min}$), which coincided with an increase in IL-4 mRNA expression and no change to IFN- γ mRNA expression [246]. This latter finding corroborates the concept that strenuous exercise favours type-2 immunity. Nevertheless, these studies did not stimulate immune cells and, therefore, the findings are not indicative of a T-cell response to an immune challenge.

Studies using acute exercise protocols and whole blood multi-antigen stimulation models have often failed to produce significant findings for cytokine production [27,28,32]. For example, 75 min of cycling at 70% $\text{VO}_{2\text{max}}$ had no effect on whole blood multi-antigen-(DTPa-HBV-IPV/Hib vaccine) stimulated IFN- γ and IL-4 production post-exercise in trained cyclists [28]; albeit, IL-4 production 2 h post-exercise was significantly lower compared to pre-exercise levels. Similarly, IFN- γ and IL-4 production was unaltered following an incremental cycling test to exhaustion using the same stimulation model [27]. Whereas, immediately following 120 min cycling at 60% $\text{VO}_{2\text{max}}$, multi-antigen-stimulated whole blood production of IFN- γ declined, whilst IL-4 remained unaltered, which persisted 2 h into recovery [247]. These latter findings suggest a suppression in the type-1 T-cell immune response; however, as the number of IFN- γ^+ T-cells within each blood sample was not measured, it is difficult to conclusively state whether this was due to a decline in IFN- γ^+ T-cells and/or a reduction in their capacity to produce IFN- γ .

3.6.1.2 Training

Following several days of intensive training, the suppressive effect of strenuous exercise on mitogen-stimulated T-cell IFN- γ production persists without an alteration to IL-4 production [24]. Whereas, several days of intensified training does not appear to influence the effect of strenuous exercise on multi-antigen-stimulated, whole blood production of IFN- γ and IL-4 [27]. Furthermore, chronic, heavy training loads are associated with a higher resting multi-antigen-stimulated, whole-blood production of IL-4 and IFN- γ than

those with low training loads and were more likely to experience URS [17,26]. However, fatigued athletes undertaking heavy training have also shown a reduction in antigen-stimulated whole blood IFN- γ production [16]. This suggests heavy training can promote both type-1 and type-2 T-cell cytokine responses to an immune challenge, but there appears to be a preference for a type-2 T-cell response, which may increase the risk of infection and viral reactivation. Nevertheless, further research is required to confirm these effects.

3.6.2 Interleukin-10

3.6.2.1 Acute exercise

The influence of acute exercise on T-cell IL-10 production is equivocal (Figure 3.4, p. 48). In stimulated whole-blood and PBMC cultures, IL-10 production either increases [247-250], decreases [251,252] or does not change [27-29]. Furthermore, unstimulated FoxP3 mRNA expression did not change within PBMCs following 1 h of cycling at 70% $\text{VO}_{2\text{max}}$ [245]. It is difficult to compare these studies due to the different exercise protocols and analytical techniques.

3.6.2.2 Training

High compared to low training loads are associated with elevated multi-antigen-stimulated, whole blood IL-10 production [26,30]. At rest, this elevated capacity to produce IL-10 is associated with higher circulating TReg cell concentrations [30], elevated whole-blood, multi-antigen-stimulated IL-4 production [17], lower salivary immunoglobulin A secretion rate and concentration [17] and increased URS incidence during a protracted training period [17]. Altogether, it appears there is an impaired pro-inflammatory response to an immune challenge when engaging in high training loads, which preferentially suppresses a type-1 T-cell immune response and likely increases illness and infection risk.

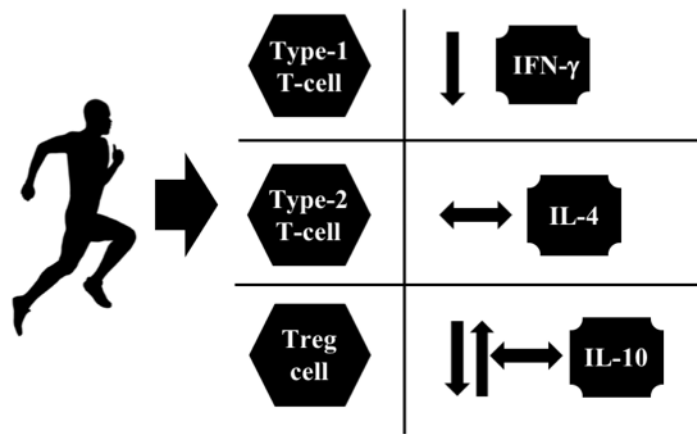


Figure 3.4. Changes in circulatory T-cell capacity to produce their characterising cytokines during the early stages of recovery following a prolonged, strenuous exercise bout. *TReg cell* Regulatory T-cell, *IFN-γ* Interferon-gamma, *IL-4* Interleukin-4, *IL-10* Interleukin-10.

3.7 Regulatory effects of stress hormones and immunometabolism on T-cell cytokine production

3.7.1 Glucocorticoids

The plasma concentrations of cortisol rise proportionately to exercise duration [253] and most markedly when exercise intensity is $>60\%$ $\text{VO}_{2\text{max}}$ [254]. Following periods of intensified training, the exercise-induced rise in cortisol is truncated [24]. Unlike catecholamines, which decline swiftly following exercise cessation, cortisol can exert its immunological effect over several hours [253].

The administration of hydrocortisone (synthetic cortisol) causes lymphopenia, which can be partly attributed to a decline in T-cells [255,256]. Nevertheless, this response only occurs when cortisol reached concentrations above those typically elicited by exercise (i.e. $\sim 1400 \text{ nmol}\cdot\text{L}^{-1}$) [255]. The effects of exercise-induced rises in cortisol on individual T-cell subset number is uncertain; however, it may explain some variation in CD4^{+} T-cell concentration [257]. In a group of seven moderately- to well-trained men ($\text{VO}_{2\text{max}}$, $\sim 4.5 \text{ L}\cdot\text{min}^{-1}$), carbohydrate intake during exercise ameliorated the exercise-induced rise in cortisol and attenuated the reduction in circulatory $\text{CD4}^{+}\text{IFN-}\gamma^{+}$ and $\text{CD8}^{+}\text{IFN-}\gamma^{+}$ cell concentration, and their $\text{IFN-}\gamma$ production following 2 h of cycling at 65% $\text{VO}_{2\text{max}}$ [25]; however, it remains uncertain if exercise-induced rises in circulating cortisol affects TReg cell number.

Additionally, *in vitro* models demonstrate that dexamethasone (a synthetic derivative of cortisol) [258-260] and supra-physiological concentrations of hydrocortisone [261] reduce the production of IL-12, a type-1 cytokine, by APCs. This can reduce the upregulation of IFN- γ and inhibition of T-cell IL-4 production; favouring a type-2 T-cell immune response. Dexamethasone also appears to directly reduce IL-12 receptors on T-cells and NK cells, which inhibits their ability to produce IFN- γ . However, the shift towards a type-2 T-cell response may be through reduced production of IL-12 by APCs [262]. Dexamethasone also upregulated IL-10 production by lymphocytes, but not monocytes [260,263]; in CD4⁺ T-cells, exposure to dexamethasone increased IL-4 production and IL-4, IL-10 and IL-12 mRNA expression [263]. Additionally, some evidence demonstrates equivocal effects on FoxP3 expression and TReg cell function following incubation with dexamethasone [264,265]. However, dexamethasone's immunological influence may differ compared to cortisol due to its greater anti-inflammatory effect, thus limiting direct comparisons. The effects of glucocorticoids on T-cell function do, however, appear to favour an anti-inflammatory T-cell cytokine response [266].

3.7.2 Catecholamines

The plasma concentrations of catecholamines of the sympathetic nervous system (SNS), adrenaline and noradrenaline, rise proportionately to exercise duration and exponentially with exercise intensity [253]. They exert their function largely via β -adrenoreceptors on leukocytes [253], with 28-61% of the exercise-induced mobilisation of lymphocytes suggested to be underpinned by β_2 -adrenoreceptor signalling [267].

The majority of studies demonstrate the effects of adrenaline and β -agonist (isoproterenol) infusion on CD4⁺ and CD8⁺ T-cells is equivocal [268]. Exercise-induced elevations in plasma adrenaline is positively associated with lymphocyte concentration, and may explain some variation in total T-cell and CD4⁺ and CD8⁺ T-cell concentration [257,269]. It has been speculated that this results from increased blood flow and mobilisation from the blood vessels of the marginalised pools [268]. However, the administration of a β -antagonist (propranolol) for a week prior to exercise reduced the exercise-induced increase in circulatory lymphocytes, CD4⁺ and CD8⁺ T-cells, without affecting the rise in catecholamines [270], suggesting an alternative or additional reason

underpinning lymphocytosis; speculatively, lymphocytes may also be released from the spleen via activation of β -adrenoceptors [271]. Periods of intensified training may also reduce lymphocyte β_2 -adrenoreceptor sensitivity, which may explain the attenuated mobilisation and redeployment of CD8⁺ and other cytotoxic T-cells [222].

In vitro models suggest glucocorticoids and catecholamines have a synergistic effect on T-cell cytokine production [266]. Adrenaline and, to a lesser extent, noradrenaline suppress IFN- γ production and enhance IL-4 and IL-10 production by PBMCs in a dose dependent manner [272]. Similarly, in whole blood cultures, adrenaline concentrations at 1 nmol·L⁻¹ reduced IL-12 production by ~50%, with 5 nmol·L⁻¹ reducing IL-12 to undetectable levels [260]. Conversely, adrenaline increased the production of IL-10 in dose dependent manner, particularly at levels above 1 nmol·L⁻¹ [260]. Not all studies suggest catecholamines influence T-cell cytokine production, which may be due to a dose response. Additionally, when six endurance trained men cycled for ~19 min at ~78% VO_{2max}, the intake of an α - and β -adrenoreceptor blockade abrogated the effect of only lymphocyte trafficking, not the magnitude of IFN- γ or IL-2 production by lymphocytes in mitogen-stimulated whole blood cultures [226], suggesting catecholamines do not influence cytokine production during exercise. TReg cell function is also mediated by activation of β_2 -adrenoreceptors in murine [273] and human [265] *in vitro* cultures, but exercise-specific effects are unclear. Altogether, it appears that catecholamines have a role in T-cell trafficking; however, their effect on cytokine production at physiological levels is uncertain.

3.7.3 Immunometabolism

T-cell activation, differentiation and function is an energy demanding process largely affected by the availability of energetic substrates [274-276]. Effector T-cells predominantly rely on anaerobic glycolysis to provide ATP irrespective of oxygen availability and exhibit high surface levels of glucose transporter, GLUT1 [277]. The stimulation of T-cells by injecting anti-CD3⁺ antibodies into mice has shown to reduce blood glucose levels, suggesting an upregulation of glucose utilisation following their activation [278]. Whereas, naïve T cells have lower glycolytic rates and GLUT-1 is undetected in unstimulated lymphocytes [277,279]. The reliance on glucose in effector T-cells is further demonstrated when its removal from the *in vitro* medium suppressed

CD8⁺ T-cell IFN- γ gene transcription [280,281]. In contrast to type-1 (and type-2) T-cells, decreased glucose and increased in FFA availability appears to favour TReg cell differentiation *in vivo* [277]. Albeit, it is uncertain if this effects stimulated IL-10 production. As an alternative fuel source, lymphocytes can produce [282] and utilise KBs [51-53], and influence the T-cell capacity to proliferate at high (nonphysiological) concentrations (16-32 mmol·L⁻¹ of β HB) [283]; whether this effects T-cell differentiation and cytokine production is unknown. Considering prolonged exercise can reduce blood glucose concentration and increase FFA and KB concentration, this may influence T-cell function within whole-blood cultures by negating a type-1 T-cell response.

3.8 Regulatory effects of carbohydrate availability and nutritional ketosis on circulating T-cell cytokine production

Nutrition is paramount to optimal immune function [284]. Nutrients, nonnutritive phytochemicals and functional foods interact with a variety of physiological states to modulate immune function, including exercise [285]. Ensuring nutritional adequacy and, in some instances, ingesting some nutrients above typical recommended daily intakes (e.g. quercetin) can support immune function in active populations and athletes [1,286,287]. Of these, macronutrient manipulation, particularly CHO intake and energy availability, is considered the most efficacious nutrition strategy to counter exercise-induced immunodepression [1,288].

3.8.1 Energy availability

Low-energy availability (LEA) is a primary risk factor for immunodepression and illness susceptibility in athletes [289]. Nevertheless, alterations to lymphocyte and T-cell trafficking and function from LEA have not been comprehensively investigated in athletes. In extreme cases, such as protein-energy malnutrition, detrimental effects pertain to almost all immune components, with T-cells a primary victim [290,291]. Protein-energy malnutrition results in the atrophy of primary and secondary lymphoid tissue and a reduction in circulating lymphocytes relative to the severity of malnutrition [292]. Whilst the extremity of malnutrition does not necessarily apply to athletes, some may engage in pathological dietary behaviours such as eating disorders or restrictive dietary behaviours, including vegetarianism, veganism, or fail to increase EI relative to rises in

training load, thus resulting in a transient or chronic state of LEA [293].

Within well-trained cadets [294], judo athletes [295] and recreationally active men [296], energy restriction and/or weight loss protocols have been associated with reduced circulating lymphocyte, CD4⁺ and CD8⁺ T-cell concentrations and the CD4⁺/CD8⁺ T-cell ratio. Further, 2 days of energy restriction in recreationally active men, reduced circulating T-cell concentration after a 30 min treadmill TT [296]. However, not all studies report a decline in circulatory T-cell concentration and the CD4/CD8 T-cell ratio with energy restriction [296]. Circulating lymphocyte concentration was also unaltered in a group of wrestlers during a 4 week weight loss period (>4% total body mass) [297]. Nonetheless, T-cell proliferation and anti-CD3⁺ antibody-stimulated PBMC IFN- γ production has been shown to decline during a 4 week weight loss period [297]. Therefore, LEA may reduce the concentrations of circulating concentrations of lymphocytes and T-cell subsets, as well as suppress a type-1 T-cell or pro-inflammatory cytokine response to an immune challenge, thus increasing the risk of viral infection and reactivation.

Possible mechanisms underpinning the effect of LEA on T-cell function are alterations to nutrient availability and hormones. Considering fasting (48 h) impairs glucose metabolism and uptake by T-cells [298], this may influence the capacity of the immune system to combat viral infection [299]. Further, very lean athletes may have lower levels of the adipokine, leptin [300,301], which supports T-cell activation and polarisation into type-1 T-cells by modulating intrinsic cell metabolism [302]. Stress hormones also link LEA and immunity. For example, cortisol was elevated in female athletes with LEA [293] and following seven days of caloric restriction in male Judokas [295]. However, whether elevated cortisol or alternative mechanisms derived from LEA/energy restriction effects lymphocyte number and function is uncertain [296].

3.8.2 Dietary carbohydrate and pre-exercise glycogen content

The effects of total dietary CHO and pre-exercise glycogen content on exercise-induced circulatory T-cell function has not been investigated. This is surprising given the influence of CHO availability on stress hormones and as a direct fuel source for T-cells. For example, 48 h of a low-CHO (0.5 g CHO·kg⁻¹) compared to high-CHO (8 g CHO·kg⁻¹) diet increased cortisol concentration, whilst reducing glucose, lactate and glutamine

concentration following a 60 min cycling bout at 70% $\text{VO}_{2\text{max}}$ [303], which would theoretically oppose a type-1 T-cell cytokine response to an immune challenge. In the same study, circulatory CD4^+ and CD8^+ T-cell concentrations were higher and lower immediately post- and 2 h post-exercise, respectively, in the low- compared to high-CHO condition; with lymphocytosis elevated immediately post-exercise and lymphopenia unaltered 2 h into recovery [303].

The amount of CHO within the hours prior to exercise, rather than the type, exerts more influence on the magnitude of exercise-induced lymphocytosis [304,305]. However, the ingestion of a low-CHO compared to higher-CHO diet (up to 3 days) and corresponding exacerbation in the rise of exercise-induced cortisol concentration did not invariably increase the exercise-induced lymphocytosis and lymphopenia conditions [306-308]. Similarly, circulatory T-cell CD4^+ and CD8^+ concentrations were unaltered following 7 weeks of training whilst ingesting a low-CHO, high-fat (21% EI from CHO and 62% EI from fat) diet compared with a high-CHO, low-fat (65% EI from CHO, 20% EI from fat) diet in untrained men [309]. Considering the potential benefits of training with low-CHO availability on subsequent endurance performance, further research examining the effect of low-CHO dietary strategies on the T-cell cytokine response following strenuous exercise seems prudent.

3.8.3 Carbohydrate intake during exercise

Carbohydrate supplementation during exercise attenuates perturbations to several immune components [1,288,310]. The primary mediating function of CHO supplementation during exercise is the maintenance of blood glucose and attenuating the rise in stress hormone concentration, particularly cortisol. In turn, the majority of studies have demonstrated a reduction in exercise-induced lymphocytosis and lymphopenia [288]. The effects of CHO supplementation on circulatory CD4^+ and CD8^+ T-cell number is less clear. The ingestion of a 200 ml of either a 6.4 or 12.8% CHO solution every 20 min during 150 min of cycling at 65% $\text{VO}_{2\text{max}}$ truncated the trafficking patterns of mitogen-stimulated $\text{CD4}^+\text{IFN-}\gamma^+$ and $\text{CD8}^+\text{IFN-}\gamma^+$ T-cells compared to placebo [25]. Furthermore, CHO (6.4% and 12.8% solutions) ingestion compared to placebo also mitigated the reduction in mitogen-stimulated CD4^+ and CD8^+ T-cell IFN- γ production; whereas, IL-4 production was unaltered [25]. In contrast, the ingestion of a high-

compared to low-CHO solution before (24 vs. 2.4 g CHO), during (60 vs. 20 g CHO·h⁻¹) and after (30 vs. 10 g CHO) each exercise bout throughout an 8 day intensified training period had no effect on resting or exercise-induced (~30 min incremental exercise test to exhaustion) perturbations to circulating concentrations of lymphocytes, CD4⁺, CD4⁺CD25⁺ and CD4⁺CD25⁺CD127^{low/-} T-cells or antigen-stimulated whole blood IL-4, IL-10 and IFN- γ production; despite total energy and CHO intake differing between dietary conditions (low-CHO, 14.7 \pm 2.7 vs. high-CHO, 17.4 \pm 3.2 MJ·day⁻¹; low-CHO, 505 \pm 107 vs. high-CHO, 679 \pm 105 g CHO·day⁻¹) [27]. This absence of effect, particularly for cytokine production, may have been due to cortisol concentration not sufficiently differing between trials.

3.8.4 Dietary fat

The effect of total dietary fat on exercise-induced circulatory T-cell concentration and function has not been investigated. This may be due to macronutrient interaction whereby increases in dietary fat simultaneously occur with a reduction in dietary CHO to maintain energy balance and, therefore, reduce CHO availability. Nevertheless, a 7 week high-fat, low-CHO (62% EI from fat, 21% EI from CHO) diet compared to an isoenergetic low-fat, high-CHO (65% EI from CHO, 20% EI from fat) diet, in conjunction with training, in a group of untrained men had no effect on resting T-cell and CD4⁺ or CD8⁺ T-cell subsets [309]. Further research on the effect of total dietary fat intake on immune function is required.

3.8.5 Nutritional ketosis

Ketone bodies appear to have a mediating role on immune function [42,311]; however, the interaction of nutritional ketosis and exercise is uncertain. From the perspective of substrate availability and metabolism, particularly CHO, it is likely that potential immune effects differ between ketone supplementation and keto-adaptation. Indeed, a 4 week KD exacerbated exercise-induced rises in cortisol concentration compared to a higher-CHO diet, despite no difference in plasma glucose concentration [312]. Whereas the coingestion of R-BD D- β HB monoester and CHO (plasma D- β HB concentration of 2.5-3.5 mmol·L⁻¹) did not affect plasma cortisol concentration, despite a reduction in plasma glucose concentration [73]. As such, the increase in cortisol following a KD and not

ketone supplementation may be underpinned by an increase in skeletal muscle IL-6 production due to reduced glycogen content [313] and/or lower blood glucose uptake [314]. The increase in IL-6 can signal to increase circulating cortisol concentration [315]. For example, a 3 week KD, compared to high-CHO diet, increased serum IL-6 concentration after 2 h of walking at $\sim 75\%$ $\text{VO}_{2\text{max}}$ in elite race walkers [316], which was confirmed in a subsequent trial using an almost identical protocol, albeit the inclusion of CHO ingestion 2 h prior and during the exercise trial [317]. Therefore, KDs may preferentially suppress a type-1 T-cell cytokine response to an immune challenge due to higher exercise-induced cortisol concentrations, whereas the effects of ketone supplements ingested in isolation are unknown.

The constituents of the *in vitro* culture medium may modulate T-cell cytokine production. Availability of energetic substrate, particularly glucose, and glucose metabolism are integral for the production of IFN- γ (see section 3.7.3). When exercising in a fasted state at submaximal intensities ($\leq 70\%$ $\text{VO}_{2\text{max}}$), prior adaptation to a KD can maintain blood glucose concentration [15,35]; however, the pre-ingestion of a high-CHO meal (2 g $\text{CHO}\cdot\text{kg}^{-1}$) and $\sim 60\text{ g}\cdot\text{h}^{-1}$ during exercise at 80% $\text{VO}_{2\text{max}}$ has been shown to increase blood glucose concentrations above a keto-adapted, fat-fed condition [14]. Furthermore, R-BD D- β HB monoester ingestion can reduce plasma glucose concentration during exercise [73], which is potentially due to a suppressive effect on hepatic glycogenolysis and/or greater glucose uptake by skeletal muscle [33,34]. Ketone bodies have also been shown to reduce glycolytic rate in neurons and cancerous pancreatic cells [43], and may exert similar effects on immune cells. Therefore, ketone supplementation and the ingestion of a KD reduce blood glucose concentration, thus lowering glucose availability within an *ex vivo* whole blood culture, and may inhibit glycolysis; theoretically, this would negate a type-1 T-cell cytokine response to an immune challenge.

As an alternative fuel source, lymphocytes can produce [282] and utilise KBs [51-53]. Whether KBs are preferentially used by type-1, type-2 or regulatory T-cells and their influence on cytokine production is unknown. β HB is also linked to immune regulation via the NLRP3 inflammasome and histone deacetylases [72,318,319]. The effects of β HB on NLRP3 inflammasome activation and monocyte production of the pro-inflammatory cytokine, IL-1 β , is conflicting [72,318]. If NLRP3 activation is suppressed, it may prevent a pro-inflammatory type-1 T-cell immune response by inhibiting the production

of IFN- γ [320]. Nevertheless, the influence of NLRP3 on infection risk is uncertain [321]. D- β HB can also act as a histone deacetylase (HDAC) inhibitor [319]. HDAC inhibitors function via various mechanisms, including the suppression of NF- κ B, similar to the action of glucocorticoids, and preferentially suppress the production of pro-inflammatory T-cell cytokines and augment TReg cell differentiation [322]. However, like NLRP3, the effect of HDAC inhibitors on T-cell function is uncertain [322,323].

Collectively, the interaction of prolonged, strenuous exercise and hyperketonaemia, either via ketone supplementation or the ingestion of a KD, appears to oppose a type-1 T-cell (IFN- γ^+) cytokine response to an immune challenge. This will shift the balance towards a type-2 T-cell (IL-4 $^+$) and TReg cell (IL-10 $^+$) cytokine response to an immune challenge. Therefore, immune protection against invading and incumbent pathogens, particularly of viral origin, may be compromised during nutritional ketosis.

3.9 Conclusion and thesis hypotheses

The body of knowledge on stimulated T-cell cytokine production in response to prolonged, strenuous exercise provides mechanistic insight into post-exercise immunodepression and illness risk. This immune marker appears to be sensitive to dietary interventions, particularly the interaction of low- vs high-CHO availability and exercise [24,25] and can provide clinically meaningful outcomes [16,17]. However, less is known about the gene expression of key T-cell cytokines, thus warranting further research. Moreover, reductions in CHO availability exacerbate exercise-induced elevations in cortisol concentration, which preferentially suppresses T-cell IFN- γ production. It is uncertain whether ketone supplementation could reduce CHO availability, particularly blood glucose concentration, to a level eliciting similar effects as dietary CHO restriction. Further, whilst KBs can be used as an energy source by lymphocytes, it is unknown whether their oxidation would compensate for reductions in CHO availability or favour the production of different cytokines.

It is, therefore, hypothesised:

- BD ingestion would negate T-cell IFN- γ , but not IL-4 and IL-10, gene expression within stimulated PBMCs at rest by reducing blood glucose concentration and, potentially, T-cell glycolysis.

- BD ingestion would impair T-cell IFN- γ , but not IL-4 and IL-10, gene expression within stimulated PBMCs following prolonged, strenuous exercise by reducing blood glucose concentration and increasing blood cortisol concentration.
- Conformity to a KD diet would impair T-cell IFN- γ , but not IL-4 and IL-10, gene expression within stimulated PBMCs following submaximal intensity, exhaustive exercise by reducing blood glucose concentration and increasing blood cortisol concentration.

In order to determine the optimal dose of BD in Chapters 5 and 6, an initial study was conducted to examine the dose response effect of BD on blood D- β HB concentration and T-cell IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs (Chapter 4). The optimal dose was required to elicit maximal blood D- β HB concentrations and have little to no effect on stimulated PBMC cytokine gene expression at rest.

The dose response effect of 1,3-butanediol on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells at rest

4.1 Abstract

This study investigated the dose response effect of the racemic ketogenic supplement, BD, on T-cell related IL-4, IL-10 and IFN- γ gene expression within stimulated PBMCs. A repeated-measures, single-blind, randomised, crossover study was conducted on six healthy, recreationally active men (age, 27.2 ± 2.7 y; body mass, 80.2 ± 10.0 kg; BMI, 23.7 ± 1.7 kg·m⁻²). Participants ingested three different doses of BD ($0.5 + 0$ g·kg⁻¹; $0.7 + 0$ g·kg⁻¹; $0.35 + 0.35$ g·kg⁻¹ BD) or placebo ($0 + 0$ g·kg⁻¹ BD), with each bolus separated by 1.5 h. Blood samples were collected at pre-supplement and 0.5, 2, 2.5 and 3.5 h after the initial bolus for analysis of D- β HB, glucose and cortisol concentration, total lymphocyte, CD4⁺, CD8⁺ and CD25⁺ T-cells, and monocyte concentration. Whole blood cultures were stimulated with SEB for 24 h to determine T-cell related IL-4, IL-10 and IFN- γ mRNA expression within isolated PBMCs *in vitro*. All doses of BD significantly increased D- β HB concentrations at 0.5, 2, 2.5 and 3.5 h (~ 0.6 - 1.0 mmol·L⁻¹) compared to placebo (all $p < 0.05$). BD had no interaction or main effects on T-cell related cytokine mRNA expression (all $p > 0.05$). In conclusion, BD ingestion does not alter *in vitro* T-cell related IL-4, IL-10 and IFN- γ gene expression to an immune challenge at rest.

4.2 Introduction

The effect of nutritional ketosis on immune function and resistance against infection is unknown. T-cells of the adaptive immune system are critical for orchestrating an immune response to invading and incumbent pathogens via the release of cytokines, particularly IL-4, IL-10 and IFN- γ [20,212]. Their function is intricately linked with immunometabolism and the availability of energetic substrate [274-276]. Following activation, stimulated effector T-cells increase their glycolytic rate and demand for glucose [274-276]. If the *in vitro* medium does not contain glucose or glycolysis is inhibited, stimulated CD8⁺ T-cell IFN- γ gene expression and production has been shown to decline [280,281]. As an alternative fuel source, lymphocytes can produce [282] and utilise KBs [51-53]; however, it is unknown if this influences T-cell cytokine production. In consideration of the therapeutic roles of KBs [65], it is important to investigate their effect on immune cells to elucidate their potential clinical applications.

When modelling an immune response to a pathogenic challenge using whole blood cultures *ex vivo*, acute dietary strategies that alter blood substrate concentrations or modulate glycolysis may influence stimulated T-cell function. For example, ketone supplementation can lower blood glucose concentration by increasing pancreatic insulin secretion [13,72] and inhibiting hepatic glycogenolysis [33,34]. Moreover, KBs can also reduce the glycolytic rate in neurons and cancerous pancreatic cells [43], and may exert similar effects on T-cells following stimulation. Therefore, if CHO availability and metabolism is suppressed by ketone supplementation, it is possible that T-cell function and the expression of their key immunomodulating cytokines, particularly IFN- γ , is altered following stimulation *in vitro*.

The racemic ketogenic supplement, BD, is a nontoxic dialcohol and is rapidly converted to β HB following ingestion. In humans, ingesting 0.25 g BD·kg⁻¹ can increase blood D- β HB concentrations to ~0.8 mmol·L⁻¹ within 30 min [90]. The R- and S- isomers of BD primarily undergo hepatic conversion to D- and L- β HB by the rate limiting enzymes, alcohol dehydrogenase and aldehyde dehydrogenase [82-85]; therefore, there may be an upper limit to BD ingestion. Excessive doses will result in enzymatic saturation and fail to further increase blood D- β HB concentration, thus resulting in blood BD accumulation [88]. However, pharmacokinetic data for BD ingestion and dose response effect on immune function are yet to be investigated. In concordance, the present study investigated

the dose response of BD on blood D- β HB concentrations and *in vitro* T-cell related IL-4, IL-10 and IFN- γ gene expression within SEB-stimulated PBMCs during 3.5 h of rest.

4.3 Methods

4.3.1 Participants

Six healthy, recreationally active males (age, 27.2 ± 2.7 y; body mass, 80.2 ± 10.0 kg; height, 1.84 ± 0.06 m; BMI, 23.7 ± 1.7 kg·m⁻²; sum of 8 (Σ 8) skinfolds, 67.0 ± 19.5 mm; weekly alcohol intake, 5.2 ± 3.7 standard units) volunteered to participate in the study. All participants were informed of the rationale of the study, the experimental procedures and possible risks before providing their written consent (Appendix C). The study was approved by the Health and Disability Ethics Committee (Wellington, New Zealand) (reference number 17/NTB/39/AM02) and prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617000731392).

Participants completed a comprehensive health and eligibility screening questionnaire (Appendix D) to ascertain if they were suitable for the study. Participants were eligible to participate if they were male, free from illness, between 18 and 35 years, nonsmokers, consumed a mixed diet and were recreationally active. Participants were excluded if they possessed cardiovascular, metabolic, neurological or immunological disorders, abnormal red blood cell or differential leukocyte counts, had taken medications or supplements known to effect immune function in the 2 weeks prior to the study, had gained or lost >2% of total body mass or had experienced illness-related symptoms in the 4 weeks prior to the study.

4.3.2 Experimental trials

Using a repeated-measures, single-blind, randomised, crossover design, participants were randomly (www.randomizer.org) assigned to either a placebo ($0 + 0$ g·kg⁻¹; PLA), medium-dose ($0.5 + 0$ g·kg⁻¹; M-BD), high-dose ($0.7 + 0$ g·kg⁻¹; H-BD) or split-dose ($0.35 + 0.35$ g·kg⁻¹; Spl-BD) of the racemic BD (proportional distribution of optical enantiomers unknown) (product code 02-59620; Penta Manufacturing Ltd, Livingston, USA), with boluses separated by 1.5 h. To standardise nutritional and physiological

status, participants were required to complete a 24 h diet record the day before the first main trial and were asked to repeat this during the 24 h preceding each subsequent trial. Additionally, participants were asked to refrain from strenuous exercise, caffeine and alcohol in the 24 h preceding each trial, and report any illness-related symptoms.

Participants arrived at the laboratory between 06:00 and 08:00 h following an overnight fast. An indwelling intravenous teflon catheter (18G, Terumo, Japan) was then inserted into the antecubital vein for serial blood sampling. After 10 min of resting, an initial (pre-supplement) blood sample was obtained, immediately followed by the ingestion of a bolus of 0, 0.5, 0.7 or 0.35 g·kg⁻¹ of BD within a 200 ml nutrient free, artificially sweetened flavoured orange drink (Thriftee, Hansells Food Group Ltd, New Zealand). Participants then rested quietly in the laboratory for 3.5 h, during which time water was consumed *ad libitum*. Participants ingested their second bolus of PLA or BD according to their trial allocation 1.5 h following their first bolus. Further blood samples were obtained 0.5, 2, 2.5 and 3.5 h after the first bolus to coincide with the time points during the exercise trials in Chapters 5 and 6 (i.e. pre-exercise, post-SS, post-TT, 1-h post-TT).

At each time point, blood was drawn into 1 x 8 ml serum and 2 x 6 ml dipotassium ethylenediamine tetra-acetic acid (K₂EDTA) vacutainers (Becton Dickinson and Co, USA) and capillary blood D-βHB concentration was measured (Freestyle Optium Neo, Abbott Diabetes Care, Victoria, Australia) using standardised techniques with the participant in a seated upright position. To identify the dose of BD eliciting maximal blood D-βHB concentrations, blood D-βHB concentration was also measured at 1 h and 1.5 h in order to more accurately calculate area under the curve (AUC) for blood D-βHB concentration between 0.5 and 2.5 h. To avoid dilution of the blood samples, the initial 3-5 ml was discarded prior to collection. Following each blood sample, and at regular 20-30 min intervals, the catheter was flushed with 3-5 ml saline (0.9% NaCl). Serum vacutainers were left to clot for 30 min at room temperature before centrifugation at 1500 g for 10 min at 4 °C. After which, isolated serum was stored at -80 °C prior to analysis of glucose and cortisol (Cobas Modular P800 Analyser, Roche Diagnostics, New Zealand).

4.3.3 Stimulated whole blood cultures

At the end of each trial, K₂EDTA treated whole blood was used for stimulated whole blood cultures in a nontreated, sterile tissue culture plate (Guangzhou Jet Bio-Filtration Co. Ltd, China) as follows: for each trial, 4 µL of 1 mg·ml⁻¹ SEB from *Staphylococcus aureus* (Merck KGaA, Darmstadt, Germany) was added to 396 µl RPMI (Thermo Fischer Scientific, USA) to give a working concentration of 10 µg·ml⁻¹, of which, 40 µL was added to 3960 µL of whole blood for each sample, giving a final stimulant concentration of 100 ng·ml⁻¹. The whole blood culture was incubated for 24 h at 37 °C and 5% CO₂ (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

4.3.4 Isolation of peripheral blood mononuclear cells

After 24 h, stimulated whole blood was diluted in a 1:1 ratio with phosphate-buffered saline (PBS) and foetal bovine serum (FBS) (2%). The diluted blood was added to SepMate tubes containing density gradient medium (Lymphoprep; density of 1.077 g·ml⁻¹) and centrifuged at 1200 g for 20 min with the brake on at room temperature according to the manufacturer's instructions (StemCell Technologies, Melbourne, Australia). The top layer was poured into a 15 ml polypropylene tube to collect the PBMCs, which were washed twice with PBS and FBS and centrifuged at 300 g for 8 min with the brake on. This is a well-documented and standardised method to collect PBMCs with high purity [324]. The supernatant was discarded and the resultant cell pellet was resuspended in 100 µL of PBS and 100 µL of Lysis/Binding buffer (MagNA Pure LC Isolation Kit – High Performance, Roche Life Science, New Zealand), then stored in 200 µL aliquots at -80 °C prior to isolation of total RNA.

4.3.5 Analysis of mRNA expression in peripheral blood mononuclear cells

Total RNA was extracted from each PBMC sample with the Roche MagNA Pure LC instrument with the MagNA Pure LC RNA isolation kit – High Performance (Roche Life Science, New Zealand). The elution volume was 50 µL, which was stored at -80 °C prior to analysis of gene expression. Relative quantification of gene expression by one step RT-PCR was performed on the LightCycler 480 instrument II (Roche Diagnostics, Auckland, New Zealand) using the LightCycler® EvoScript RNA SYBR® Green I Master (Roche

Diagnostics, Auckland, New Zealand), primers (Integrated DNA Technologies, Inc., Singapore) at a working concentration of 0.9 $\mu\text{mol}\cdot\text{L}^{-1}$ and RNA in a final volume of 20 μL . There was no need for adjustment of magnesium as the master mix was optimised with a fixed concentration of $\text{Mg}(\text{OAc})_2$. The reactions were performed using the following thermocycling conditions; 60 °C for 15 min (reverse transcription/cDNA synthesis), 95 °C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95 °C for 15 sec for denaturation and 60 °C for 1 min for annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melt analyses in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). The housekeeping gene used for mRNA expression analysis was β_2 -microglobulin (β_2 -MG) due to its stability within mitogen-stimulated PBMCs [325]. All primer sequences were acquired from previously published sources [325] and can be referred to in Table 4.1. The relative gene expression levels were calculated using the comparative Ct ($\Delta\Delta\text{Ct}$) equation [326], where the relative expression was calculated as $2^{-\Delta\Delta\text{Ct}}$ and expressed as a fold-change. mRNA for all target genes were normalised to the reference gene (β_2 -MG) within the same participant, trial and time point, and to a calibrator of 0.5 h Ct values of the PLA trial within the same participant.

Table 4.1. Primer sequences used for PBMC cytokine gene expression.

Gene Name		5'-3' primer sequence
IL-4	FW	AACAGCCTCACAGAGCAGAAGAC
	RW	GCCCTGCAGAAGGTTTCCTT
IL-10	FW	GCTGGAGGACTTTAAGGGTTACCT
	RW	CTTGATGTCTGGGTCTTGGTTCT
IFN- γ	FW	GTTTTGGGTTCTCTTGGCTGTTA
	RW	AAAAGAGTTCCATTATCCGCTACATC
β_2 -MG	FW	AATTGAAAAAGTGGAGCATTCAGA
	RW	GGCTGTGACAAAGTCACATGGTT

FW *forward primer*, RW *reverse primer*, IL-4 *interleukin-4*, IL-10 *interleukin-10*, IFN- γ *interferon- γ* , β_2 -MG *β_2 -microglobulin*. Primer sequences were acquired from published sources [325].

4.3.6 Lymphocytes, monocytes and T-cell subsets

K₂EDTA treated whole blood was used to determine total circulating leukocyte and differential cell concentration (Sysmex XT-2000i Automated Haematology Analyzer, Sysmex Corporation, Auckland, New Zealand) and CD4⁺, CD8⁺ and CD25⁺ lymphocyte subset percentage of total lymphocytes (Muse Cell Analyzer, Merck Millipore, Abacus Dx, New Zealand). The Muse Cell Analyzer features miniaturised fluorescence detection and microcapillary technology for rapid, accurate and reliable quantitative cell analysis. All kits were used according to the manufacturer's instructions for processing whole blood. Cell concentrations for CD4⁺, CD8⁺ and CD25⁺ lymphocytes were calculated by multiplying the percentage of these cells with the total circulating lymphocyte cell concentration.

4.3.7 Statistical analysis

All data are expressed as mean \pm standard deviation (SD) unless otherwise stated. Data were checked for normality as indicated by the Shapiro-Wilk score and, where appropriate, statistical analysis was performed on the logarithmic transformation of the data (i.e. D- β HB, glucose and cortisol concentrations; CD4⁺, CD8⁺, CD25⁺ T-cell subset concentrations; lymphocytes and monocyte concentrations; and IL-4, IL-10, IFN- γ mRNA expression and the IFN- γ /IL-4 mRNA expression ratio). A two-way (trial x time) repeated-measures ANOVA was performed (IBM SPSS Statistics software version 21, IBM Corp.) and if Mauchly's test of sphericity was violated, adjustments to the degrees of freedom were made for the ANOVA using Greenhouse-Geisser ϵ . Where a significant effect was observed, post hoc analysis was conducted using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons applied to the unadjusted *p* value to locate specific differences. Significance level was accepted at an alpha of $p < 0.05$.

4.4 Results

4.4.1 D- β HB, glucose and cortisol

Pre-supplement blood D- β HB concentrations, serum glucose concentration and serum cortisol concentrations did not differ between trials (all $p > 0.05$) (Table 4.2, p. 66). There

was a significant trial x time interaction for blood D-βHB concentration ($p < 0.001$), with elevations at 0.5, 2, 2.5 and 3.5 h in M-BD, H-BD and Spl-BD trials compared to PLA (all $p < 0.01$) (Table 4.2). However, no differences were observed at these time points between the M-BD, H-BD and Spl-BD trials (all $p > 0.05$). Nor were there differences in AUC for blood D-βHB concentration between 0.5 and 2.5 h (M-BD, 87.50 ± 9.57 ; H-BD, 105.75 ± 24.75 ; Spl-BD, 92.25 ± 21.27 ; all $p > 0.05$). Peak D-βHB concentrations did not differ between the M-BD, H-BD or Spl-BD trials (all $p > 0.05$). There was no trial x time interaction ($p = 0.07$) or main effects of trial ($p = 0.07$) or time ($p = 0.20$) for serum glucose concentration. There was no trial x time interaction ($p = 0.11$) or main effect of trial ($p = 0.07$) on serum cortisol concentration. However, there was an effect of time on serum cortisol concentration ($p < 0.001$), with lower concentrations at 0.5, 2, 2.5 and 3.5 h compared to pre-supplement (all $p < 0.01$) and lower concentrations at 2, 2.5 and 3.5 h compared to 0.5 h (all $p < 0.001$) (Table 4.2).

Table 4.2. Capillary blood D-βHB and serum glucose and cortisol concentrations during the PLA, M-BD, H-BD and Spl-BD trials.

	Pre-supplement	0.5 h	2 h	2.5 h	3.5 h
<i>Capillary blood D-βHB concentration (mmol·L⁻¹)</i>					
PLA	0.17 ± 0.05	0.17 ± 0.05	0.18 ± 0.04	0.17 ± 0.05	0.17 ± 0.08
M-BD	0.13 ± 0.05	0.57 ± 0.04*	0.83 ± 0.15*	0.80 ± 0.09*	0.82 ± 0.15*
H-BD	0.15 ± 0.05	0.65 ± 0.16*	0.98 ± 0.29*	1.03 ± 0.27*	1.02 ± 0.26*
Spl-BD	0.15 ± 0.05	0.55 ± 0.18*	0.85 ± 0.29*	0.97 ± 0.27*	1.02 ± 0.35*
<i>Serum glucose (mmol·L⁻¹)</i>					
PLA	4.58 ± 0.44	4.70 ± 0.55	4.72 ± 0.35	4.83 ± 0.34	4.98 ± 0.24
M-BD	4.50 ± 0.41	4.45 ± 0.36	4.42 ± 0.28	4.30 ± 0.32	4.52 ± 0.29
H-BD	4.63 ± 0.54	4.67 ± 0.58	4.07 ± 0.48	4.33 ± 0.58	4.28 ± 0.31
Spl-BD	4.87 ± 0.53	4.48 ± 0.56	4.52 ± 0.42	4.40 ± 0.31	4.52 ± 0.19
<i>Serum cortisol (nmol·L⁻¹ ^{a,b})</i>					
PLA	439 ± 65	397 ± 93	319 ± 133	352 ± 127	291 ± 86
M-BD	437 ± 147	370 ± 171	294 ± 90	271 ± 67	210 ± 35
H-BD	423 ± 100	338 ± 88	207 ± 79	249 ± 99	243 ± 79
Spl-BD	400 ± 135	380 ± 179	277 ± 121	238 ± 118	178 ± 42

Values are presented as mean ± SD. PLA *placebo*, M-BD *medium dose*, H-BD *high dose* and Spl-BD *split dose* of R,S-1,3-butanediol. Significantly higher compared to PLA for time point (* $p < 0.01$). Main effect for time; significantly lower at 0.5, 2, 2.5 and 3.5 h compared to pre-supplement (^a $p < 0.001$). Main effect for time; significantly lower at 2, 2.5 and 3.5 h compared to 0.5 h (^b $p < 0.001$).

4.4.2 Total circulating lymphocytes, monocytes and T-cell subsets

No significant interaction or main effects were observed for total circulating lymphocytes, CD4⁺ or CD25⁺ T-cells (all $p > 0.05$) (Table 4.3, p. 68). However, there was an effect of time for circulating CD8⁺ T-cell concentration ($p = 0.026$) and monocyte concentration ($p = 0.001$). Circulating monocyte concentration was significantly lower at 2 h ($p = 0.003$) and 2.5 h ($p < 0.001$) compared to 0.5 h, and lower at 3.5 h compared to 2 h ($p = 0.01$). CD8⁺ T-cell concentration was lower at 3.5 h compared to pre-supplement ($p = 0.02$), and lower at 2.5 ($p = 0.04$) and 3.5 h ($p = 0.02$) compared to 0.5 h (Table 4.3, p. 68).

Table 4.3. Total circulating lymphocyte, monocyte and CD4⁺, CD8⁺ and CD25⁺ T-cell subset concentrations during the PLA, M-BD, H-BD and Spl-BD trials.

	Pre-supplement	0.5 h	2 h	2.5 h	3.5 h
<i>Total lymphocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i>					
PLA	2.18 \pm 0.34	2.06 \pm 0.36	1.94 \pm 0.45	1.96 \pm 0.59	2.09 \pm 0.66
M-BD	2.07 \pm 0.32	1.99 \pm 0.39	1.89 \pm 0.53	1.79 \pm 0.42	1.75 \pm 0.32
H-BD	1.96 \pm 0.18	1.94 \pm 0.37	1.91 \pm 0.31	2.02 \pm 0.27	1.88 \pm 0.50
Spl-BD	2.18 \pm 0.65	1.94 \pm 0.34	1.89 \pm 0.36	1.77 \pm 0.33	1.95 \pm 0.39
<i>Monocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)^{a,c}</i>					
PLA	0.44 \pm 0.10	0.52 \pm 0.09	0.45 \pm 0.09	0.47 \pm 0.10	0.44 \pm 0.09
M-BD	0.52 \pm 0.16	0.50 \pm 0.09	0.47 \pm 0.12	0.44 \pm 0.13	0.42 \pm 0.10
H-BD	0.47 \pm 0.07	0.52 \pm 0.09	0.48 \pm 0.12	0.45 \pm 0.12	0.41 \pm 0.08
Spl-BD	0.51 \pm 0.08	0.55 \pm 0.14	0.52 \pm 0.10	0.45 \pm 0.06	0.50 \pm 0.12
<i>CD4⁺ cells ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i>					
PLA	1.00 \pm 0.41	0.96 \pm 0.40	0.88 \pm 0.33	0.91 \pm 0.31	0.88 \pm 0.29
M-BD	0.94 \pm 0.33	0.88 \pm 0.24	0.82 \pm 0.24	0.82 \pm 0.17	0.83 \pm 0.25
H-BD	0.92 \pm 0.31	0.88 \pm 0.24	0.82 \pm 0.24	0.82 \pm 0.17	0.83 \pm 0.25
Spl-BD	1.17 \pm 0.25	0.99 \pm 0.20	0.91 \pm 0.09	0.92 \pm 0.12	0.99 \pm 0.13
<i>CD8⁺ cells ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)^{b,d}</i>					
PLA	0.59 \pm 0.22	0.55 \pm 0.22	0.50 \pm 0.20	0.50 \pm 0.22	0.52 \pm 0.31
M-BD	0.63 \pm 0.25	0.61 \pm 0.30	0.52 \pm 0.27	0.52 \pm 0.30	0.48 \pm 0.22
H-BD	0.51 \pm 0.21	0.53 \pm 0.26	0.51 \pm 0.24	0.51 \pm 0.28	0.47 \pm 0.28
Spl-BD	0.55 \pm 0.16	0.52 \pm 0.19	0.48 \pm 0.19	0.52 \pm 0.26	0.50 \pm 0.20
<i>CD25⁺ cells ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i>					
PLA	0.20 \pm 0.10	0.19 \pm 0.09	0.18 \pm 0.12	0.19 \pm 0.12	0.20 \pm 0.12
M-BD	0.16 \pm 0.03	0.18 \pm 0.05	0.17 \pm 0.04	0.16 \pm 0.05	0.16 \pm 0.05
H-BD	0.16 \pm 0.06	0.15 \pm 0.05	0.15 \pm 0.06	0.17 \pm 0.06	0.15 \pm 0.07
Spl-BD	0.22 \pm 0.14	0.19 \pm 0.08	0.20 \pm 0.09	0.18 \pm 0.09	0.20 \pm 0.10

Values are presented as mean \pm SD. PLA *placebo*, M-BD *medium dose*, H-BD *high dose* and Spl-BD *split dose* of R,S-1,3-butanediol. Main effect for time; significantly lower at 2.5 and 3.5 h compared to 0.5 h (^a $p < 0.01$; ^b $p < 0.05$). Main effect for time; significantly lower at 3.5 h compared to 2 h (^c $p = 0.01$). Main effect for time; significantly lower at 3.5 h compared to pre supplement (^d $p < 0.05$).

4.4.3 T-cell related cytokine mRNA expression within SEB-stimulated peripheral blood mononuclear cells

Quantifiable mRNA expressions of PBMC β_2 -MG, IFN- γ , IL-4 and IL-10 were detected in all SEB-stimulated whole blood samples. No significant interaction or main effects were observed for IL-4, IL-10 or IFN- γ mRNA expression (all $p > 0.05$), except for a main effect for time on IL-10 ($p = 0.002$). However, post hoc analysis could only locate a trend towards a reduction in IL-10 mRNA expression at 3.5 h compared to 2 h ($p = 0.06$) (Figure 4.1).

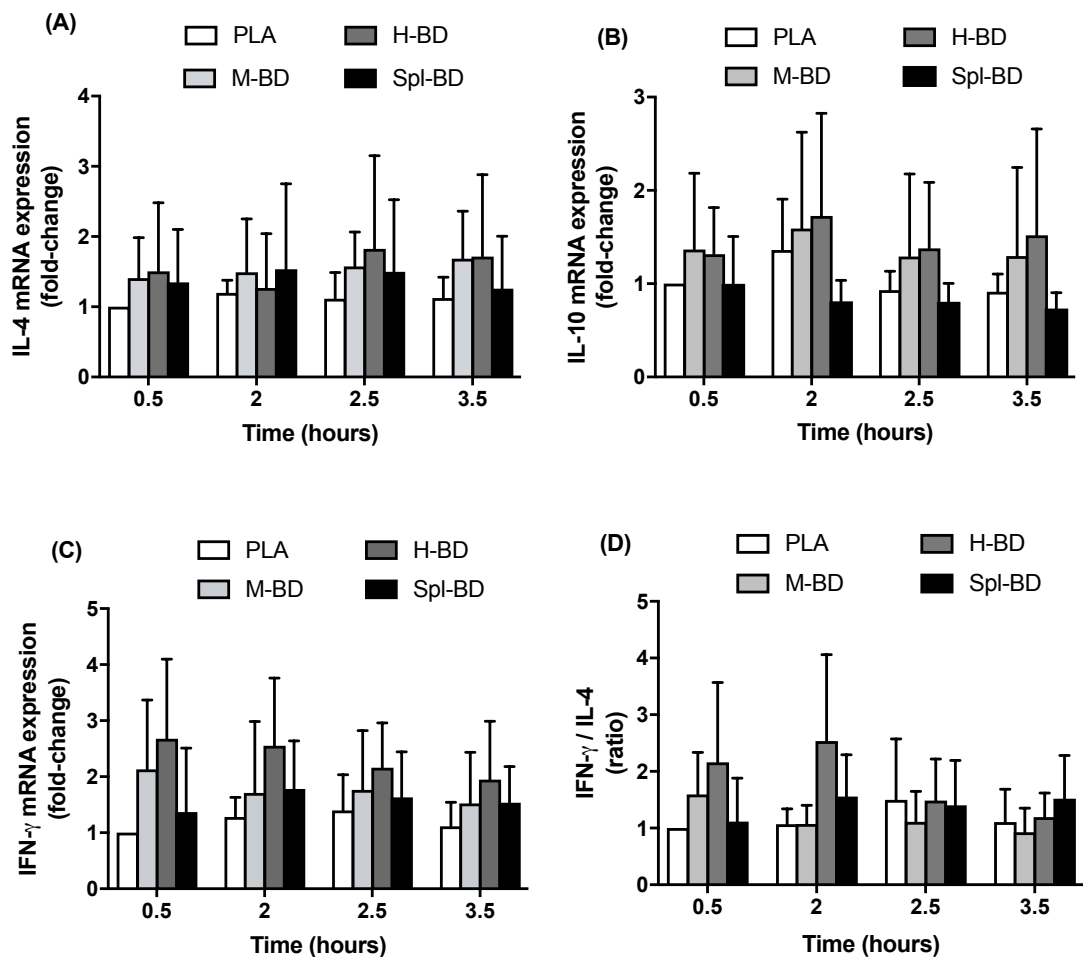


Figure 4.1. T-cell related cytokine mRNA expression within PBMCs following 24 h whole blood SEB-stimulation for (A) IL-4, (B) IL-10, (C) IFN- γ and (d) IFN- γ /IL-4 ratio expressed as fold change during the PLA, M-BD, H-BD and Spl-BD trials. Values are presented as mean + SD. PLA placebo, M-BD medium dose, H-BD high dose and Spl-BD split dose of R,S-1,3-butanediol.

4.5 Discussion

The present study investigated the effects of BD ingestion on blood D- β HB concentration and gene expression of the key immunomodulating T-cell cytokines, IL-4, IL-10 and IFN- γ , within SEB-stimulated PBMCs. Blood D- β HB concentration increased to ~ 0.6 mmol·L⁻¹ after 0.5 h, with peak concentrations of ~ 1 mmol·L⁻¹ occurring in all trials between 2 and 3.5 h. However, there were no differences in blood D- β HB or serum glucose and cortisol concentrations between the M-BD, H-BD and Spl-BD trials. Despite elevated blood D- β HB concentrations, T-cell related IL-4, IL-10 and IFN- γ gene expression and the IFN- γ /IL-4 ratio did not differ compared to placebo. These findings suggest acute increases in blood D- β HB concentration up to ~ 1 mmol·L⁻¹ do not alter the initiation of an inflammatory T-cell cytokine response to an immune challenge *in vitro* at rest.

In the present study, blood D- β HB concentrations may have been insufficient to exert a meaningful effect on metabolism and immune function. For example, BD ingestion did not alter serum glucose concentration, despite blood D- β HB concentrations mirroring racemic ketone salt ingestion (~ 1 mmol·L⁻¹), which have been shown to increase insulin secretion and reduce blood glucose concentration [13]. Therefore, differences in substrate metabolism (i.e. BD conversion to β HB) or increased L- β HB (via conversion of S-BD) may have interfered with the suppressive action of KBs on CHO metabolism [92]. Nonetheless, whilst glucose availability and metabolism are critical for the T-cell IFN- γ production *in vitro* [280,281], it may not be possible for ketone supplements to reduce blood glucose concentration to levels that affect stimulated T-cell cytokine production. Therefore, KBs may influence immune function via mechanisms other than via modulating glucose metabolism.

Ketone bodies may directly provide an energetic substrate for stimulated T-cells [51-53]; however, this is only speculative as the role - if any - of KB oxidation in T-cell function and stimulated cytokine production of IL-4, IL-10 and IFN- γ is unknown. Nevertheless, as T-cell subsets can favour the oxidation of specific substrates, such as cytotoxic T-cells (IFN- γ producers) and glucose [280,281] or TReg cells (IL-10 producers) and FFA [277], it is possible KB oxidation favours the production of a specific cytokine. Moreover, KBs can act as both pro- [72] and anti- [318] inflammatory mediators of the innate immune

system. Recently, the ingestion of the racemic ketone salts and R-BD D- β HB monoester, which raised blood D- β HB concentration to ~ 0.9 and ~ 3.2 mmol \cdot L $^{-1}$ within 30 min, respectively, both increased *ex vivo* LPS-stimulated (i.e. monocyte driven) whole blood production of IL-1 β , a pro-inflammatory cytokine [72]. The authors suggested the increase in IL-1 β production was due to NLRP3 activation [72], although this conflicts with an earlier study [318]. The influence of NLRP3 on T-cell cytokine production has not been established and its effect on infection risk is unclear due to a high-level of redundancy from other inflammasomes [321]. As such, further studies should explore potential mechanisms that KBs can modulate T-cell function and cytokine production to an immune challenge.

4.6 Conclusion

Hyperketonaemia induced via BD ingestion eliciting blood D- β HB concentrations up to ~ 1 mmol \cdot L $^{-1}$ does not appear to influence *in vitro* T-cell related IL-4, IL-10 and IFN- γ gene expression within SEB-stimulated PBMCs at rest. However, BD ingestion or acute hyperketonaemia could influence on the T-cell cytokine response to an immune challenge under different metabolic or pathologic conditions, which warrants further research.

4.7 Epilogue

Hyperketonaemia following BD ingestion may alter the metabolic response to exercise, particularly the regulation of substrate availability and metabolism. For example, acute ketone supplementation can reduce blood glucose, FFA and lactate concentrations, as well as suppress skeletal muscle glycolysis during exercise [11,12]. This may have downstream effects on exercise-induced immune perturbations due to shifts in CHO availability and, potentially, blood cortisol concentration. Therefore, the effect of BD ingestion on high-intensity endurance performance and exercise-induced immune perturbations (i.e. T-cell related cytokine gene expression within SEB-stimulated PBMCs) is examined in Chapters 5 and 6, respectively. Based on the results of Chapter 4, the Spl-BD dose was chosen for use in Chapters 5 and 6 as it elicited similar increases in blood D- β HB concentration as the M-BD and H-BD trials, whilst having no measurable effect on T-cell related cytokine gene expression within SEB-stimulated

PBMCs at rest. As such, it will be assumed that any changes in cytokine gene expression in Chapter 6 is because of the additional influence of exercise.

Chapter 5

The effect of 1,3-butanediol on cycling time-trial performance

5.1 Abstract

This study investigated the effect of the racemic ketogenic supplement, BD, on cycling TT performance and tolerability. A repeated-measures, randomised, crossover study was conducted in nine trained male cyclists (age, 26.7 ± 5.2 y; body mass, 69.6 ± 8.4 kg; height, 1.82 ± 0.09 m; BMI, 21.2 ± 1.5 kg·m⁻²; $\text{VO}_{2\text{peak}}$, 63.9 ± 2.5 ml·kg⁻¹·min⁻¹; W_{max} , 389.3 ± 50.4 W). Participants ingested 0.35 g·kg⁻¹ of BD or PLA 30 min before and 60 min during 85 min of SS exercise at 85% of the power eliciting their secondary ventilatory threshold, which preceded a ~25-35 min TT (i.e. 7 kJ·kg⁻¹). BD ingestion increased blood D-βHB concentration throughout exercise (0.44-0.79 mmol·L⁻¹) compared to PLA (0.11-0.16 mmol·L⁻¹) (all $p < 0.001$), which peaked 1 h following the TT (1.38 ± 0.35 vs. 0.34 ± 0.24 mmol·L⁻¹) ($p < 0.001$). Serum glucose and blood lactate concentrations were not different between trials (all $p > 0.05$). BD ingestion increased oxygen consumption and carbon-dioxide production after 20 min of SS exercise ($p = 0.002$ and $p = 0.032$, respectively); however, no further effects on cardiorespiratory parameters were observed. Within the BD trial, moderate to severe gastrointestinal symptoms were reported in five participants and low levels of dizziness, nausea and euphoria were reported in two participants. However, this had no effect on TT duration (PLA, 28.50 ± 3.59 ; BD, 28.72 ± 3.23 min; $p = 0.62$) and average power output (PLA, 290.10 ± 53.70 ; BD, 286.42 ± 45.88 W; $p = 0.50$). These results suggest BD has no benefit for endurance performance.

5.2 Introduction

The interaction of energetic substrates during exercise has been investigated for over 100 years [327]. Endurance performance up to ~3 h appears to be CHO-dependent (skeletal muscle and hepatic glycogen, blood glucose, lactate and exogenous sources) [115], with the contribution of fat-derived fuel largely influenced by exercise intensity, training status and diet [123]. These events require acute dietary interventions to sustain endogenous fuel supply, with the most common and effective approaches including CHO-loading and supplementation [5]. However, recent scientific enquiry has focused on the metabolic and performance effects of an additional energetic substrate, KBs [11,12]. The oral ingestion of ketone supplements, specifically salts and esters, have shown to induce hyperketonaemia within 30 min [13], thus allowing the effect of KBs to be delineated from the interference to changes in substrate availability and metabolism resulting from alternative ketogenic strategies.

The ergogenic potential of KBs is linked to their direct contribution to the production of ATP and ability to regulate substrate metabolism. Following entry into the TCA cycle, D- β HB liberates 13.0 ATP·mole⁻¹ per C₂ unit, compared to 12.67 and 10 ATP·mole⁻¹ per C₂ unit for glucose and pyruvate, respectively [137], whilst increasing mitochondrial efficiency and the Gibbs free energy of ATP hydrolysis [65]. This suggests that KBs are a more efficient substrate than CHO. Although their energy yield is less than palmitate (i.e. 16.13 ATP·mole⁻¹ per C₂ unit), fat utilisation declines from moderate- to high-intensity exercise [328]; whereas, absolute KB utilisation increases [73]. The ingestion of ketone esters has also been shown to attenuate CHO utilisation as demonstrated by reduced glycolytic rates [73] and reduced lactate accumulation during moderate- to high-intensity exercise [67,73,74]. Concomitantly, there is a decline in adipose tissue lipolysis [67,73], whereas intramuscular triacylglycerol lipolysis may increase [73]. Collectively, this presents a unique metabolic profile to examine endurance performance.

The impact of ketone and ketogenic supplementation on endurance performance remains equivocal, with beneficial [73], detrimental [62,67] and trivial [63,74] effects reported. Recently, the effects of 0.5 g·kg⁻¹ of BD (split into 0.25 + 0.125 + 0.125 g·kg⁻¹ doses) in combination with CHO on a 5 km TT preceded by 60 min of running at 75% VO_{2max} was investigated in trained runners [90]. Blood D- β HB concentration ranged between ~0.8

and $\sim 1.1 \text{ mmol}\cdot\text{L}^{-1}$ throughout exercise; however, there was no effect on TT performance or cardiorespiratory variables [90]. The authors suggested coingesting higher doses of BD with $<60 \text{ g}\cdot\text{h}^{-1}$ of CHO may be necessary to promote gastrointestinal absorption of BD and elevate blood D- β HB to concentrations that elicit an ergogenic effect [90]. Moreover, performance protocols within previous studies may have been of insufficient duration ($\sim 90 \text{ min}$) and intensity to elucidate an effect of acute hyperketonaemia. Therefore, future studies are warranted to examine the effect of different ketone and ketogenic supplements with varying conditions of CHO availability and endurance performance protocols. In concordance, the present study investigated the effect of $0.7 \text{ g}\cdot\text{kg}^{-1}$ of BD split into two equal doses without the interference of CHO coingestion on a cycling TT ($\sim 30 \text{ min}$) preceded by 85 min of glycogen-lowering SS exercise in trained cyclists.

5.3 Methods

5.3.1 Participants

Nine trained male cyclists, consuming a mixed diet for at least 12 months and without a history of recurrent gastrointestinal symptoms volunteered to participate in the study (age, $26.7 \pm 5.2 \text{ y}$; body mass, $69.6 \pm 8.4 \text{ kg}$; height, $1.82 \pm 0.09 \text{ m}$; BMI, $21.2 \pm 1.5 \text{ kg}\cdot\text{m}^{-2}$; $\Sigma 8$ skinfolds, $53 \pm 10 \text{ mm}$; $\text{VO}_{2\text{peak}}$, $63.9 \pm 2.5 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; W_{max} , $389.3 \pm 50.4 \text{ W}$; hours training per week, $12.3 \pm 2.3 \text{ h}$; weekly alcohol intake 1.4 ± 1.2 standard units). All participants were informed of the rationale of the study, experimental procedures and possible risks before providing their written consent (Appendix E). The study was approved by the Health and Disability Ethics Committee (Wellington, New Zealand) (reference number 17/NTB/39/AM02) and prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617001347358).

Participants completed a comprehensive health and eligibility screening questionnaire (Appendix D) to ascertain if they were suitable for the study. Participants were eligible to participate if they were male, healthy, between 18 and 35 years, nonsmokers, consumed a mixed diet for at least 12 months and had a $\text{VO}_{2\text{peak}} > 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. Participants were excluded if they possessed cardiovascular, metabolic, neurological or immunological disorders, abnormal red blood cell or differential leukocyte counts, had taken medications

or supplements known to effect immune function in the previous 2 weeks, or had experienced symptoms of infection or illness in the 4 weeks prior to the study.

5.3.2 Preliminary testing and familiarisation

Participants presented to the laboratory on two occasions prior to the experimental trials. On the first visit, participants arrived at a time of convenience, having fasted for a minimum of 4 h and refrained from caffeine, alcohol and strenuous exercise for the preceding 24 h. Participants' body mass (shorts only), height and $\Sigma 8$ skinfolds were measured by an accredited anthropometrist (ISAK, level 1, skinfold CV of 3.8%). To determine $\text{VO}_{2\text{peak}}$, VT_2 and maximal power output (W_{max}), participants performed a continuous incremental cycling protocol on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) commencing cycling at 95 W with increments of 35 W every 3 min until either; 1) volitional exhaustion or; 2) cadence could not be maintained above 60 rpm. Ratings of perceived exertion (RPE) (Borg 6-20 scale [329]) and heart rate (HR) using short range telemetry (Garmin Fenix 3, Garmin, Kansas, USA) were noted during the final 30 sec of each stage. The expired gas was collected and analysed continuously using a computerised metabolic system with mixing chamber (Parvo Medics TrueOne 2400, Salt Lake City, Utah, USA) and W_{max} was calculated according to the formula; $W_{\text{max}} = W_{\text{final}} + (t/T) \times W_{\text{inc}}$. Where W_{final} is the power output (W) of the final completed stage, t is the time achieved in the final uncompleted stage (s), T is the duration of each stage (180 sec) and W_{inc} is the workload increment (35 W). The power eliciting VT_2 was determined using the V-slope method [330] by two researchers in a pro-rata manner and $\text{VO}_{2\text{peak}}$ was determined by the highest averaged 30 sec. To familiarise participants with the TT, participants remounted the cycle ergometer after 15-20 min of rest, which was switched from hyperbolic to linear mode and commenced a TT equivalent to 7 $\text{kJ}\cdot\text{kg}^{-1}$ (~25-35 min). The power output in linear mode is cadence (rpm) dependent, with power (W) calculated according to the formula; $W = L \times (\text{rpm})^2$. The linear factor (L) was calculated to elicit a power output of 70% W_{max} at an rpm of 90. For all cycling tests, bike dimensions were set to the participants' preferences and were repeated for subsequent trials. The participants returned 3-7 days later to perform a familiarisation protocol, which involved completing the requirements of the experimental trial without the ingestion of BD, fluid restriction or blood collection (described below).

5.3.3 Experimental trial

Using a repeated-measures, randomised, crossover design, participants were randomly (www.randomizer.org) assigned to either PLA or BD (proportional distribution of optical isomers unknown; product code 02-59620; Penta Manufacturing Ltd, Livingston, USA). Participants were not informed of their trial allocation. However, due to the difficulty masking the bitter taste of BD, achieving successful blinding was deemed unlikely. For the day prior to each experimental trial, participants were prescribed a diet consisting of $6 \text{ g}\cdot\text{kg}^{-1}$ of CHO based on their dietary preferences by an experienced registered dietitian (RD), which was to be consumed by 23:00 h, and were asked to avoid caffeine and alcohol for the day prior to each experimental trial. Compliance was confirmed using an image-assisted weighed dietary record reported remotely in real-time via a mobile phone application (WhatsApp, Facebook, California, USA). Additionally, participants were asked to refrain from strenuous exercise for the preceding 48 h and to consume 500 ml of water prior to arrival. The purpose of standardising CHO intake and exercise before each trial was to normalise CHO availability to promote similar rates of muscle glycogen utilisation prior to the TT. This was estimated at ~50% based on the participant characteristics, CHO intake and exercise protocol [331]. Participants arrived at the laboratory between 07:00 and 08:00 h having fasted from 23:00 h the previous day. An indwelling intravenous teflon catheter (18G, Terumo, Japan) was inserted into the antecubital vein for serial blood sampling. This was followed by the ingestion of a bolus of 0 or $0.35 \text{ g}\cdot\text{kg}^{-1}$ BD within a $2 \text{ ml}\cdot\text{kg}^{-1}$ artificially sweetened, orange flavoured drink (Thriftee, Hansells Food Group Ltd, New Zealand) (pre-supplement). After 30 min (pre-exercise), participants commenced SS cycling at the power output eliciting 85% of their VT_2 ($240.8 \pm 28.3 \text{ W}$; $62.0 \pm 2.4\% \text{ W}_{\text{max}}$; $73.0 \pm 5.2\% \text{ VO}_{2\text{peak}}$) for 85 min. Every 20 min, HR and expired gas was collected, with participants providing their RPE. After 60 min of cycling (i.e. 60-min SS), participants ingested a second bolus of PLA or $0.35 \text{ g}\cdot\text{kg}^{-1}$ BD according to their trial allocation. Following completion (post-SS), participants rested for 5 min, then were instructed to complete the TT (as previously described) as fast as possible while remaining in a seated position. Participants were blinded to their power output and elapsed time; however, they were notified at each quarter of completion and were counted down from 100 kJ in 10 kJ decrements. No respiratory or blood samples were collected during the TT; however, HR was collected at each quarter of completion. All trials were conducted by the same researcher and standardised encouragement was

provided. Fluid intake was restricted to 2 ml·kg⁻¹ of water every 15 min during the SS and TT phases. Following the TT (post-TT), participants removed wet clothing and towel dried themselves prior to having their body mass measured. Participants then completed a customised questionnaire adapted from published sources [68,332], including 27 items pertaining to systemic (13), upper abdominal (6) and lower abdominal (8) symptoms, and prior tested by two experienced RDs for understanding and literacy. Participants were prompted for additional symptoms not stated on the questionnaire and were asked to identify their trial allocation. They were then provided with 5 ml·kg⁻¹ of water and rested for 60 min prior to departing. All exercise trials throughout the study were performed in standard laboratory conditions of 18.3 ± 0.6 °C and 67.6 ± 7.4% relative humidity, and were separated by 7-10 days.

5.3.4 Blood sampling and analysis

Capillary blood D-βHB concentration was measured (Freestyle Optium Neo, Abbott Diabetes Care, Victoria, Australia) and venous blood samples were collected at pre-supplement, pre-exercise, 30-min SS, 60-min SS, post-SS, post-TT and 1-h post-TT into 8 ml serum vacutainers (Becton Dickinson and Co, USA) with the participants seated in an upright position. Serum vacutainers were left to clot for 30 min at room temperature prior to centrifugation at 1500 g for 10 min at 4 °C and separation into two 1.5 ml aliquots to be stored at -80 °C prior to the analysis of glucose concentration (Cobas Modular P800 Analyser, Roche Diagnostics, New Zealand). Capillary blood lactate concentration was measured (Lactate 2 Pro, Akray, Japan) at pre-exercise, post-SS and post-TT. All capillary blood samples were collected from participants' finger tips using standardised techniques.

5.3.5 Data analysis

All data are expressed as mean ± SD unless otherwise stated. Data were checked for normality as indicated by the Shapiro-Wilk score and, where appropriate, statistical analysis was performed on the logarithmic transformation of the data (i.e. D-βHB and glucose concentrations; RPE; VCO₂; and RER). Paired *t*-tests were used to compare TT duration, average TT power output and HR, and change in body mass between trials. A two-way (trial x time) repeated-measures ANOVA was performed for glucose, D-βHB, lactate, cardiorespiratory and RPE data (IBM SPSS Statistics software version 21, IBM

Corp., Chicago IL, USA). If Mauchly's test of sphericity was violated, adjustments to the degrees of freedom were made for the ANOVA using Greenhouse-Geisser ϵ . Where a significant effect was observed, post hoc analysis was conducted using Student's paired t -tests with Holm-Bonferroni adjustments for multiple comparisons applied to the unadjusted p value to locate specific differences. Significance level was accepted at an alpha of $p < 0.05$. To interpret the magnitude of effect and identify trends within nonsignificant data, Cohen's d effect sizes (ES) (\pm 90% confidence limits (CL)) were estimated using a purpose-built spreadsheet [333], with ES thresholds set at <0.2 , >0.2 , >0.6 , >1.2 , >2.0 and >4.0 for *trivial*, *small*, *moderate*, *large*, *very large* and *extremely large* effects, respectively [334]. However, as Cohen's d may over-emphasise the effect of BD on blood D- β HB concentration compared to ketone supplements with greater rates of conversion, a novel approach was used to determine the magnitude of effect whereby the possible range of change was transformed into a full scale of deflection (FSD) [335]. The deflection (\pm 90% CL) was estimated by the difference in D- β HB concentration between the PLA and BD trials for each time point and the range was calculated by subtracting D- β HB concentration of the PLA trial for each time point from $3.5 \text{ mmol}\cdot\text{L}^{-1}$, which is approximately the highest blood D- β HB concentration reported during exercise of a similar intensity following the ingestion of a ketone supplement [73]. Each range was set at 0 to 100% and the magnitude thresholds were defined as >10 , >30 , >50 , >70 and $>90\%$ for *small*, *moderate*, *large*, *very large* and *extremely large* effects, respectively. If the 90% CLs overlapped 0 for either ES statistics, the magnitude of effect was deemed *unclear*.

5.4 Results

5.4.1 D- β HB, glucose and lactate concentration

Pre-supplement blood D- β HB concentration (PLA, 0.12 ± 0.04 ; BD, $0.14 \pm 0.05 \text{ mmol}\cdot\text{L}^{-1}$) and serum glucose concentration (PLA, 4.33 ± 0.30 ; BD, $4.49 \pm 0.26 \text{ mmol}\cdot\text{L}^{-1}$) did not differ between trials (all $p > 0.05$). A significant trial x time interaction was observed for blood D- β HB concentration ($p < 0.001$), with increased concentrations from pre-exercise to 1-h post-TT in the BD compared to PLA trial (all $p < 0.001$) (Figure 5.1A, p. 82). However, the *small* increase in blood D- β HB concentration at pre-exercise ($FSD = 12.57 \pm 2.09$) declined to a *trivial* effect at 30-min SS ($FSD = 9.84 \pm 1.16$) and 60-min SS (FSD

= 9.52 ± 1.59). The second bolus of BD at 60-min SS led to a *small* increase in blood D- β HB concentration at post-SS ($FSD = 15.38 \pm 3.65$) and post-TT ($FSD = 19.41 \pm 3.35$) which elevated to a *moderate* increase at 1-h post-TT ($FSD = 32.45 \pm 6.41$) compared to PLA, coinciding with peak concentrations of $1.38 \pm 0.35 \text{ mmol}\cdot\text{L}^{-1}$. Despite a significant trial x time interaction being observed for serum glucose concentration ($p = 0.027$), post hoc analysis could only locate a trend for a *small* reduction in serum glucose concentration in the BD compared to PLA trial at post-TT ($p = 0.43$; $d = -0.48 \pm 0.46$). A significant effect for time was also observed for serum glucose concentration ($p < 0.001$), with higher concentrations from 30-min SS to post-TT compared to pre-exercise and at post-TT compared to post-SS (all $p < 0.001$) (Figure 5.1B, p. 82). Pre-exercise blood lactate concentration did not differ between trials (PLA, 2.33 ± 0.62 ; BD, $2.02 \pm 0.52 \text{ mmol}\cdot\text{L}^{-1}$; $p = 0.37$) and BD had no effect on blood lactate concentration during exercise (all $p > 0.05$). However, there was a significant effect for time ($p < 0.001$), with blood lactate concentration elevated at post-SS compared to pre-exercise and at post-TT compared to post-SS (Figure 5.1C, p. 82).

5.4.2 Steady-state exercise cardiorespiratory variables and perceived exertion

There was a significant trial x time interaction for VO_2 and VCO_2 ($p = 0.021$ and $p = 0.032$, respectively). Post hoc analysis revealed *small* increases in VO_2 and VCO_2 in the BD trial compared to PLA at 20 -min SS ($p = 0.002$; $d = 0.32 \pm 0.10$ and $p = 0.032$; $d = 0.29 \pm 0.20$, respectively); however, no other differences were located (Table 5.1, p. 83). A trial x time interaction was also observed for relative intensity ($p = 0.026$), with a *small* increase occurring at 20-min SS in the BD compared to PLA trial ($p = 0.001$; $d = 0.45 \pm 0.13$). There were no significant effects of BD on RER, HR or RPE (all $p > 0.05$). However, there was a trend for *moderately* higher RPE in the BD trial compared to PLA at 80-min SS ($d = 0.65 \pm 0.56$) (Table 5.1). Significant effects for time were observed for HR ($p < 0.001$), which increased from 20-min SS to 40-min SS, then from 60-min SS to 80-min SS (all $p < 0.05$), and RPE ($p = 0.004$), which increased between subsequent time points (all $p < 0.05$) (Table 5.1).

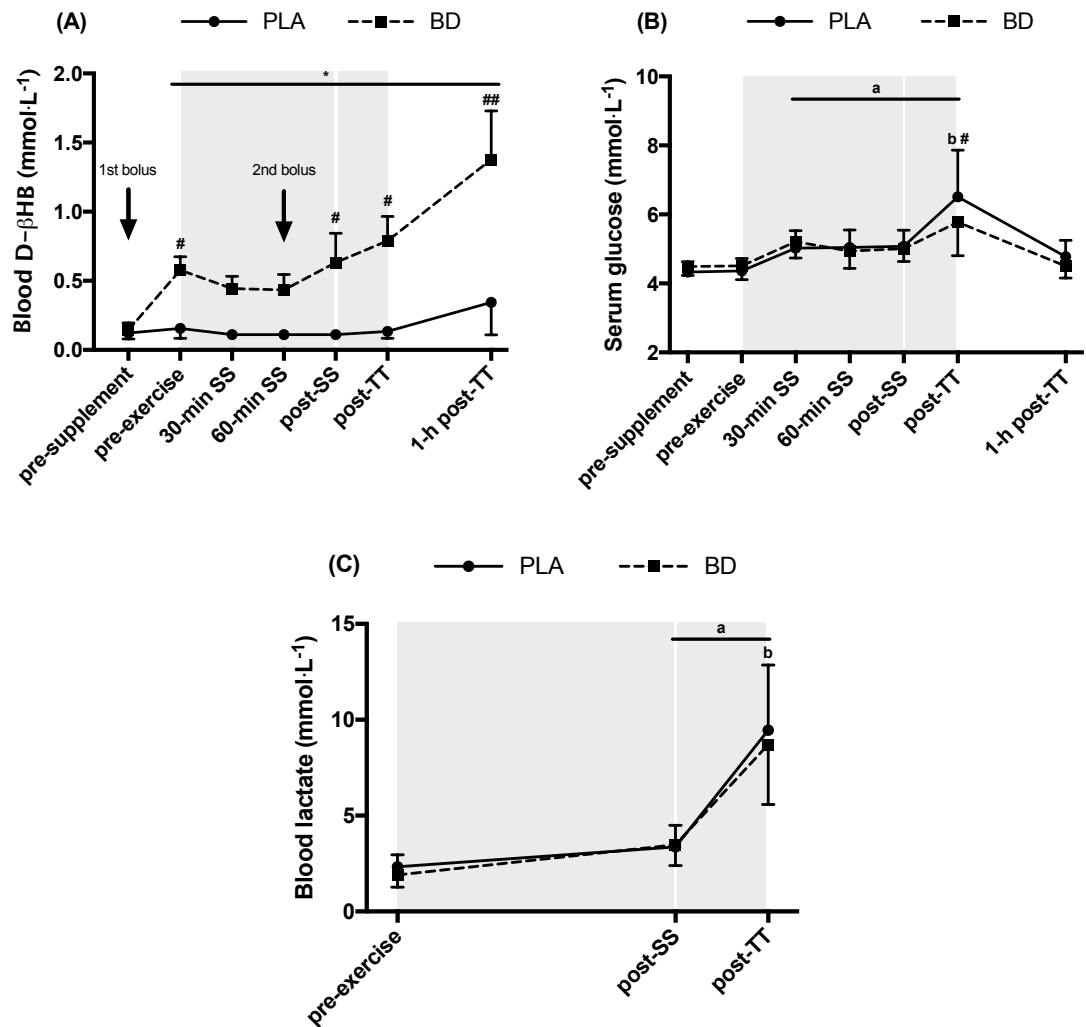


Figure 5.1. (A) capillary blood D-βHB, (B) serum glucose and (C) capillary blood lactate concentrations during the PLA and BD trials. Values are presented as mean ± SD. The grey area denotes the steady-state and time-trial phases. Significantly higher in BD compared to PLA at time point ($*p < 0.001$). Effect size (d or FSD); [#]*small* and ^{##}*moderate* effects for BD compared to PLA at time point. Main effect for time; significantly higher to pre-exercise (^a $p < 0.001$). Main effect for time; significantly higher to post-SS (^b $p < 0.001$).

Table 5.1. Cardiorespiratory variables and perceived exertion during steady-state cycling during the PLA and BD trials.

	Pre-exercise	20-min SS	40-min SS	60-min SS	80-min SS
VO₂ (L·min⁻¹)					
PLA		3.15 ± 0.30	3.22 ± 0.32	3.24 ± 0.26	3.28 ± 0.32
BD		3.26 ± 0.32**	3.25 ± 0.25	3.21 ± 0.21	3.23 ± 0.30
ES (± 90% CL)		0.32 ± 0.10	0.07 ± 0.20	-0.11 ± 0.21	-0.14 ± 0.23
VCO₂ (L·min⁻¹)					
PLA		2.91 ± 0.26	2.95 ± 0.27	2.93 ± 0.26	2.95 ± 0.27
BD		2.99 ± 0.27*	2.97 ± 0.23	2.90 ± 0.25	2.91 ± 0.24
ES (± 90% CL)		0.29 ± 0.16	0.07 ± 0.22	-0.09 ± 0.17	-0.12 ± 0.32
RER					
PLA		0.92 ± 0.03	0.92 ± 0.03	0.90 ± 0.03	0.90 ± 0.03
BD		0.92 ± 0.04	0.91 ± 0.03	0.91 ± 0.04	0.90 ± 0.02
ES (± 90% CL)		-0.16 ± 0.30	-0.07 ± 0.41	0.02 ± 0.33	0.05 ± 0.64
% VO_{2peak}					
PLA		71.21 ± 4.69	72.75 ± 5.47	73.29 ± 6.16	73.99 ± 5.64
BD		73.57 ± 4.42*	73.39 ± 5.11	72.59 ± 5.95	72.95 ± 5.66
ES (± 90% CL)		0.45 ± 0.13	0.11 ± 0.28	-0.10 ± 0.20	-0.17 ± 0.31
HR (beats·min⁻¹)^{a,b}					
PLA	59 ± 11	154 ± 16	158 ± 14	158 ± 14	160 ± 14
BD	59 ± 6	157 ± 14	159 ± 14	158 ± 13	160 ± 13
ES (± 90% CL)	0.03 ± 0.68	0.16 ± 0.15	0.08 ± 0.18	0.01 ± 0.16	0.01 ± 0.20
RPE^{a,c,d}					
PLA		12.9 ± 0.9	13.6 ± 1.1	13.9 ± 0.9	14.3 ± 0.9
BD		12.9 ± 1.6	13.7 ± 1.6	14.0 ± 1.6	15.0 ± 1.4
ES (± 90% CL)		-0.06 ± 0.74	0.05 ± 0.71	0.05 ± 0.87	0.65 ± 0.56

Values are presented as mean ± SD. VO₂ volume of oxygen consumed, VCO₂ volume of carbon dioxide produced; RER respiratory exchange ratio, VO_{2peak} peak oxygen uptake, HR heart rate and RPE ratings of perceived exertion. Significantly higher in BD compared to PLA at time point (* $p < 0.05$; ** $p < 0.01$). Main effect for time; significantly higher at 40-min SS compared to 20-min SS (^a $p < 0.001$). Main effect for time; significantly higher at 80-min SS compared to 60-min SS (^b $p < 0.05$; ^c $p < 0.001$). Main effect for time; significantly higher at 60-min SS compared to 40-min SS (^d $p < 0.05$).

5.4.3 Time-trial performance

BD had no effect on TT duration (PLA, 28.49 ± 3.59 vs. BD, 28.72 ± 3.23 min; $p = 0.62$; $d = 0.06 ± 0.20$) (Figure 5.2A, p. 84), average power output (PLA, 290.10 ± 53.70 vs. BD, 286.42 ± 45.88 watts; $p = 0.50$; $d = -0.06 ± 0.16$) (Figure 5.2B, p. 84) or average HR (PLA, 175 ± 11 vs. BD, 173 ± 7 beats·min⁻¹; $p = 0.38$; $d = -0.13 ± 0.26$). Nor was there a carry-over effect between trials ($p > 0.05$). Five participants believed they performed better during the BD trial compared to PLA; however, only three were correct.

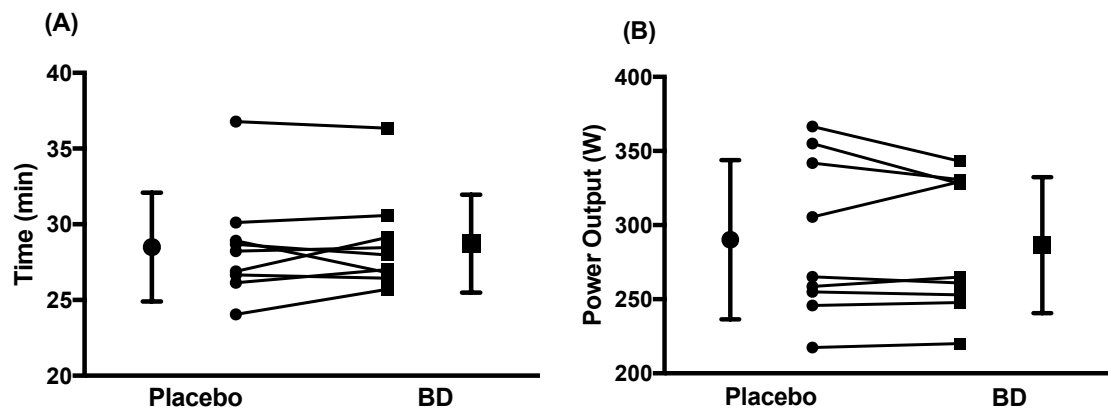


Figure 5.2. Time-trial performance presented as (A) mean \pm SD and individual time and (B) mean \pm SD and individual power output for the PLA and BD trials.

5.4.4 Tolerability

There was no difference in the change of body mass (corrected for fluid intake) between trials (PLA, -2.14 ± 0.48 kg; BD, -2.07 ± 0.42 kg; $p = 0.15$; $d = 0.13 \pm 0.15$). Within the BD trial, two participants experienced transient symptoms of low levels of nausea, euphoria and dizziness, which they related to a state of alcohol intoxication. Five participants reported low to moderate levels of belching and burping, and one participant reported severe abdominal pain. No participants reported similar symptoms during the PLA trial. Everyone disliked the taste of BD, which resulted in retching for four participants. Everyone correctly identified their trial allocation, which was likely due to the difficulty masking the taste of BD and subsequent gastrointestinal effects.

5.5 Discussion

The main finding of the present study was despite BD increasing blood D- β HB concentration, there were no differences TT performance or metabolic and cardiorespiratory variables during SS exercise. This supports previous evidence suggesting ketone supplements eliciting blood D- β HB concentrations up to ~ 1 mmol·L⁻¹ do not benefit endurance performance. Furthermore, BD elicited gastrointestinal distress, in particular belching and burping, as well as symptoms of nausea, euphoria and dizziness in some participants, indicating BD has an upper limit of tolerance.

The increase in BD-induced blood D- β HB concentration paralleled other racemic ketone and ketogenic supplements. For example, the ingestion of 0.5 g·kg⁻¹ BD [90], ~ 0.8 g·kg⁻¹

¹ of a racemic β HB salt solution (total of 24-37 g D,L- β HB) [61,63] and 0.5 g·kg⁻¹ of a R,S-1,3-butanediol acetoacetate diester [67], typically ingested as a split dose, elicited blood D- β HB concentration up to ~1 mmol·L⁻¹. The ingestion of racemic ketone supplements also increase blood L- β HB, which does not directly contribute to energy production [13,82], but may negate the suppressive effect of D- β HB on CHO metabolism [92]; however, this may depend on blood D- and L- β HB concentration. In the present study, peak blood D- β HB concentrations occurred at 1-h post-TT, which was substantially higher than values observed in the resting study (see Chapter 4, Table 4.2); however, post-exercise hepatic ketogenesis was a likely contributor [49]. In contrast, the ingestion of the nonracemic R-BD D- β HB monoester can increase blood D- β HB to 3.5 mmol·L⁻¹ during exercise of similar intensity [73]. This highlights the differences in componentry and metabolism of racemic ketone and ketogenic supplements compared to the nonracemic R-BD D- β HB monoester.

Following the onset of exercise, blood D- β HB concentration declined and plateaued until after the second bolus of BD ingestion at 60-min SS. Compared to the resting study (Chapter 4), blood D- β HB concentrations were ~0.2-0.3 mmol·L⁻¹ lower from 30-min SS to post-TT. It would, therefore, seem that D- β HB was continuously released into the circulation, whilst being taken up by contracting muscle cells via monocarboxylate transporters [41]; although, this was not able to be confirmed. Whilst a shift in the RER towards D- β HB and AcAc's respiratory quotient of 0.89 and 1.0, respectively, may be indicative of KB oxidation, this effect can be abrogated at exercise intensities above 60% VO_{2max} [61]. Further, it is possible to estimate D- β HB oxidation rates by comparing iAUC for blood D- β HB concentrations between resting and exercising conditions, as previously conducted [73]; however, resting blood D- β HB concentrations were not measured in the present study and the validity of these calculations have not been confirmed using direct calorimetry. Therefore, the contribution of KBs to EE and how this differs between different forms of ketone supplements remains unknown.

Exercise efficiency did not appear to be altered in the present study as demonstrated by no sustained difference in oxygen utilisation during SS exercise. Similar findings have been reported following the ingestion of racemic ketone β HB salts [61-63], BD [90] and ketone esters [67,73]. Therefore, the enhanced metabolic efficiency of KBs, often referred to in an isolated rat's perfused heart [139], may not translate to whole body exercise in

humans. There was also no effect of BD on blood lactate accumulation, corroborating studies using BD [90] and racemic β HB salts [61-63], but not ketone esters [67,73,74], which could be due to the interference of L- β HB. Nor were there differences in serum glucose concentration between trials, which is in contrast to other studies using racemic β HB salts [62,63] and ketone esters [67,73,74]. However, there was a trend towards a *small* reduction in serum glucose concentration immediately following the TT in the BD trial, which may be due to an attenuation of hepatic glycogenolysis. Nonetheless, this did not appear to effect performance.

Clearly, the physiological conditions suitable for ketone supplements to augment performance remain difficult to identify. Considering the potential for hyperketonaemia to downregulate glycolysis and suppress adipocyte lipolysis, the optimal range of KB concentration to enhance substrate provision and energy production remains obscure. This is exacerbated by differences in the conversion rate of various ketone supplements to D- β HB and measurement discrepancy of point-of-care versus laboratory-based methods [67,97], thus making comparisons between studies difficult. For example, following the ingestion of the R-BD D- β HB monoester within a CHO drink, plasma D- β HB was maintained above $\sim 2\text{-}3\text{ mmol}\cdot\text{L}^{-1}$ and improved performance during a preloaded 30 min cycling TT by $\sim 2\%$ compared to the ingestion of an isoenergetic CHO only drink [73]. However, these performance benefits have not been replicated and may be abrogated in exercise trials 90 min or less proceeding the ingestion of recommended CHO intakes [67,73,74]. It is also possible that the interaction between KB concentration and CHO availability has a mediating role on KB metabolism [101], with high rates of KB oxidation requiring high CHO availability to maintain the anaplerotic flux in the TCA cycle [102,103]. However, how this translates to real world performance is yet to be elucidated.

The ingestion of BD also led to symptoms synonymous with low levels of alcohol intoxication within two participants. Typically, BD is rapidly converted to β HB via hepatic alcohol and aldehyde dehydrogenases [82]; however, these steps are rate limiting and may be influenced by previous ethanol exposure [86]. In the present study, participants habitually consumed low levels of alcohol (~ 1.5 standard drinks per week); therefore, their maximal capacity to metabolise BD into β HB could have been below the dose ingested, resulting in the accumulation of BD in the circulation. As such, there was a trend towards a *moderate* increase in RPE nearing the end of SS cycling in the BD trial;

whereas, significant differences could have been elucidated if localised RPE measures were employed [78]. Moreover, five participants reported moderate to severe gastrointestinal symptoms, which was not reported previously with BD ingestion [90]; therefore, symptoms could have been exacerbated by ingesting BD in a fasted state or without coingesting CHO. In turn, adverse gastrointestinal and/or systemic symptoms would restrict the application of some ketone and ketogenic supplements in endurance events whereby exogenous substrate provision and management of gastrointestinal distress are critical factors for performance.

5.6 Conclusions

Similar to other ketone supplements eliciting blood D- β HB concentrations up to ~ 1 mmol·L⁻¹, BD does not benefit endurance performance. However, it is uncertain whether this absence of effect persists in events >2-3 h. BD may also induce symptoms related to a low level of alcohol intoxication, including nausea, euphoria and dizziness, and moderate to severe gastrointestinal symptoms, suggesting its ingestion should be avoided at higher doses. Further attempts to identify the ergogenic properties of ketone supplements need to focus on products increasing blood D- β HB concentrations >1.5 mmol·L⁻¹ and their interaction with varying levels of CHO availability.

The effect of 1,3-butanediol on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells following prolonged, strenuous exercise

6.1 Abstract

This study investigated the effect of the racemic ketogenic supplement, BD, on T-cell related cytokine gene expression within SEB-stimulated PBMCs following prolonged, strenuous exercise. A repeated-measures, randomised, crossover study was conducted in nine healthy, trained male cyclists (age, 26.7 ± 5.2 y; body mass, 69.6 ± 8.4 kg; height, 1.82 ± 0.09 m; $\text{VO}_{2\text{peak}}$, 63.9 ± 2.5 ml·kg⁻¹·min⁻¹). Participants ingested 0.35g·kg⁻¹ of BD or placebo 30 min before and 60 min during 85 min of SS exercise, which preceded a ~30 min TT (7 kJ·kg⁻¹). Blood samples were collected at pre-supplement, pre-exercise, post-SS, post-TT and 1-h post-TT. Whole blood cultures were stimulated with SEB for 24 h to determine T-cell related IL-4, IL-10 and IFN- γ mRNA expression within isolated PBMCs *in vitro*. BD ingestion increased blood D- β HB concentration from pre-exercise to post-TT (0.44-0.79 mmol·L⁻¹) compared to placebo (0.11-0.16 mmol·L⁻¹) (all $p < 0.001$), which peaked at 1-h post-TT (1.38 ± 0.35 vs. 0.34 ± 0.24 mmol·L⁻¹) ($p < 0.001$). Serum glucose and cortisol concentrations did not differ between trials (all $p > 0.05$). BD ingestion increased T-cell related IFN- γ mRNA expression throughout the trial ($p = 0.011$); however, no significant effects were observed for IL-4 and IL-10 mRNA expression or the IFN- γ /IL-4 ratio (all $p > 0.05$). In conclusion, hyperketonaemia appears to transiently amplify the initiation of pro-inflammatory T-cell related IFN- γ response to an immune challenge *in vitro* following prolonged, strenuous exercise, suggesting enhanced type-1 T-cell immunity. Nevertheless, further research is warranted to confirm these findings and identify the potential impact on illness and infection risk.

6.2 Introduction

Prolonged, strenuous exercise bouts are associated with a transient state of immunodepression and increased illness and infection risk, particularly URS and URTI [1]. Recurrent illness detracts from training availability [3] and is associated with impaired performance in pinnacle events [3,336]; therefore, dietary strategies preventing illness are invaluable. Maintaining CHO availability during exercise (i.e. skeletal muscle and hepatic glycogen, and blood glucose) is one of the most efficacious dietary strategies to support immune function [1]. Ketone supplements, which induce hyperketonaemia (blood KBs $>0.5 \text{ mmol}\cdot\text{L}^{-1}$) within minutes following ingestion, can reduce blood lactate accumulation [67,73], suppress skeletal muscle glycolysis and lower blood glucose concentration [73] during exercise. As such, ketone supplements have been investigated for their impact on performance [62,63,67,73,74] and recovery [75,76] and overreaching [77]. However, no studies have investigated the effects of ketone supplementation on exercise-induced immune perturbations.

Acute prolonged, strenuous exercise perturbs several immune components [1]; of which, T-cells are highly sensitive to exercise stress [18]. T-cells orchestrate an immune response to invading and incumbent pathogens and comprise ~60-80% of the lymphocyte population [18,19]; their exercise-induced trafficking patterns and capacity to mount an inflammatory cytokine response probably influences illness risk [18,185,205]. Type-1 T-cells primarily produce IFN- γ , a pro-inflammatory cytokine that protects against intracellular pathogens (e.g. viruses) [20]. Following acute prolonged, strenuous exercise, circulatory type-1 T-cell mitogen-stimulated production of IFN- γ *in vitro* appears to decline [24,25,225]. However, the ingestion of CHO during exercise attenuates this effect, potentially by increasing blood glucose and reducing the rise in cortisol concentrations [24,25]. Alternatively, type-2 T-cells and regulatory T-cells (TReg cells) produce the anti-inflammatory cytokines IL-4 and IL-10, respectively [20,212]. Interleukin-4 cross-regulates IFN- γ and protects against extracellular pathogens (e.g. bacteria and fungi) [20]. Whereas, IL-10 exhibits an immunosuppressive action on both type-1 and, to a lesser extent, type-2 T-cells [212]. Mitogen- and multi-antigen-stimulated circulatory T-cell IL-4 and IL-10 production *in vitro* appears less affected following acute prolonged, strenuous exercise [27,28,205,247], thus favouring an anti-inflammatory T-cell cytokine response to an immune challenge.

Measuring T-cell cytokine gene expression can characterise how exercise alters the initial stages of immune regulation [337]. An single study has investigated the effects of strenuous exercise on stimulated (influenza- and tetanus toxoid-stimulated) T-cell cytokine gene expression *in vitro* [32]; however, there was no influence of exercise or CHO supplementation. These findings contradict the more pronounced alterations to stimulated T-cell cytokine protein production following exercise, which could be due to differences in the exercise stress (i.e. intermittent high-intensity vs. SS continuous exercise) and immune cell stimulation model; therefore, more research is required to elucidate the effects of exercise and factors influencing CHO availability and/or metabolism on T-cell cytokine gene expression to an immune challenge.

As ketone bodies can modulate CHO metabolism, it is possible they have a mediating effect on the immune response following exercise. BD is a nontoxic dialcohol and can increase blood D-βHB to ~0.8 mmol·L⁻¹ within 30 min in humans [90]. In concordance, the present study investigated the effect of acute hyperketonaemia via BD ingestion on *in vitro* T-cell related IL-4, IL-10 and IFN-γ gene expression within stimulated PBMCs in response to prolonged, strenuous exercise.

6.3 Methods

6.3.1 Participants

Participant characteristics are detailed in Chapter 5, section 5.3.1.

6.3.2 Preliminary testing and familiarisation

Preliminary testing and familiarisation are detailed in Chapter 5, section 5.3.2.

6.3.3 Experimental trial

The experimental trial is detailed in Chapter 5, section 5.3.3.

6.3.4 Blood sampling and analysis

Venous blood samples were collected at pre-supplement, pre-exercise, post-SS, post-TT

and 1-h post-TT into an 8 ml serum and two 6 ml K₂EDTA vacutainers (Becton Dickinson and Co, USA) with the participants seated in an upright position. Due to the rapid exercise-induced lymphocyte kinetics [338], all blood samples were collected within 1 min of the specified time points. The cannula was flushed with 3-4 ml of saline every 30 min to maintain patency. Serum vacutainers were left to clot for 30 min at room temperature prior to centrifugation at 1500 g for 10 min at 4 °C. After which, samples were separated into two 1.5 ml aliquots and stored at -80 °C prior to the analysis of glucose and cortisol concentration (Cobas Modular P800 Analyser, Roche Diagnostics, New Zealand). Capillary blood D-βHB concentration was measured (Freestyle Optium Neo, Abbott Diabetes Care, Victoria, Australia) at pre-supplement, pre-exercise, post-SS, post-TT and 1-h post-TT from a fingertip blood sample using standardised techniques.

6.3.5 Haematological analysis

K₂EDTA treated whole blood was used to determine total circulating leukocyte and differential cell concentration (Sysmex XT-2000i Automated Haematology Analyzer, Sysmex Corporation, Auckland, New Zealand). All cell concentrations were adjusted for plasma volume changes from the initial (pre-supplement) blood sample, with changes being estimated according to Dill and Costill (1974) [339].

6.3.6 T-cell subsets

Measurement of blood T-cell subset concentrations are detailed in Chapter 4, section 4.3.6. All cell concentrations were adjusted for plasma volume changes from the initial (pre-supplement) blood sample, with changes being estimated according to Dill and Costill (1974) [339].

6.3.7 Stimulated whole blood cultures

Stimulation of whole blood samples is detailed in Chapter 4, section 4.3.3.

6.3.8 Isolation of peripheral blood mononuclear cells

Isolation of PBMCs is detailed in Chapter 4, section 4.3.4.

6.3.9 Analysis of mRNA expression in peripheral blood mononuclear cells

The procedures for extracting and amplifying PBMC RNA are detailed in Chapter 4, section 4.3.5. However, in the present study, RNA for all target genes were normalised to the reference gene (β_2 -MG) within the same participant, condition and time point, and to a calibrator of *pre-exercise* Ct values of the PLA trial within the same participant.

6.3.10 Statistical analysis

All data are expressed as mean \pm SD unless otherwise stated. Data were checked for normality as indicated by the Shapiro-Wilk score and, where appropriate, statistical analysis was performed on the logarithmic transformation of the data (i.e. D- β HB and glucose concentration; WBC, granulocyte and monocyte concentrations; CD8⁺ and CD25⁺ T-cell subset concentrations and the CD4⁺/CD8⁺ T-cell subset ratio; IL-4, IL-10, IFN- γ mRNA expression and the IFN- γ /IL-4 mRNA expression ratio). A two-way (trial x time) repeated-measures ANOVA was performed for D- β HB, glucose and cortisol concentration, cell counts and cytokine gene expression (IBM SPSS Statistics software version 21, IBM Corp.). If Mauchly's test of sphericity was violated, adjustments to the degrees of freedom were made for the ANOVA using Greenhouse-Geisser ϵ . Where a significant effect was observed, post hoc analysis was conducted using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons applied to the unadjusted *p* value to locate specific differences. Significance level was accepted at an alpha of $p < 0.05$. To interpret the magnitude of effect and identify trends between trials within nonsignificant data, ES statistics were implemented as detailed in Chapter 5, section 5.3.5.

6.4 Results

6.4.1 Time-trial performance, cardiorespiratory variables and perceived exertion

Time-trial performance, cardiorespiratory variables and perceived exertion are detailed in Chapter 5, sections 5.4.2 and 5.4.3, respectively.

6.4.2 Changes in body mass and plasma volume

There was no difference in the exercise-induced change of body mass (corrected for fluid intake) (PLA, -2.14 ± 0.48 ; BD, -2.07 ± 0.42 kg; $p = 0.15$; $d = 0.13 \pm 0.15$). Despite a significant trial x time interaction being observed for percentage plasma volume change compared to the initial blood sample ($p = 0.011$), post hoc analysis could not locate specific differences between trials (all $p > 0.05$). However, there was a trend for a *moderate* increase and decrease in plasma volume in the BD trial compared to PLA at pre-exercise ($p = 0.21$; $d = 0.95 \pm 0.78$) and 1-h post-TT ($p = 0.28$; $d = -0.66 \pm 0.63$), respectively. Within each trial, plasma volume declined from pre-exercise to post-SS and post-TT, and then increased from post-TT to 1-h post-TT (all $p < 0.05$). Plasma volume declined by $7.6 \pm 6.4\%$ and $8.8 \pm 6.1\%$ at post-TT in the PLA and BD trials, respectively ($p = 0.53$; $d = -0.17 \pm 0.47$).

6.4.3 D-βHB, glucose and cortisol concentration

Pre-supplement blood D-βHB concentration (PLA, 0.12 ± 0.04 vs. BD, 0.14 ± 0.05 mmol·L⁻¹), serum glucose concentration (PLA, 4.33 ± 0.30 vs. BD, 4.49 ± 0.26 mmol·L⁻¹) and serum cortisol concentration (PLA, 463.8 ± 66.9 vs. BD, 460.0 ± 86.6 nmol·L⁻¹) did not differ between trials (all $p > 0.05$). A significant trial x time interaction was observed for blood D-βHB concentration ($p < 0.001$), with significant *small* to *moderate* increases occurring in the BD compared to PLA trial at pre-exercise, post-SS, post-TT and 1-h post-TT (all $p < 0.001$) (Figure 6.1A, p. 96). A significant trial x time interaction was observed for serum glucose concentration ($p = 0.024$); however, post hoc analysis could only locate a trend for a *small* reduction in serum glucose concentration at post-TT in the BD compared to PLA trial ($p = 0.60$; $d = -0.48 \pm 0.46$), which coincided with an increase in serum glucose concentration from post-SS to post-TT within the PLA ($p = 0.01$) but not the BD trial ($p = 0.11$). A main effect for time was also observed for serum glucose concentration ($p < 0.001$), with concentrations increasing from pre-exercise to post-SS and post-TT (all $p < 0.001$), which subsequently declined from post-TT to 1-h post-TT ($p < 0.001$) (Figure 6.1B, p. 96). There was no trial x time interaction for serum cortisol concentration ($p = 0.985$); however, there was a main effect for time ($p < 0.001$), with higher concentrations at post-SS ($p = 0.002$), post-TT ($p < 0.001$) and 1-h post-TT

($p = 0.001$) compared to pre-exercise and a lower concentration at 1-h post-TT compared to TT ($p < 0.001$) (Figure 6.1C, p. 96).

6.4.4 Circulating leukocyte and differential cell concentrations

There was no difference between trials for pre-supplement and pre-exercise concentrations for circulating leukocyte, total lymphocyte, monocyte or granulocyte concentrations, or the relative contribution of total lymphocytes to the total leukocyte population (all $p > 0.05$) (Table 6.3, p. 97). There were no trial x time interactions for circulating leukocyte, total lymphocyte or granulocyte concentrations (all $p > 0.05$); however, there was a trial x time interaction for monocyte concentration ($p = 0.048$). Albeit, post hoc analysis could not locate significant differences in monocyte concentration between trials (all $p > 0.05$), there were trends towards a *small* increase at pre-exercise ($p = 0.39$; $d = 0.51 \pm 0.51$) and a *moderate* reduction at 1-h post-TT ($p = 0.18$; $d = -0.65 \pm 0.48$) in the BD compared to PLA trial. Main effects of time were observed for circulating leukocyte ($p < 0.001$), total lymphocyte ($p < 0.001$), monocyte ($p < 0.001$) and granulocyte ($p < 0.001$) cell concentrations (Table 6.3). At the onset of exercise, all differential leukocyte populations increased, resulting in elevated cell concentrations at post-SS and post-TT compared to pre-exercise (all $p < 0.05$). All differential leukocyte concentrations remained higher at 1-h post-TT compared to pre-exercise (all $p < 0.05$), except for total lymphocytes, which declined to below pre-exercise concentrations ($p < 0.001$).

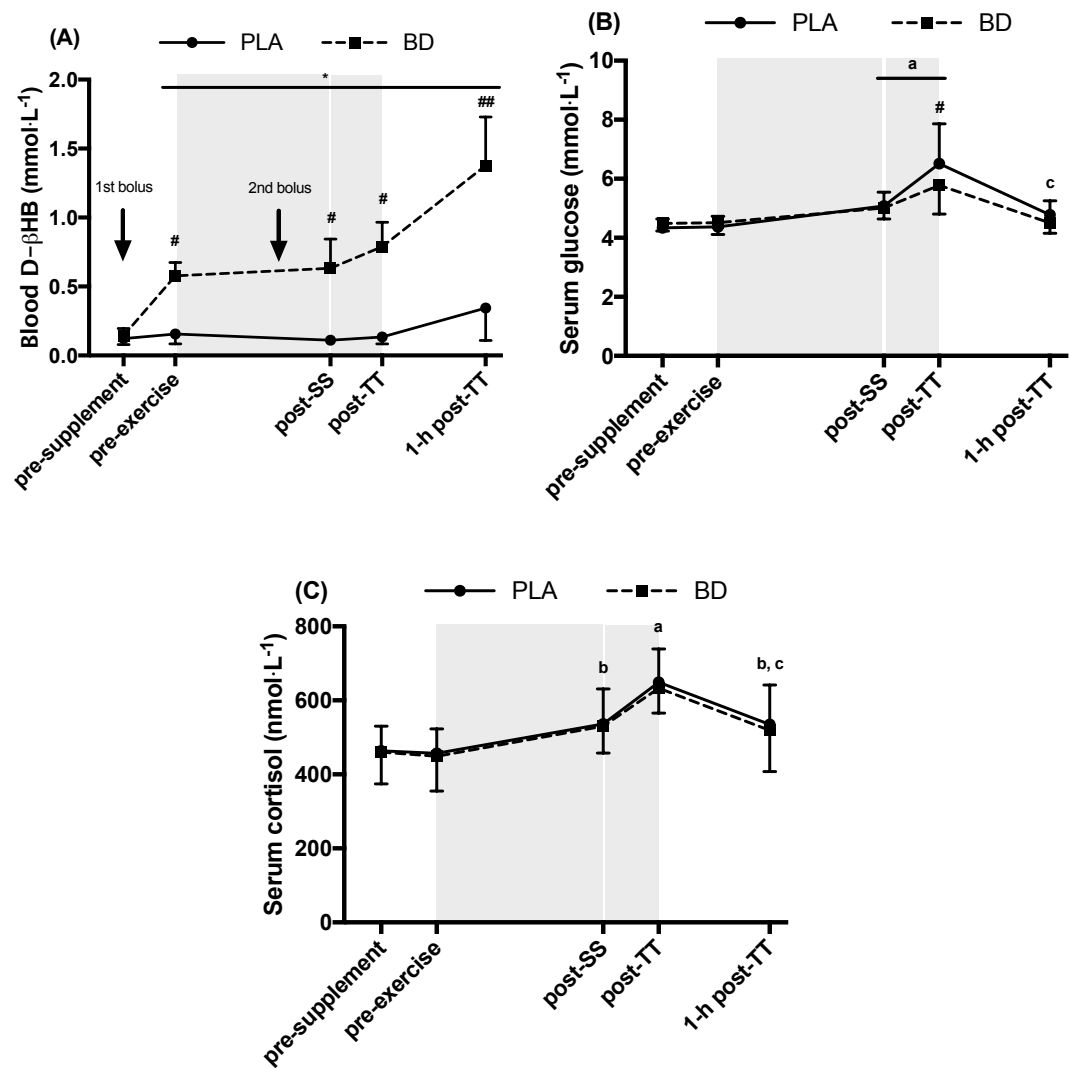


Figure 6.1. (A) capillary blood D-βHB, (B) serum glucose and (C) serum cortisol concentrations during the PLA and BD trials. Values are presented as mean ± SD. The grey areas indicate the steady-state (SS) and time-trial (TT) phases. *Significantly higher in BD compared to PLA for time point ($p < 0.001$). Effect size (d or FSD); [#]*small* and ^{##}*moderate* effects for BD compared to PLA at time point. Main effect for time; significantly higher compared to pre-exercise (^a $p < 0.001$; ^b $p < 0.05$). Main effect for time; significantly lower compared post-TT (^c $p < 0.001$).

Table 6.1. Circulating leukocyte, total lymphocyte, monocyte and granulocyte cell concentrations during the PLA and BD trials.

	Pre-supplement	Pre-exercise	Post-SS	Post-TT	1-h post-TT
<i>Leukocytes ($\times 10^9$ cells·L⁻¹)^{a, c, d}</i>					
PLA	5.09 ± 0.95	4.96 ± 0.92	7.56 ± 2.71	10.94 ± 3.37	11.46 ± 2.53
BD	5.38 ± 1.08	5.40 ± 1.25	7.66 ± 1.90	11.08 ± 1.95	11.58 ± 2.89
ES (± 90% CL)	0.26 ± 0.21	0.39 ± 0.54	0.08 ± 0.41	0.12 ± 0.40	0.00 ± 0.70
<i>Lymphocytes ($\times 10^9$ cells·L⁻¹)^{a, c, e}</i>					
PLA	1.83 ± 0.44	1.65 ± 0.42	2.19 ± 0.60	3.11 ± 0.72	1.25 ± 0.21
BD	1.96 ± 0.48	1.84 ± 0.42	2.42 ± 0.60	3.11 ± 0.61	1.30 ± 0.26
ES (± 90% CL)	0.25 ± 0.30	0.41 ± 0.46	0.34 ± 0.55	0.00 ± 0.44	0.22 ± 0.49
<i>Monocytes ($\times 10^9$ cells·L⁻¹)^{b, c, d}</i>					
PLA	0.53 ± 0.15	0.48 ± 0.11	0.58 ± 0.11	0.76 ± 0.24	0.82 ± 0.15
BD	0.54 ± 0.14	0.54 ± 0.13 [#]	0.59 ± 0.17	0.78 ± 0.19	0.72 ± 0.16
ES (± 90% CL)	0.12 ± 0.15	0.51 ± 0.51	0.00 ± 0.60	0.14 ± 0.45	-0.65 ± 0.48
<i>Granulocytes ($\times 10^9$ cells·L⁻¹)^{a, c, d}</i>					
PLA	2.79 ± 0.82	2.89 ± 0.85	5.20 ± 2.62	7.51 ± 2.90	9.53 ± 2.49
BD	3.12 ± 1.19	3.12 ± 1.14	4.90 ± 1.66	7.50 ± 2.10	9.72 ± 2.80
ES (± 90% CL)	0.26 ± 0.32	0.25 ± 0.41	0.00 ± 0.38	0.06 ± 0.39	0.03 ± 0.69

Values are presented as mean ± SD. PLA *placebo* and BD *R,S-1,3-butanediol*. Main effect for time; significantly higher at post-SS compared to pre-exercise (^a $p < 0.001$; ^b $p < 0.05$). Main effect for time; significantly higher at post-TT compared to pre-exercise (^c $p < 0.001$). Main effect for time; significantly higher at 1-h post-TT compared to pre-exercise (^d $p < 0.001$). Main effect for time; significantly lower at 1-h post-TT compared to pre-exercise (^e $p < 0.001$).

6.4.5 Circulating T-cell subset concentrations

There was no difference for pre-supplement and pre-exercise circulating CD4⁺, CD8⁺ and CD25⁺ T-cell concentrations between trials (all $p > 0.05$) (Table 6.4, p. 98). There were no trial x time interactions for circulating CD4⁺, CD8⁺ and CD25⁺ T-cell concentrations (all $p > 0.05$). Similarly, the CD4⁺/CD8⁺ cell ratio did not differ between trials at pre-exercise or elicit a trial x time interaction (all $p > 0.05$). Main effects of time were observed for circulating CD4⁺ ($p < 0.001$), CD8⁺ ($p < 0.001$) and CD25⁺ ($p < 0.001$) T-cell concentrations, and the CD4⁺/CD8⁺ T-cell ratio ($p < 0.001$) (Table 6.4). Circulating CD4⁺, CD8⁺ and CD25⁺ T-cell were higher at post-SS ($p < 0.05$) and post-TT ($p < 0.001$) compared to pre-exercise, then declined to below pre-exercise concentrations at 1-h post-

TT ($p < 0.001$). This reduced the $CD4^+/CD8^+$ T-cell ratio at post-SS and post-TT compared to pre-exercise (all $p < 0.001$), and a higher $CD4^+/CD8^+$ T-cell ratio at 1-h post-TT ($p < 0.001$).

Table 6.2. Circulating T-cell subset concentrations during the PLA and BD trials.

	Pre-supplement	Pre-exercise	Post-SS	Post-TT	1-h post-TT
<i>CD4⁺ cells ($\times 10^9$ cells·L⁻¹)^{a, c, d}</i>					
PLA	0.82 ± 0.23	0.74 ± 0.22	0.87 ± 0.27	1.11 ± 0.22	0.56 ± 0.10
BD	0.87 ± 0.26	0.79 ± 0.21	0.96 ± 0.32	1.09 ± 0.25	0.58 ± 0.14
ES (± 90% CL)	0.19 ± 0.31	0.23 ± 0.43	0.29 ± 0.45	-0.10 ± 0.61	0.20 ± 0.45
<i>CD8⁺ cells ($\times 10^9$ cells·L⁻¹)^{b, c, d}</i>					
PLA	0.62 ± 0.21	0.54 ± 0.19	0.76 ± 0.30	1.23 ± 0.52	0.35 ± 0.10
BD	0.65 ± 0.25	0.61 ± 0.20	0.82 ± 0.36	1.13 ± 0.44	0.34 ± 0.08
ES (± 90% CL)	0.11 ± 0.32	0.32 ± 0.37	0.16 ± 0.40	-0.13 ± 0.31	-0.06 ± 0.26
<i>CD25⁺ cells ($\times 10^9$ cells·L⁻¹)^{a, c, d}</i>					
PLA	0.19 ± 0.11	0.17 ± 0.11	0.20 ± 0.10	0.27 ± 0.10	0.12 ± 0.09
BD	0.18 ± 0.12	0.18 ± 0.11	0.21 ± 0.11	0.24 ± 0.09	0.12 ± 0.07
ES (± 90% CL)	-0.12 ± 0.56	0.03 ± 0.50	0.03 ± 0.65	-0.44 ± 0.67	-0.09 ± 0.45
<i>CD4⁺/CD8⁺ (ratio)^{e, f}</i>					
PLA	1.38 ± 0.35	1.41 ± 0.32	1.22 ± 0.33	1.01 ± 0.30	1.67 ± 0.42
BD	1.41 ± 0.47	1.38 ± 0.41	1.28 ± 0.54	1.06 ± 0.38	1.76 ± 0.47
ES (± 90% CL)	0.03 ± 0.27	-0.16 ± 0.28	0.05 ± 0.38	0.11 ± 0.21	0.17 ± 0.22

Values are presented as mean ± SD. PLA *placebo* and BD *R,S-1,3-butanediol*. Main effect for time; significantly higher at post-SS compared to pre-exercise (^a $p < 0.05$; ^b $p < 0.001$). Main effect for time; significantly higher at post-TT compared to pre-exercise (^c $p < 0.001$). Main effect for time; significantly lower at 1-h post-TT compared to pre-exercise (^d $p < 0.001$). Main effect for time; significantly lower at post-SS and post-TT compared to pre-exercise (^e $p < 0.001$). Main effect for time; significantly higher at 1-h post-TT compared to pre-exercise (^f $p < 0.001$).

6.4.6 T-cell related cytokine mRNA expression within SEB-stimulated PBMCs

Quantifiable mRNA expressions of PBMC β_2 -MG, IFN- γ , IL-4 and IL-10 were detected in all SEB-stimulated whole blood samples. There were no significant trial x time interactions for PBMC IL-4, IL-10 or IFN- γ mRNA expression (all $p > 0.05$) (Figures 6.2A-C, p. 100). However, there were trends towards *moderate* to *large* increases in IFN- γ mRNA expression in the BD compared to PLA trial at post-SS ($d = 1.62 \pm 1.32$), post-

TT ($d = 0.77 \pm 0.68$) and 1-h post-TT ($d = 1.61 \pm 1.01$) (all $p > 0.05$) (Figure 6.2C). These effects underpinned a main effect of trial, with IFN- γ mRNA expression elevated in the BD compared to PLA trial ($p = 0.01$; $d = 0.38 \pm 0.20$). There was a trend for a trial x time interaction for the IFN- γ /IL-4 ratio ($p = 0.07$), with trends towards *moderate* increases in the IFN- γ /IL-4 ratio in the BD compared to PLA trial at pre-exercise ($d = 1.07 \pm 0.65$) and 1-h post-TT ($d = 0.91 \pm 0.50$) (Figure 6.2D, p. 100). Furthermore, main effects of time were observed for IL-4 mRNA expression ($p < 0.001$), IL-10 mRNA expression ($p < 0.001$), IFN- γ mRNA expression ($p < 0.001$) and the IFN- γ /IL-4 ratio ($p < 0.001$) (Figures 6.2A-D). Compared to pre-exercise, IL-4 mRNA expression was lower at post-SS ($p = 0.02$), post-TT ($p < 0.001$) and 1-h post-TT ($p < 0.001$). Whereas, IFN- γ mRNA expression and the IFN- γ /IL-4 ratio increased at post-SS and post-TT compared to pre-exercise, then declined to below pre-exercise values at 1-h post-TT (all $p < 0.001$). IL-10 mRNA expression remained stable throughout exercise, then declined to below pre-exercise values at 1-h post-TT ($p = 0.004$).

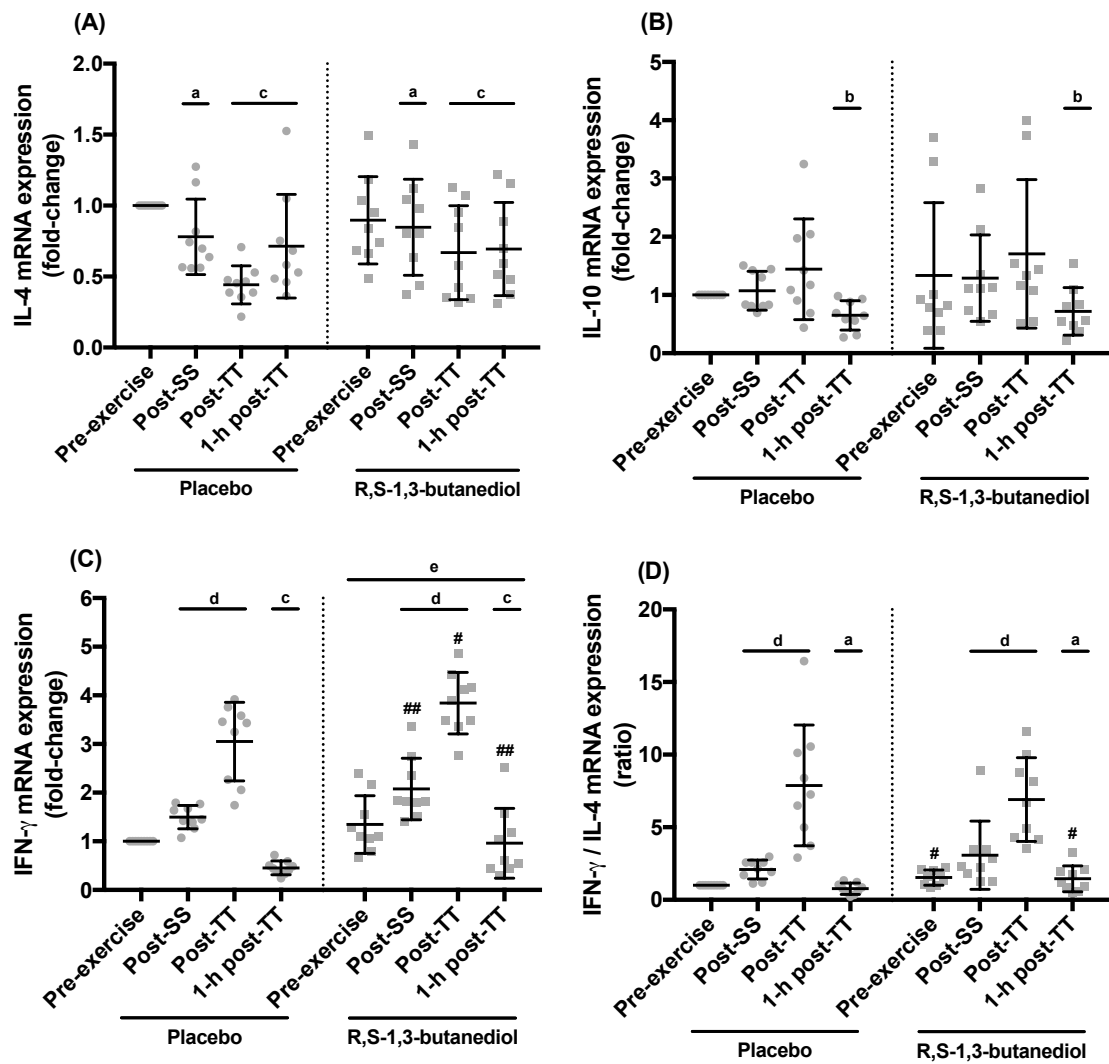


Figure 6.2. T-cell related cytokine mRNA expression within PBMCs following 24 h whole blood SEB-stimulation for (A) IL-4, (B) IL-10, (C) IFN- γ and (D) IFN- γ /IL-4 ratio during the PLA and BD trials. Values are presented as mean \pm SD and individual responses. Significantly higher in BD compared to PLA for time point ($*p < 0.05$). Effect size (d); #*moderate* and ##*large* effects for BD compared to PLA at time point. Main effect for time; significantly lower compared to pre-exercise ($^ap < 0.05$; $^bp < 0.01$; $^cp < 0.001$). Main effect for time; significantly higher compared to pre-exercise ($^dp < 0.001$). Main effect for trial; significantly higher compared to PLA ($^ep = 0.01$).

6.5 Discussion

The present study investigated the effects of BD ingestion and prolonged, strenuous exercise on T-cell related cytokine gene expression within stimulated PBMCs. The main findings were: 1) BD ingestion increased blood D- β HMB concentration; 2) BD ingestion increased T-cell related IFN- γ gene expression, with the magnitude of change greatest at post-SS, post-TT and 1-h post-TT; 3) BD ingestion did not alter T-cell related IL-4 or IL-10 gene expression. Moreover, exercise appeared to lead to the ingress of T-cells with a

higher capacity for IFN- γ gene expression into the blood, followed by their rapid egress during recovery. In contrast, T-cell related IL-4 and IL-10 gene expression declined following the onset and cessation of exercise, respectively. As such, exercise shifted the IFN- γ /IL-4 ratio towards IFN- γ predominance, which normalised after 1 h of recovery. Therefore, it appears hyperketonaemia transiently amplifies the type-1 T-cell cytokine response to an immune challenge at the gene level immediately following exercise.

Theoretically, the present findings would suggest acute hyperketonaemia via ketone supplementation acts as an immunological adjuvant following exhaustive exercise due to an enhanced activation of a pro-inflammatory T-cell related cytokine response. Typically, stimulated T-cell IFN- γ production declines during the early stages of recovery [205], which may increase susceptibility to viral infections and reactivation. This pro-inflammatory action of hyperketonaemia to an immune challenge in the present study is surprising considering KBs are often referred to as anti-inflammatory mediators of the immune system in nonexercising and disease models [43]. Further, KBs typically have a suppressive effect on CHO metabolism; therefore, on the basis that type-1 T-cell activation upregulates glucose metabolism, hyperketonaemia would have been expected to negate a pro-inflammatory cytokine response. Nevertheless, the increase in T-cell related IFN- γ gene expression within SEB-stimulated PBMCs in the present study appeared transient as it declined to below pre-exercise levels 1 h into recovery.

The mechanisms augmenting T-cell related IFN- γ gene expression following BD ingestion are difficult to identify. T-cell activation is highly dependent on energy derived from glucose [274-276] and elevated concentrations of stress hormones appear to suppress stimulated IFN- γ production [266,340]. However, no difference in serum glucose concentration was observed, with the exception of a trend towards a *small* reduction immediately following the TT in the BD trial. Additionally, serum cortisol concentration and RPE did not differ, indicating similar levels of metabolic stress between trials. Speculatively, elevated KBs could act as an additional energy source for immune cells, as lymphocytes can both produce [282] and oxidise KBs [51-53]. Hyperketonaemia could also exert its influence beyond immunometabolism, as recent evidence has shown ketone supplementation can increase pro-inflammatory LPS-stimulated monocyte IL-1 β production, which was attributed to NLRP3 activation [72].

However, not all studies are in agreement [318] and the role of NLRP3 activity in T-cell function and cytokine production is largely unknown.

Moreover, BD did not increase blood D- β HB to concentrations reciprocating nonracemic ketone supplements. This was observed by *small* effects on D- β HB concentration during exercise compared to the R-BD D- β HB monoester, which can increase blood D- β HB concentrations to $>2\text{-}3\text{ mmol}\cdot\text{L}^{-1}$ during exercise [73]. Similarly, the monoester did not alter cortisol concentration during 2 h of cycling at 70% $\text{VO}_{2\text{max}}$ when coingested with CHO compared to an isoenergetic CHO only solution, despite lowering plasma glucose concentration [73]. Therefore, it is possible small reductions in blood glucose concentration caused by hyperketonaemia are insufficient to alter the exercise-induced stress response; alternatively, the coingestion of CHO could have negated the exacerbating effect of reduced blood glucose concentration on the exercise-induced rise in cortisol in the aforementioned study. Hence, it is uncertain if nonracemic ketone supplements eliciting greater effects on blood D- β HB concentration and CHO metabolism exert a greater effect on the immune response to exercise.

Another interesting finding was the increase T-cell related IFN- γ gene expression following the SS and TT phases in both trials, despite elevated cortisol concentrations. In comparison, IL-4 gene expression declined following the onset of exercise and into recovery, with IL-10 gene expression lower 1 h into recovery compared to pre-exercise. This indicates that the exercise-induced rise in cortisol concentration may not suppress pro-inflammatory T-cell cytokine gene expression to an immune challenge, as originally hypothesised. Furthermore, elevations in cortisol concentration may not favour T-cell IL-4 and IL-10 production, which is in contrast to current paradigms [36]. The effect of exercise-induced rises in cortisol concentration on immune function is becoming less clear, as previous studies have suggested cortisol does not suppress immune markers using *in vitro* [341] and *in vivo* models [197,342]. Clearly, further research is required to understand the relationship between exercise, cortisol and immune function, and the modulating effect of CHO availability.

A more likely cause for the increase in T-cell related IFN- γ gene expression within SEB-stimulated PBMCs was due to T-cell subset trafficking patterns. In the present study, there was an increase in circulatory CD4^{+} and CD8^{+} lymphocyte concentration

immediately following exercise, which was likely due to an influx of cytotoxic (IFN- γ^+) T-cells from secondary lymphoid tissue [219,343]. As type-2 T-cell trafficking appears unaffected by the onset of exercise [24,25] and TReg cells may oppose the trafficking patterns of type-1 T-cells [31], the make-up of the PBMC population can transiently shifted towards a IFN- γ^+ T-cell phenotype immediately following exercise. Nevertheless, the increase in cortisol concentration could have altered T-cell function prior to their egress from the blood, which could impair immunosurveillance and cytotoxicity in peripheral tissues during recovery [185]; however, this is only speculative and requires further research.

A strength of the current study was the use of whole blood stimulation to accommodate for the hormonal, substrate and cellular shifts resulting from exercise. Compared to isolated cell cultures, this provides an *in vitro* model that more closely reflects *in vivo* immune regulation [22]. Furthermore, SEB as an immunological stimulus has been well established to model a lymphocyte-driven cytokine response in whole blood cultures [243]. SEB binds outside of the major histocompatibility complex class II and *nonspecifically* activates up to 20-25% of the T-cell population displaying the V β T-cell receptor [242,244]. This interaction triggers a pro-inflammatory response and the proliferation of T-cells bearing specific V β subgroups [242-244]. The concentration of SEB (100 ng·ml⁻¹) was chosen based on an earlier study which demonstrated a plateau in whole blood stimulated IFN- γ production at 100 ng·ml⁻¹ [243].

By measuring cytokine gene expression relative to the housekeeping gene, β_2 -MG, shifts in total cell number within the PBMC population were able to be accommodated. However, β_2 -MG is not specific to T-cells as it is an integral component of the major histocompatibility complex class I molecule, which is present on all nucleated cells of the human body [344]. As the cellular make-up of the PBMCs includes various other lymphocyte subsets (B-lymphocytes, gamma delta T-cells, T-helper 17 cells and natural killer cells) and monocytes, gene expression levels in the present study cannot be exclusively extrapolated to the T-cell population. Nevertheless, T-cells are the most abundant lymphocyte subset within the PBMC population and are the primary producers of the cytokines under investigation; therefore, it is reasonable to suggest any shifts in cytokine gene expression are underpinned by changes to circulatory T-cell function or phenotype.

6.6 Conclusion

Acute hyperketonaemia via BD ingestion transiently amplified T-cell related IFN- γ gene expression within SEB-stimulated PBMCs during and following prolonged, strenuous exercise; this suggests an enhanced initiation of a pro-inflammatory type-1 T-cell response to an immune challenge. As cortisol concentration did not differ between the BD and PLA trials, it is possible the increased availability of energetic substrate from elevated blood D- β HB concentrations altered T-cell function *in vitro*. Whether this relates to cytokine production and illness risk *in vivo* is uncertain, but it does suggest ketone and/or ketogenic supplements that increase blood D- β HB concentrations up to ~ 1 mmol·L⁻¹ may be used potentially without exacerbating exercise-induced immunodepression. Nevertheless, further research into the effects of acute hyperketonaemia on immune function is warranted, particularly following the ingestion of ketone supplements that markedly alter CHO metabolism.

6.7 Epilogue

Based on Chapters 5 and 6, it appears that acutely elevated blood D- β HB concentration to ~ 1 mmol·L⁻¹ via BD ingestion has minimal effect on endurance performance and may transiently augment a pro-inflammatory T-cell related cytokine response to an immune challenge following exercise. Whether adaptation to a KD exerts similar effects on immune function is uncertain as it not only increases blood KB concentration, but also increases fat oxidation rates to compensate for reductions in CHO availability (i.e. skeletal muscle and hepatic glycogen). As there is an increase in blood cortisol concentration following exercise compared to higher-CHO conditions [312], stimulated T-cell IFN- γ production may be preferentially suppressed. However, adaptation to a KD reduces CHO oxidation during exercise, thus lowering the rate of glycogen utilisation [15,35]; therefore, when submaximal intensity exercise is performed to exhaustion, compared to matched-workload protocols, post-exercise CHO availability can be similar [15], and may influence post-exercise cortisol concentrations towards unity. These adaptations may also preserve exercise capacity at submaximal exercise intensities [15]; albeit, this is yet to be confirmed using acute fuelling strategies typically implemented in real world competition. Therefore, the effect of a 31-day KD on submaximal intensity endurance capacity and exercise-induced immune perturbations (i.e. T-cell related

cytokine gene expression within stimulated PBMCs and SIgA) is examined in Chapters 7 and 8, respectively.

The effect of a 31-day ketogenic diet on
submaximal-intensity exercise capacity and
efficiency

7.1 Abstract

This study investigated the effect of a 31-day KD on submaximal exercise capacity and efficiency. A repeated-measures, crossover study was conducted in eight trained male endurance runners ($\text{VO}_{2\text{max}}$, $59.4 \pm 5.2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Participants ingested their habitual diet (HD) (13.1 MJ, 43% [$4.6 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 38% [$1.8 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) or an iso-energetic KD (13.7 MJ, 4% [$0.5 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 78% [$4 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) from day 0 to 31 ($p < 0.001$). On days -2 and 29, participants undertook a fasted, graded metabolic test ($\sim 25 \text{ min}$); and on days 0 and 31, participants completed a run-to-exhaustion trial at 70% $\text{VO}_{2\text{max}}$ ($\sim 12.9 \text{ km}\cdot\text{h}^{-1}$) following the ingestion of a high-CHO meal ($2 \text{ g CHO}\cdot\text{kg}^{-1}$) or an iso-energetic low-CHO, high-fat meal ($<10 \text{ g CHO}$), with CHO ($\sim 55 \text{ g}\cdot\text{h}^{-1}$) or iso-energetic fat (coconut oil) supplementation ($0 \text{ g CHO}\cdot\text{h}^{-1}$) during exercise. Training load did not differ between trials and there was no effect of diet on $\text{VO}_{2\text{max}}$ (all $p > 0.05$). The KD impaired exercise efficiency, particularly at $>70\%$ $\text{VO}_{2\text{max}}$, as evidenced by oxygen uptake that could not be explained by shifts in RER and increased EE (all $p < 0.05$). However, exercise efficiency was maintained on a KD when exercising at $<60\%$ $\text{VO}_{2\text{max}}$ (all $p > 0.05$). There was no effect of diet on TTE (pre-HD, 237 ± 44 vs. post-HD, $231 \pm 35 \text{ min}$; $p = 0.44$ and pre-KD, 239 ± 27 vs. post-KD, $219 \pm 53 \text{ min}$; $p = 0.36$). Following keto-adaptation, an RER >1.0 compared to <1.0 at $\text{VO}_{2\text{max}}$ coincided with the preservation and reduction in TTE, respectively. In conclusion, a 31-day KD preserved mean submaximal exercise capacity in trained endurance athletes without necessitating acute CHO fuelling strategies.

7.2 Introduction

Humans elicit numerous metabolic adaptations in response to shifts in dietary macronutrient intake to reconcile substrate availability with EE. During continuous submaximal endurance exercise (>3-4 h), exhaustion appears to be associated with depleted endogenous CHO stores (i.e. skeletal muscle [119] and hepatic [120] glycogen) and the inability to maintain the CHO oxidation rates exhibited during the early stages of exercise [119,121]. As such, various dietary-training strategies have been proposed to spare finite glycogen stores (~700 g) and to optimise competition fuelling [8]. Carbohydrate loading and supplementation appear to be the most efficacious strategies for prolonging exercise capacity and improving endurance performance by maximising glycogen content and preserving CHO oxidation rates throughout exercise [5,119,121]. However, interest has persisted in chronic adaptation to low-CHO diets in an attempt to spare endogenous CHO stores by maximising fat oxidation rates and increasing hepatic production of fatty acid-derived KB as an additional fuel source [345].

Very low-CHO, high-fat, KDs contrast typical recommendations for endurance athletes. These are characterised by CHO intake <50 g·day⁻¹ and elevated circulating KB (primarily D-βHB) concentrations >0.5 mmol·L⁻¹ [9], although concentrations >0.2 mmol·L⁻¹ may be accepted [10]. The term *keto-adaptation* has been used to encompass the metabolic adaptations resulting from a KD, which include; 1) an increase in MFO to >1 g·min⁻¹ [14,15,48]; 2) a reduction in blood glucose utilisation [15] and; 3) a reduction in muscle [15,35] and hepatic [35] glycogen utilisation. The importance of KBs to EE is uncertain; however, it is postulated as the defining feature differentiating adaptation to keto- versus nonketogenic, lower-CHO (~2.5 g·kg⁻¹·day⁻¹ or <25 % EI), higher-fat diets (i.e. fat-adaptation). It appears a minimum of 3-4 weeks is required to overcome the initial performance decrement associated with a KD [14,15] and, despite suggestion that several months is required to optimise keto-adaptation, the only studies having investigated athletes ingesting a KD for this duration did not examine performance [35,48] or failed to rigorously monitor dietary intake and training volume [174].

Recently, a 3 week KD was shown to negate high-intensity exercise performance in a ~45 min TT following 3 weeks of intensified training in elite race walkers [14]. However, performance during prolonged, high-intensity events (<2-3 h) demands high rates of

CHO, not fat, oxidation [5,8]. As such, keto- (or fat-) adaptation is more likely to benefit submaximal exercise events lasting several hours as the practically infinite fat stores become the preferred energetic substrate. A single study has investigated the effect of a KD on submaximal exercise capacity (62-64% $\text{VO}_{2\text{max}}$), with the researchers employing a single-arm design with the pre-test acting as the CHO-diet trial and the post-test following 4 weeks of ingesting a KD [15]. Of the five trained cyclists, three improved and two impaired their TTE, resulting in no overall difference between dietary conditions (~ 147 vs. ~ 151 min). However, there was the potential of an order- or training-effect and the results appeared heavily skewed by the improvement of a single participant from 148-232 min. Additionally, for the CHO-diet trial, participants commenced exercise following an overnight fast and did not supplement with CHO during exercise, which is incongruent with recommended performance nutrition strategies [5,173]. Therefore, the study design appeared to favour the keto-adapted trial.

In the same study, the researchers stated the efficiency of substrate oxidation improved following keto-adaptation due to a similar oxygen uptake at the same absolute workload [15]. As the stoichiometry of fat compared to CHO oxidation requires more oxygen for combustion to generate an identical energy yield, it would have been expected that oxygen uptake increased following the KD if exercise efficiency was maintained. This increased oxygen cost of exercise following keto- or fat-adaptation may impair exercise efficiency during prolonged, high-intensity endurance exercise [14]. However, the shift in oxygen uptake at submaximal intensities may simply be a reflection of substrate preference, not exercise efficiency [154]. More appropriate measures of exercise efficiency may be the energy cost of exercise [154] and the discrepancy between measured and predicted oxygen uptake based on shifts in the RER [157,158]. Although differences in these measures tend to be subtle, they may elicit significant effects on submaximal exercise capacity.

To date, no studies have investigated the effect of keto-adaptation on submaximal exercise capacity in trained endurance athletes using a repeated-measures, crossover design and acute fuelling strategies to polarise substrate availability and metabolism. In concordance, the aim of the present study was to examine the effect of a 31-day KD on submaximal endurance capacity, substrate utilisation and exercise efficiency.

7.3 Methods

7.3.1 Study design

This study was conducted during the maintenance training phase for all participants. Participants underwent two 31-day experimental conditions (i.e. KD or their mixed HD) with a testing block immediately prior and during the final three days of the intervention in a randomised (www.randomizer.org), counterbalanced, crossover design, with a 14-21 day washout period between dietary interventions. A schematic overview of the study design is shown in Figure 7.1.

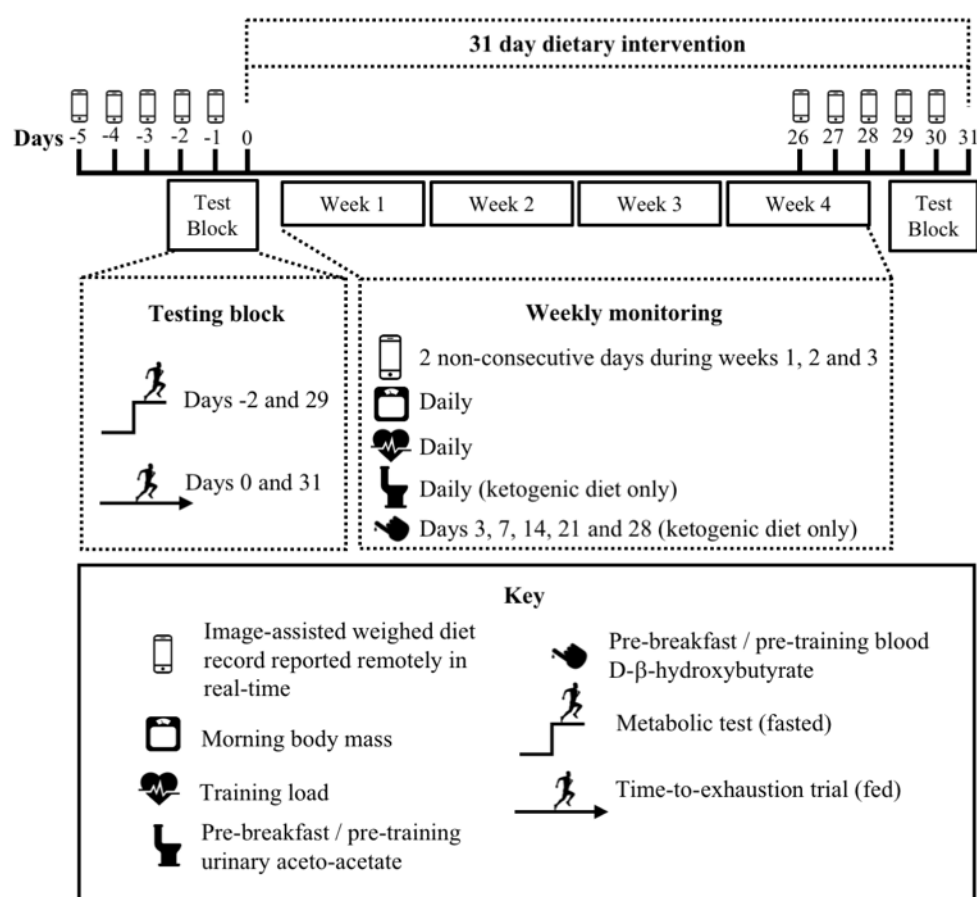


Figure 7.1. An overview of the study design showing testing blocks, dietary and training monitoring, and the dietary intervention period.

7.3.2 Participants

Participants were required to have been; 1) habitually consuming a mixed diet for >12 months; 2) weight stable for >1 month; 3) running >50 km·week⁻¹ and; 4) able to run a marathon in <3.5 h. Participants were excluded if they; 1) had a history of fat- or keto-adaptation; 2) previously ingested ketone supplements; 3) were currently or recently injured; 4) experienced moderate-to-severe gastrointestinal symptoms or illness within the previous 4 weeks; 5) had a history of irritable bowel syndrome; 6) habitually smoked or; 7) had been ingesting dietary supplements or medications known to effect performance within the previous 2 weeks, with the exception of caffeine, protein and CHO supplements. Ten eligible healthy, trained, male endurance athletes (two marathoners, five ultra-marathoners and three long-distance triathletes) volunteered to participate in the study. Participants were comprehensively screened by an experienced RD following their expression of interest and completed a comprehensive health and eligibility screening questionnaire (Appendix D) to ascertain if they were suitable for the study. Participants were fully informed of the rationale of the study and possible risks of the experimental procedures before providing their written consent (Appendix F). However, participants were not informed of the potential performance effects of a KD and were requested to refrain from personal investigation to prevent biasing their results. The study was approved by the Auckland University of Technology Ethics Committee (Auckland, New Zealand).

7.3.3 Testing block

7.3.3.1 Metabolic and body composition testing (Days -2 and 29)

Participants presented to the laboratory between 06:00 and 07:00 h having fasted from 23:00 h the previous day and abstained from caffeine, alcohol and strenuous exercise for the previous 24 h. Participants' body mass (shorts only), height and $\Sigma 8$ skinfolds were measured by an accredited anthropometrist (ISAK, level 1, skinfold CV of 3.8%). To determine $\text{VO}_{2\text{max}}$, maximal aerobic EE (EE-aero_{max}), MFO and Fat_{max}, participants performed a graded metabolic test to volitional exhaustion on a motorised treadmill (h/p/cosmos, Germany). Participants ran for 3 min stages at 7.5, 9, 10.5, 12, 13.5 and 15 km·h⁻¹ against a 1% gradient to simulate the energetic cost of level-gradient outdoor

running [346]. If RER was ≥ 1.0 at $13.5 \text{ km}\cdot\text{h}^{-1}$, the $15 \text{ km}\cdot\text{h}^{-1}$ stage was excluded. Expired gas was collected and analysed continuously using a computerised metabolic system with mixing chamber (Parvo Medics TrueOne 2400, Salt Lake City, Utah, USA), with the final 30 sec of each stage averaged to calculate VO_2 and VCO_2 . The second ventilatory threshold (VT_2) was determined using the V-slope method [330]. Ratings of perceived exertion (Borg 6-20 scale [329]) and HR using short range telemetry (Garmin Fenix 3, Garmin, Kansas, USA) were recorded during the final 30 sec of each stage. Following the completion of the final stage, treadmill speed was reduced to $11 \text{ km}\cdot\text{h}^{-1}$ and subsequently increased by $0.5 \text{ km}\cdot\text{h}^{-1}$ every 30 sec until the attainment of volitional exhaustion. $\text{VO}_{2\text{max}}$ was determined by averaging the highest 30 sec period and accepted if there was at least a 30 sec plateau in VO_2 and one of the following two criteria; 1) $\text{RER} > 1.10$ or; 2) $\text{HR} \pm 10 \text{ beats}\cdot\text{min}^{-1}$ age predicted maximum ($220 - \text{age}$). Simple regression equations were used to estimate the speed required to elicit 70% $\text{VO}_{2\text{max}}$ for the use in the run-to-exhaustion (RTE) trial, until which participants were requested to refrain from strenuous exercise ($\sim 48 \text{ h}$).

7.3.3.2 Run-to-exhaustion trial (days 0 and 31)

Participants presented to the laboratory between 06:00 and 07:00 h having abstained from caffeine and alcohol for the previous 24 h. Following the metabolic test on either day -2 or 29, participants were requested to ingest a high-CHO diet and consume adequate fluid to replicate their typical race preparation. Participants ingested a prescribed breakfast containing $2 \text{ g}\cdot\text{kg}^{-1}$ of CHO 90 min prior to arrival to replicate typical race-day nutrition. In the post-KD trial, participants continued ingesting a KD following the metabolic test and were prescribed an isoenergetic LCHF breakfast ($< 10 \text{ g CHO}$). Participants collected their first morning void to measure hydration status via urine specific gravity (USG) with a pre-exercise hypohydration threshold set at > 1.025 . Soon after arrival, an indwelling intravenous teflon catheter (18G, Terumo, Japan) was inserted into the antecubital vein for serial venous blood sampling and body mass was measured (shorts only). Participants then commenced running at the speed estimated to elicit 70% $\text{VO}_{2\text{max}}$ until volitional exhaustion (i.e. 120-135 min post-breakfast). Treadmill speed was matched during the pre- and post-diet trials. Participants were prescribed $4 \text{ ml}\cdot\text{kg}^{-1}$ of a 7.2% CHO-electrolyte drink (4:1 glucose to fructose ratio; Replace, Horleys, New Zealand) every 20 min, which was adjusted based on each participant's tolerance and replicated in their post-diet trial.

During the post-KD trial, participants received a combination of artificially sweetened fluids (electrolyte drink and cordial), water and coconut oil (100% energy derived from fat) (Blue Coconut Oil, Blue Coconut, New Zealand) at a rate reciprocating the fluid and energy ingested during the pre-KD trial. Expired gas was collected for 4-5 min every 30 min and at exhaustion, with the final 1 min averaged to calculate VO_2 and VCO_2 , alongside RPE [329] and HR (Garmin Fenix 3, Garmin, Kansas, USA). On attainment of volition exhaustion, treadmill speed was reduced to $4.4 \text{ km}\cdot\text{h}^{-1}$ for 2 min, and then restored to the speed eliciting 70% $\text{VO}_{2\text{max}}$ until the participant indicated volitional exhaustion. This process was repeated so at the third attainment of volitional exhaustion the test was terminated. The walking time was excluded from the total TTE. This protocol has a lower CV for measuring exercise capacity compared to traditional single exhaustion protocols (5.4%, 1.4-9.6 [95% CI]) [347]. Stimulatory aids (i.e. television, music and conversation) were provided until the end of the first exhaustion phase to reduce boredom. Immediately following exercise cessation, participants removed wet clothing and towel dried themselves prior to measuring their body mass. All trials were conducted by the same researcher and standardised encouragement was provided. To ensure maximal effort in each trial, a substantial monetary incentive was awarded to the participant who accumulated the highest TTE following completion of the four trials. Participants were blinded to elapsed time during each trial and were not notified of their results until study completion. Participants were then provided with $5 \text{ ml}\cdot\text{kg}^{-1}$ of water and rested for 1 h prior to departing

7.3.4 Dietary intervention and monitoring

Participants commenced their dietary allocation (KD or HD) immediately upon completion of their initial RTE trial (i.e. day 0) as theoretically the ability to restore depleted muscle and hepatic glycogen led to rapid differentiation between the two dietary conditions. To ensure immediate and ongoing compliance to the KD, participants undertook a comprehensive education session with a RD following the metabolic test to provide sufficient preparation time. The education session included the following; 1) provision of a KD information booklet specifically developed for this study (Appendix G); 2) three-day menu plan specific to the participant's energy requirements, dietary preferences and tolerances as determined by prior dietary review; 3) 7-day example menu plan for additional meal ideas; 4) extensive list of snack ideas and; 5) lifestyle, dining-

out, shopping, cooking and budgetary advice. To further support dietary compliance, participants were required to have daily contact with a RD, which included unlimited daily access (phone and email). The prescribed KD contained ≤ 50 g·day⁻¹ CHO, 15-20% EI from protein and 75-80% EI from fat. Each participant was provided with coconut oil, extra virgin olive oil, LCHF cereal, and (for both dietary conditions) discounted fruit and vegetables. Participants were requested to refrain from alcohol and dietary supplement use for the duration of the study.

To monitor compliance to each dietary intervention, participants were trained in dietary reporting and provided an image-assisted (alongside a fiducial marker) weighed dietary record reported remotely in real-time to a RD via a mobile phone application (WhatsApp, Facebook, California, USA) for the 5 days preceding each RTE trial, a minimum of two nonconsecutive days between days 1-7, 8-14 and 15-21, and the morning of the trial to confirm the ingestion of the prescribed breakfast (Appendix H). Where under-reporting was suspected, participants were required to provide a 24 h dietary recall or repeat the dietary report the subsequent day. Each dietary record was coded (FoodWorks Professional Edition, Version 8, Xyris Software, Australia), with images validating the reported intakes, by a RD and checked for accuracy by a second RD. To help maintain energy balance, participants reported their morning body mass daily and were advised to prevent a >2% fluctuation. Verification of compliance to the KD was via daily self-measurement of urinary AcAc concentrations with a semi-quantitative (colour range) strip (Ketostix, Bayer) and capillary blood D- β HB concentrations on days 3, 7, 14, 21 and 28 prior to ingesting breakfast and exercising. Capillary blood D- β HB concentrations were also measured immediately before the post-KD metabolic test by the primary researcher. Images of the results were immediately sent to the primary researcher for quantification of ketosis. Colour comparisons were made using the urinary AcAc strips in a subgroup (n = 4) of participants when ingesting a HD to dismiss the potential of false positives.

7.3.5 Training monitoring

Participants designed their own 28-day training schedule and were asked to replicate this during each diet. This included a combination of running and cycling. Participants reported their resting morning HR (HR_{rest}) and training data for each session, which included session duration (min), average HR and RPE. These variables were used to calculate Banister training impulse (TRIMP) and session RPE (sRPE) [348]. Because the intervention was applied under free-living conditions, all other lifestyle choices were allowed to vary naturally.

7.3.6 Blood sampling and analysis

Capillary blood D- β HB was measured (Freestyle Optium Neo, Abbott Diabetes Care, Victoria, Australia) and venous blood samples were collected at pre-exercise, 1-h exercise, 2-h exercise, exhaustion and 1-h post exhaustion into 8 ml serum vacutainers (Becton Dickinson and Co, USA) with the participants seated in an upright position. The cannula was flushed with 3-4 ml of saline every 30 min to maintain patency. Each serum vacutainer was left to clot for 30 min prior to centrifugation at 1500 g for 10 min at 4 °C and separation into two 1.5 ml aliquots to be stored at -80 °C prior to the analysis of glucose concentration (Cobas Modular P800 Analyser, Roche Diagnostics, New Zealand). Capillary blood lactate (Lactate 2 Pro, Akray, Japan) concentration was measured at pre-exercise, 1-h exercise, 2-h exercise and exhaustion. All capillary blood samples were collected from participants' fingertips using standardised techniques.

7.3.7 Calculation of whole body rates of substrate oxidation and energy expenditure

Whole body rates of CHO oxidation, fat oxidation and EE were calculated from SS VO_2 and VCO_2 using nonprotein RER values [151]. During the KD trials, no corrections to the equations were implemented as the oxidation of fat-derived KBs do not alter the stoichiometry [151]. To ascertain $EE_{aero-max}$, VO_{2max} was multiplied by 21.745 $J \cdot ml^{-1} O_2$, assuming glucose was the only substrate oxidised [349]. Fat oxidation rates obtained during the metabolic test were depicted graphically as a function of exercise intensity (% VO_{2max}) and a 3rd order polynomial curve with intersection in (0,0) was constructed to

determine MFO and Fat_{max} . A 3rd order polynomial curve with intersection was chosen based on the best curve fit and with the assumption that if VO_2 was zero, no fat oxidation would be observed.

To compare exercise efficiency between the pre- and post-KD conditions, calculations were adapted from previously published sources [151] to predict VO_2 based on shifts in RER with unchanged efficiency (see equation below). Discrepancies between predicted and measured VO_2 were used to quantify unaccounted oxygen uptake (i.e. oxygen uptake that cannot be explained by shifts in RER alone).

$$EE \text{ (kcal)} = (0.55 \times VCO_2) + (4.471 \times VO_2)$$

Therefore,

$$1) \quad EE / VO_2 = ([0.55 \times VCO_2] / VO_2) + 4.471$$

Since $RER = VCO_2 / VO_2$

$$2) \quad EE / VO_2 = (0.55 \times RER) + 4.471$$

$$3) \quad EE = ([0.55 \times RER] + 4.471) \times VO_2$$

Therefore, assuming exercise efficiency is maintained

$$4) \quad \text{Predicted } VO_2 = EE / ([0.55 \times \text{Post-KD RER}] + 4.471)$$

$$5) \quad \text{Predicted } VO_2 = ([0.55 \times \text{Pre-KD } VCO_2] + [4.471 \times \text{Pre-KD } VO_2]) / ([0.55 \times \text{Post-KD RER}] + 4.471)$$

Energy conversion: 1 kcal = 4.18 kJ

7.3.8 Data analysis

All data are expressed as mean (\pm SD) unless otherwise stated. The mean difference (Δ) (\pm 90% CL) between interventions and pre- to post-intervention are also expressed. Data were checked for normal distribution using the Shapiro-Wilk tests and, where appropriate, statistical analysis was performed on the logarithmic transformation of the data (i.e. Metabolic test: RPE; RER; relative VO_2 ; and relative EE; and RTE trial: glucose and lactate concentrations; RER; CHO oxidation; and absolute EE). For cardiorespiratory data, values were averaged for each hour and only used in analysis if $n = 100\%$ for all pre- and post-intervention trials. For urinary AcAc concentrations, weekly values were averaged for each participant. A three-way (diet \times adaptation \times intensity / time for

cardiorespiratory and metabolic variables) or two-way (diet x adaptation for exercise capacity and body composition variables or adaptation x intensity / time for predicted vs. measured oxygen uptake) repeated-measures ANOVA was performed and, if Mauchly's test of sphericity was violated, adjustments to the degrees of freedom were made for the ANOVA using Greenhouse-Geisser ϵ (IBM SPSS Statistics software, version 21, IBM Corp.). Where a significant effect was observed, post-hoc analysis was conducted using Holm-Bonferroni adjustments for multiple comparisons. Within-group changes from pre- to post-intervention were examined using adjusted Student's paired t -tests for dependant variables. Significance level was accepted at an alpha of $p < 0.05$. To interpret the magnitude of effect and to identify trends within nonsignificant data, Cohen's d ES ($\pm 90\%$ CL) were estimated using a purpose-built spreadsheet [333], with ES thresholds set at <0.2 , >0.2 , >0.6 , >1.2 , >2.0 and >4.0 for *trivial*, *small*, *moderate*, *large*, *very large* and *extremely large* effects, respectively. If the 90% CLs overlapped 0, the magnitude of effect was deemed *unclear*.

7.4 Results

7.4.1 Participants

Two participants were excluded during the study due to a combination of poor dietary reporting, poor dietary compliance to the KD and low training load, thus giving a sample size of $n = 8$ (two marathoners, four ultra-marathoners and two long-distance triathletes) (age, 29.6 ± 5.1 y; body mass, 73.1 ± 6.9 kg; height, 1.81 ± 0.05 m; BMI, 22.4 ± 1.7 kg·m²; VO_{2max}, 59.4 ± 5.2 ml·kg⁻¹·min⁻¹; hours training per week, 10.4 ± 1.6 h; years training, 9.0 ± 3.5 y).

7.4.2 Dietary intake and training load

There was no diet x adaptation interaction for body mass ($p = 0.083$); however, main effects for diet ($p = 0.011$) and adaptation ($p < 0.001$) were observed. Body mass was lower in the KD compared to HD ($p < 0.002$) and after, compared to before, adaptation ($p = 0.015$). There was a significant diet x adaptation interaction for $\Sigma 8$ skinfolds ($p = 0.05$), with a significant *moderate* reduction at post- compared to pre-KD ($\Delta = -5.2 \pm 2.7$ mm; $p = 0.028$; $d = -0.60 \pm 0.35$). There was no difference for pre-intervention dietary

macronutrient and energy intake between groups (all $p > 0.05$; $d = \text{unclear or trivial}$) (data not shown). Dietary intake during each intervention is summarised in Table 7.1 (p. 121). There was no difference in daily energy intake between diets ($p = 0.49$; $d = 0.35 \pm 0.92$). Protein intake did not differ between diets ($p = 0.99$, $d = 0.01 \pm 1.08$). However, in the KD compared to HD, there was a significant *large* reduction in CHO intake ($\Delta = -302 \pm 49 \text{ g}\cdot\text{day}^{-1}$; $p < 0.001$; $d = -3.44 \pm 0.64$) and a significant *extremely large* increase in fat intake ($\Delta = 152 \pm 35 \text{ g}\cdot\text{day}^{-1}$; $p < 0.001$; $d = 4.57 \pm 1.20$). During the KD, all participants demonstrated positive urinary ketones by day three and for 95.5% of participant days. For blood D- β HB, all participants had elevated concentrations of $\geq 0.3 \text{ mmol}\cdot\text{L}^{-1}$ by day three and $\geq 0.5 \text{ mmol}\cdot\text{L}^{-1}$ by day seven. Participants demonstrated blood D- β HB concentrations $\geq 0.5 \text{ mmol}\cdot\text{L}^{-1}$ on 87.5% of participant days from day three onwards (Figure 7.2). Training load did not differ between diets as determined by total TRIMP ($p = 0.39$; $d = 0.25 \pm 0.51$) and total sRPE ($p = 0.59$; $d = -0.17 \pm 0.56$) (Table 7.2, p. 122). However, there was a trend towards a *small* increase in total running TRIMP during the KD compared to HD ($\Delta = 279 \pm 220$; $p = 0.08$; $d = 0.27 \pm 0.25$), despite total running distance not differing (253.7 ± 112.8 vs. $249.7 \pm 104.9 \text{ km}$; $p = 0.71$; $d = 0.03 \pm 0.16$).

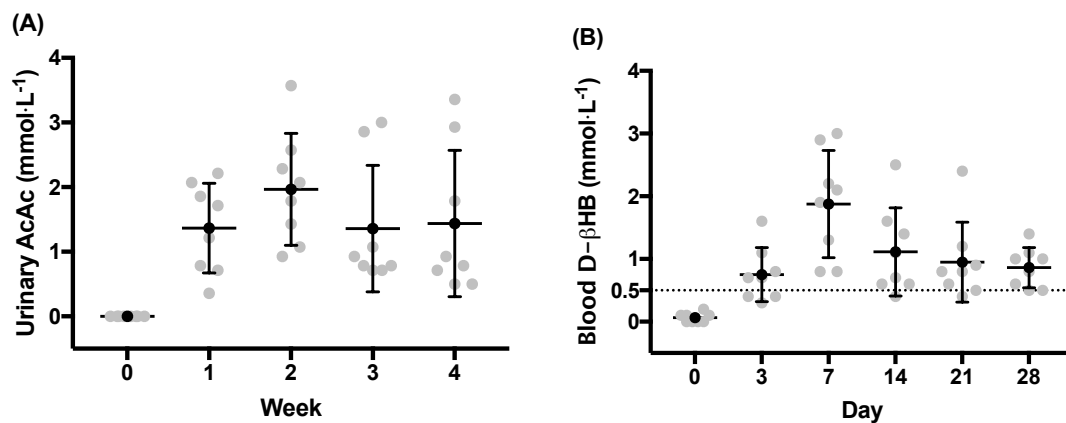


Figure 7.2. Ketone body concentrations during keto-adaptation. Presented as (A) mean \pm SD and individual weekly urinary acetoacetate (AcAc) concentration and (B) mean \pm SD and individual capillary blood D- β -hydroxybutyrate (D- β HB) concentration.

7.4.3 Cardiorespiratory variables, perceived exertion and substrate oxidation during the metabolic test

For the post-KD test, blood D- β HB concentration was $0.94 \pm 0.45 \text{ mmol}\cdot\text{L}^{-1}$ on arrival to the laboratory. There were no diet x adaptation interactions or main effects for $\text{VO}_{2\text{max}}$, $\text{EE}_{\text{aero-max}}$, VT_2 or HR_{max} (all $p > 0.05$; all $d = \text{trivial}$ or *unclear*) (Table 7.3, p. 123; Figure 7.3, p. 124). However, there were diet x adaptation interaction for $\nu\text{VO}_{2\text{max}}$ ($p = 0.01$), with a significant *moderate* reduction in the post- compared to pre-KD test ($\Delta = -1.0 \pm 0.8 \text{ km}\cdot\text{h}^{-1}$; $p = 0.025$; $d = -0.92 \pm 0.52$). There were no interactions or main effects for HR or RPE (all $p > 0.05$). A significant diet x adaptation x intensity interaction was observed for absolute VO_2 ($p = 0.003$), with a *small* nonsignificant increase at $12 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 0.13 \pm 0.11 \text{ L}\cdot\text{min}^{-1}$; $p = 0.22$; $d = 0.34 \pm 0.31$) and a significant *moderate* increase at $13.5 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 0.29 \pm 0.10 \text{ L}\cdot\text{min}^{-1}$; $p = 0.011$; $d = 0.66 \pm 0.27$) in the post- compared to pre-KD test (Table 7.3). Relative to body mass, there was a significant diet x adaptation x intensity interaction for VO_2 ($p = 0.011$), with a trend towards a *moderate* increase at $12 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 2.7 \pm 1.5 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.082$; $d = 0.70 \pm 0.47$) and a significant *large* increase at $13.5 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 5.0 \pm 1.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.009$; $d = 1.30 \pm 0.52$) during the post- compared to pre-KD test (Figure 7.3A).

There were significant diet x adaptation interactions for RER during the metabolic test ($p = 0.001$) and RER at $\text{VO}_{2\text{max}}$ ($p = 0.004$) (Table 7.3). In the post- compared to pre-KD test, there was a significant reduction in RER across all intensities ($p < 0.001$) and a *very large* reduction in RER at $\text{VO}_{2\text{max}}$ ($\Delta = -0.14 \pm 0.04$; $p = 0.002$; $d = -2.84 \pm 1.07$). No differences were observed between the pre- and post-HD tests for RER (all $p > 0.05$). There were significant diet x training interactions for MFO and FAT_{max} (all $p < 0.001$), with a significant *extremely large* increase in MFO (0.57 ± 0.10 vs. $1.12 \pm 0.10 \text{ g}\cdot\text{min}^{-1}$; $p < 0.001$; $d = 4.95 \pm 1.07$) and FAT_{max} (43 ± 5 vs. $70 \pm 4\% \text{ VO}_{2\text{max}}$; $p < 0.001$; $d = 5.05 \pm 0.82$) in the post- compared to pre-KD test. The HD intervention had no effect on MFO (0.57 ± 0.11 vs. $0.60 \pm 0.12 \text{ g}\cdot\text{min}^{-1}$; $p = 0.43$; $d = 0.20 \pm 0.25$) or FAT_{max} (45 ± 7 vs. $44 \pm 3\% \text{ VO}_{2\text{max}}$; $p = 0.42$; $d = -0.18 \pm 0.39$). All participants exhibited MFO rates $> 1.0 \text{ g}\cdot\text{min}^{-1}$ and $\text{FAT}_{\text{max}} > 65\% \text{ VO}_{2\text{max}}$ in the post-KD test.

7.4.4 Exercise efficiency during the metabolic test

In the post-KD test, there was a significant adaptation x intensity interaction for predicted compared to measured VO_2 ($p < 0.001$), with a significant *large* increase in measured VO_2 at $13.5 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 4.4 \pm 1.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.01$, $d = 1.25 \pm 0.53$) (Figure 7.3C, p. 124). The shift in RER from the pre- to post-KD trial explained $14 \pm 8\%$ of the increase in VO_2 at $13.5 \text{ km}\cdot\text{h}^{-1}$. Furthermore, there were significant diet x adaptation x intensity interactions for absolute EE ($p < 0.001$) and EE relative to body mass ($p = 0.011$) (Table 7.3, p. 123 and Figure 7.3D, p. 124; respectively). There was a significant *small* increase in absolute EE at $13.5 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 5.1 \pm 2.2 \text{ kJ}\cdot\text{min}^{-1}$; $p = 0.026$; $d = 0.56 \pm 0.28$) and, when relative to body mass, a *small* nonsignificant increase at $12 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 0.02 \pm 0.02 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.18$; $d = 0.56 \pm 0.47$) and a significant *moderate* increase at $13.5 \text{ km}\cdot\text{h}^{-1}$ ($\Delta = 0.04 \pm 0.02 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.018$; $d = 1.14 \pm 0.73$) in the post- compared to pre-KD test.

Table 7.1. Summary of dietary intake during the 31-day dietary adaptation periods.

	Energy		Carbohydrate			Fat			Protein		
	MJ·day ⁻¹	kJ·kg·day ⁻¹	g·day ⁻¹	g·kg·day ⁻¹	% EI	g·day ⁻¹	g·kg·day ⁻¹	% EI	g·day ⁻¹	g·kg·day ⁻¹	% EI
Habitual Diet	13.07 ± 1.67	178.1 ± 27.1	336 ± 78	4.6 ± 1.3	42.9 ± 7.8	134 ± 30	1.8 ± 0.3	38.5 ± 7.1	145 ± 18	2.0 ± 0.3	18.6 ± 1.4
Ketogenic Diet	13.73 ± 2.43	190.6 ± 45.4	34 ± 11*	0.5 ± 1.1*	4.1 ± 0.8*	286 ± 60*	4.0 ± 1.1*	77.7 ± 3.9*	145 ± 21	2.0 ± 0.3	18.2 ± 3.5
ES	0.35	0.41	-3.44	-2.94	-4.45	4.57	5.65	4.91	0.01	0.10	-0.30
(± 90% CI)	± 0.82	± 0.85	± 0.64	± 0.62	± 0.64	± 1.20	± 0.81	± 0.78	± 1.09	± 1.11	± 1.18

Values are presented as mean ± SD. MJ *megajoules*; kJ *kilojoules* and; EI *energy intake*. Significantly different to habitual diet (* $p < 0.001$).

Table 7.2. Summary of accumulated training load during the 31-day dietary adaptation periods.

	Running			Cycling		Total	
	Distance (km)	TRIMP	sRPE	TRIMP	sRPE	TRIMP	sRPE
Habitual Diet	249.7 ± 104.9	2304 ± 918	6840 ± 3146	373 ± 692	1799 ± 3583	2677 ± 629	8665 ± 3120
Ketogenic Diet	253.7 ± 112.8	2582 ± 1131	7109 ± 3337	271 ± 392	969 ± 1517	2854 ± 885	8078 ± 2427
ES (± 90% CI)	0.03 ± 0.16	0.27 ± 0.25	0.08 ± 0.32	-0.13 ± 0.31	-0.21 ± 0.38	0.25 ± 0.51	-0.17 ± 0.56

Values are presented as mean ± SD. TRIMP *training impulse*; sRPE *session ratings of perceived exertion*.

Table 7.3. Summary of metabolic variables and perceived exertion during the pre- and post-diet metabolic tests.

		Habitual Diet					Ketogenic Diet				
		Stage 1	Stage 2	Stage 3	Stage 4	Max	Stage 1	Stage 2	Stage 3	Stage 4	Max
Velocity (km·h⁻¹)	Pre	9	10.5	12	13.5	18.81 ± 0.88	9	10.5	12	13.5	19.31 ± 0.96
	Post					18.93 ± 0.94					18.31 ± 1.07*
	ES (± 90% CI)					0.13 ± 0.15					-0.92 ± 0.52
VO₂^b (L·min⁻¹)	Pre	2.14 ± 0.18	2.50 ± 0.14	2.88 ± 0.22	3.25 ± 0.27	4.35 ± 0.49	2.16 ± 0.26	2.47 ± 0.27	2.79 ± 0.35	3.08 ± 0.39	4.30 ± 0.55
	Post	2.14 ± 0.18	2.47 ± 0.20	2.85 ± 0.26	3.27 ± 0.26	4.32 ± 0.46	2.13 ± 0.25	2.50 ± 0.19	2.93 ± 0.23	3.37 ± 0.27*	4.38 ± 0.43
	ES (± 90% CI)	0.00 ± 0.41	-0.20 ± 0.73	-0.16 ± 0.51	0.07 ± 0.37	-0.05 ± 0.14	-0.10 ± 0.48	0.09 ± 0.40	0.34 ± 0.31	0.66 ± 0.22	0.13 ± 0.25
RER^{a,b}	Pre	0.84 ± 0.03	0.87 ± 0.04	0.89 ± 0.04	0.93 ± 0.04	1.12 ± 0.05	0.85 ± 0.05	0.88 ± 0.03	0.90 ± 0.04	0.93 ± 0.04	1.14 ± 0.04
	Post	0.84 ± 0.04	0.88 ± 0.04	0.90 ± 0.03	0.94 ± 0.04	1.13 ± 0.06	0.74 ± 0.17	0.76 ± 0.03	0.78 ± 0.02	0.81 ± 0.02	1.00 ± 0.05
	ES (± 90% CI)	-0.03 ± 0.59	0.06 ± 0.57	0.18 ± 0.52	0.07 ± 0.30	0.16 ± 0.44	-1.90 ± 0.72	-3.43 ± 1.06	-3.01 ± 0.78	-3.23 ± 0.76	-2.84 ± 1.06
EE^b (kJ·min⁻¹)	Pre	44.2 ± 3.8	51.7 ± 3.0	59.9 ± 4.5	67.7 ± 5.6	94.4 ± 10.5	44.6 ± 5.5	51.2 ± 5.5	58.0 ± 7.3	64.2 ± 8.1	93.5 ± 12.0
	Post	44.1 ± 3.8	51.1 ± 4.2	59.1 ± 5.3	68.2 ± 5.5	94.0 ± 10.1	43.5 ± 5.2	51.1 ± 3.9	59.9 ± 4.7	69.3 ± 5.6*	95.3 ± 9.4
	ES (± 90% CI)	-0.01 ± 0.37	-0.19 ± 69	-0.15 ± 0.48	0.07 ± 0.37	-0.03 ± 0.16	-0.18 ± 0.46	-0.02 ± 0.40	0.24 ± 0.31	0.56 ± 0.28	0.13 ± 0.25
HR^b (beats·min⁻¹)	Pre	120 ± 6	134 ± 8	143 ± 7	154 ± 10	186 ± 8	123 ± 11	135 ± 12	145 ± 11	155 ± 12	186 ± 10
	Post	121 ± 7	132 ± 7	143 ± 10	156 ± 11	184 ± 10	124 ± 12	137 ± 10	148 ± 11	160 ± 10	188 ± 11
	ES (± 90% CI)	0.07 ± 0.30	-0.22 ± 0.26	-0.05 ± 0.41	0.11 ± 0.28	-0.19 ± 0.16	0.07 ± 0.33	0.15 ± 0.31	0.26 ± 0.21	0.32 ± 0.24	0.16 ± 0.28
RPE^b	Pre	9.0 ± 0.8	10.6 ± 0.9	12.0 ± 0.8	13.3 ± 1.0		9.3 ± 0.7	10.5 ± 0.8	11.8 ± 0.7	13.3 ± 0.9	
	Post	9.3 ± 0.7	10.8 ± 0.9	11.8 ± 1.0	13.0 ± 1.2		9.4 ± 0.7	10.9 ± 1.4	12.5 ± 0.9	14.1 ± 1.0	
	ES (± 90% CI)	0.29 ± 0.54	0.12 ± 0.79	-0.32 ± 0.56	-0.23 ± 0.40		0.15 ± 0.84	0.35 ± 1.23	0.91 ± 0.38	0.87 ± 0.23	

Values are presented as mean ± SD. ES = Cohen's *d* effect size as interpreted according to <0.2, >0.2, >0.6, >1.2, >2.0 and >4.0 for *trivial*, *small*, *moderate*, *large*, *very large* and *extremely large*, respectively. VO₂ oxygen uptake; RER respiratory exchange ratio; EE energy expenditure; HR heart rate; RPE ratings of perceived exertion. Significantly different in the post-KD compared to pre-KD test (**p* < 0.05; ***p* < 0.01). Diet x training interaction; lower in the post-KD compared to pre-KD test across stages 1-4 (^a*p* < 0.001). Main effect of intensity; incremental increase from stages 1-4 (^b*p* < 0.001).

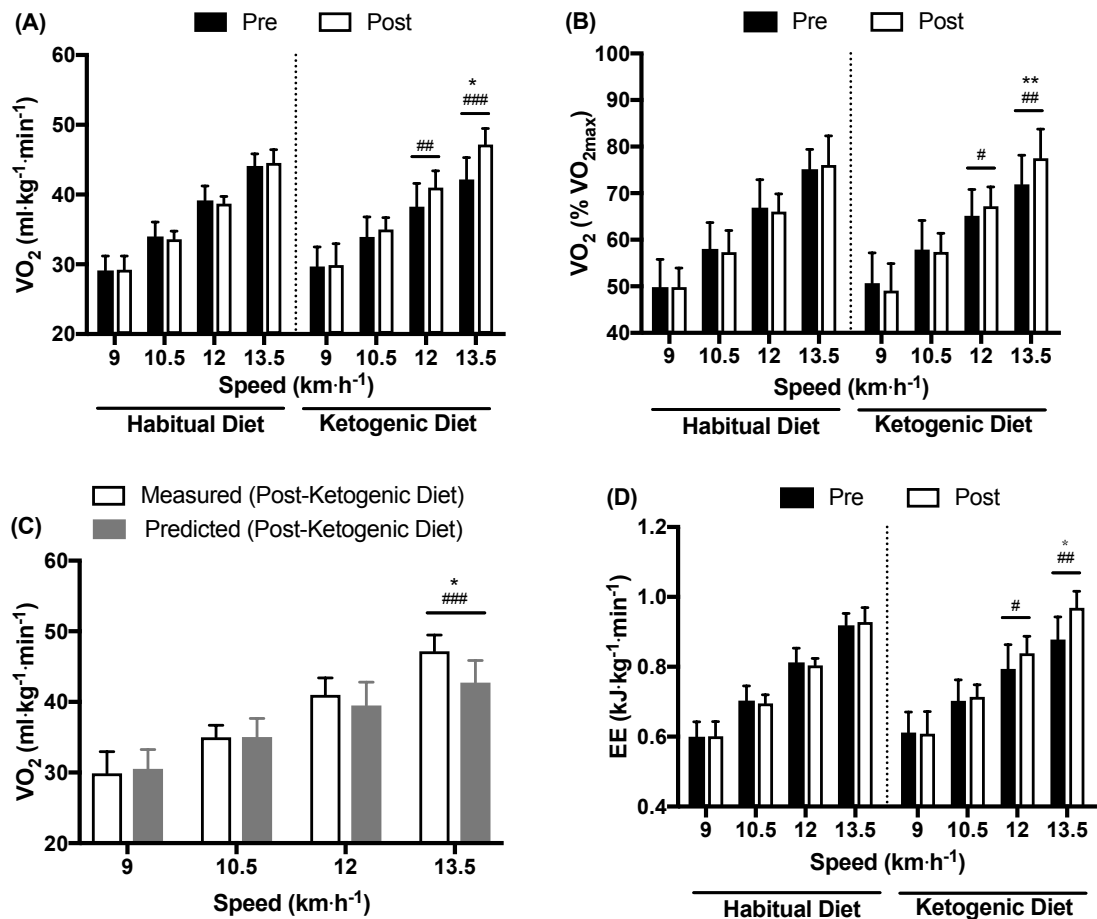


Figure 7.3. Oxygen uptake and exercise efficiency during the pre- and post-diet metabolic tests presented as (A) oxygen uptake relative to body mass ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), (B) oxygen uptake relative to $\text{VO}_{2\text{max}}$, (C) oxygen uptake in the post-KD test compared to predicted values based on shifts in respiratory exchange ratio from the pre-KD test, whilst assuming no difference in exercise efficiency and (D) energy expenditure relative to body mass. Values are mean \pm SD. Significantly different to pre-diet values (* $p < 0.05$; ** $p < 0.01$). Effect size (d); #small, ##moderate and ###large.

7.4.5 Environmental and dietary conditions during the run-to-exhaustion trial

Trials were performed within standard laboratory conditions of 17 ± 1 °C and $45 \pm 3\%$ humidity. There was no difference in running speed between the HD and KD trials (12.9 ± 0.7 vs. 12.9 ± 0.8 $\text{km}\cdot\text{h}^{-1}$, $p = 0.89$) and all participants commenced each trial with USG values < 1.025 . The exercise-induced reduction in body mass did not differ between trials (all $p > 0.05$, all $d = \text{trivial}$ or *unclear*). Macronutrient composition of the pre-HD and post-HD trial meals were identical (148 ± 14 g CHO, 7 ± 1 g fat, 23 ± 4 g protein) and similar to the pre-KD trial meal (146 ± 14 g CHO, 7 ± 1 g fat and 23 ± 4 g protein). However, CHO and fat content were lower and higher, respectively, in the post- compared to pre-KD trial meal (8 ± 1 g CHO and 67 ± 6 g fat), whereas protein content was similar

(26 ± 6 g). There was no difference in CHO ingestion rate during the pre-HD, post-HD and pre-KD trials (55.7 ± 6.0 , 53.4 ± 2.8 and 55.3 ± 8.4 g·h⁻¹, respectively; all $p > 0.05$; all $d = \text{trivial}$ or *unclear*). Coconut oil was ingested during the post-KD trial at a rate identical to the energy intake in the pre-KD trial (26.0 ± 4.0 g·h⁻¹).

7.4.6 Submaximal exercise capacity

There were no diet x adaptation interactions or main effects for TTE ($p = 0.557$) or distance-to-exhaustion (DTE) ($p = 0.508$) (Figures 7.4A and 7.4B). Furthermore, there was no difference in mean change between diets for TTE ($p = 0.56$; $d = 0.25 \pm 0.60$) or DTE ($p = 0.51$; $d = 0.26 \pm 0.60$). The range within the 90% confidence intervals (CI) for TTE and DTE increased ~2-fold in the post- compared to pre-KD trial, whereas, there was a reduction from the pre- to post-HD trial ([pre-HD, 112-263 min vs. post-HD, 211-252 min and pre-KD, 223-254 min vs. post-KD, 188-250 min] and [pre-HD, 45.8-56.4 km vs. post-HD, 45.7-53.7 km and pre-KD, 47.8-54.6 km vs. post-KD, 40.5-53.1 km]).

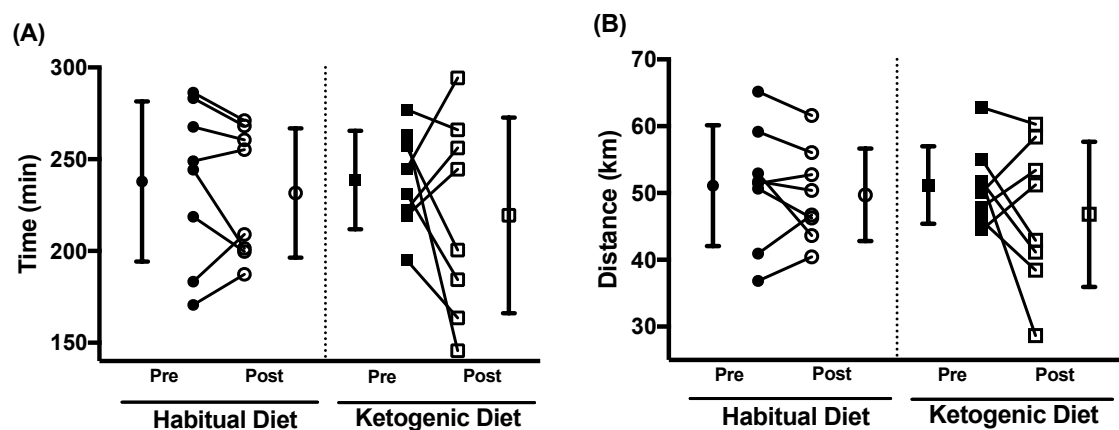


Figure 7.4. Submaximal exercise capacity for the pre- and post-diet RTE trials. Values presented as (A) mean \pm SD and individual time-to-exhaustion and (B) mean \pm SD and individual distance-to-exhaustion.

7.4.7 D-βHB, glucose and lactate concentration during the run-to-exhaustion trial

There was a significant diet x adaptation x time interaction for blood D-βHB concentration ($p < 0.001$), with significant *large* to *extremely large* increases in the post- compared to pre-KD trial for all time points (all $p < 0.05$) (Figure 7.5A, p. 127). No differences in blood D-βHB concentration were observed between the pre- and post-HD trials (all $p > 0.05$, all $d = \text{trivial}$ or *unclear*). There was a significant diet x adaptation x time interaction for serum glucose concentration ($p = 0.032$); however, post hoc analysis could only locate a *moderate* nonsignificant increase at pre-exercise ($p = 0.21$, $d = 0.92 \pm 0.62$) and a *moderate* nonsignificant reduction at 2-h exercise ($p = 0.49$, $d = -0.93 \pm 0.82$) in the post- compared to pre-KD trial (Figure 7.5B, p. 127). Serum glucose concentration was elevated from 1-h exercise to 1-h post-exhaustion compared to pre-exercise in all trials (all $p < 0.05$), except for the post-KD trial, which only exhibited an increase from pre-exercise to 1 h exercise ($p = 0.005$). There was no diet x adaptation x time interaction for blood lactate concentration ($p = 0.061$). However, there was a significant effect for time ($p = 0.03$), with blood lactate concentrations lower at 2-h exercise compared to exhaustion ($p = 0.02$) (Figure 7.5C, p. 127).

7.4.8 Substrate oxidation during the run-to-exhaustion trial

There was a significant diet x adaptation interaction for the rate and percentage contribution to total EE of CHO and fat oxidation (all $p < 0.001$) (Figures 7.6A-D, p. 128). Carbohydrate oxidation was 2.5-fold higher in the pre- compared to post-KD trial and fat oxidation was ~3.5-fold higher in the post- compared to pre-KD trial (all $p < 0.001$). In the post-KD trial, fat oxidation rates ranged between 0.88-1.51 g·min⁻¹ for all time points. There were no differences in substrate oxidation between the pre- and post-HD trials (all $p > 0.05$; all $d = \text{trivial}$ or *unclear*).

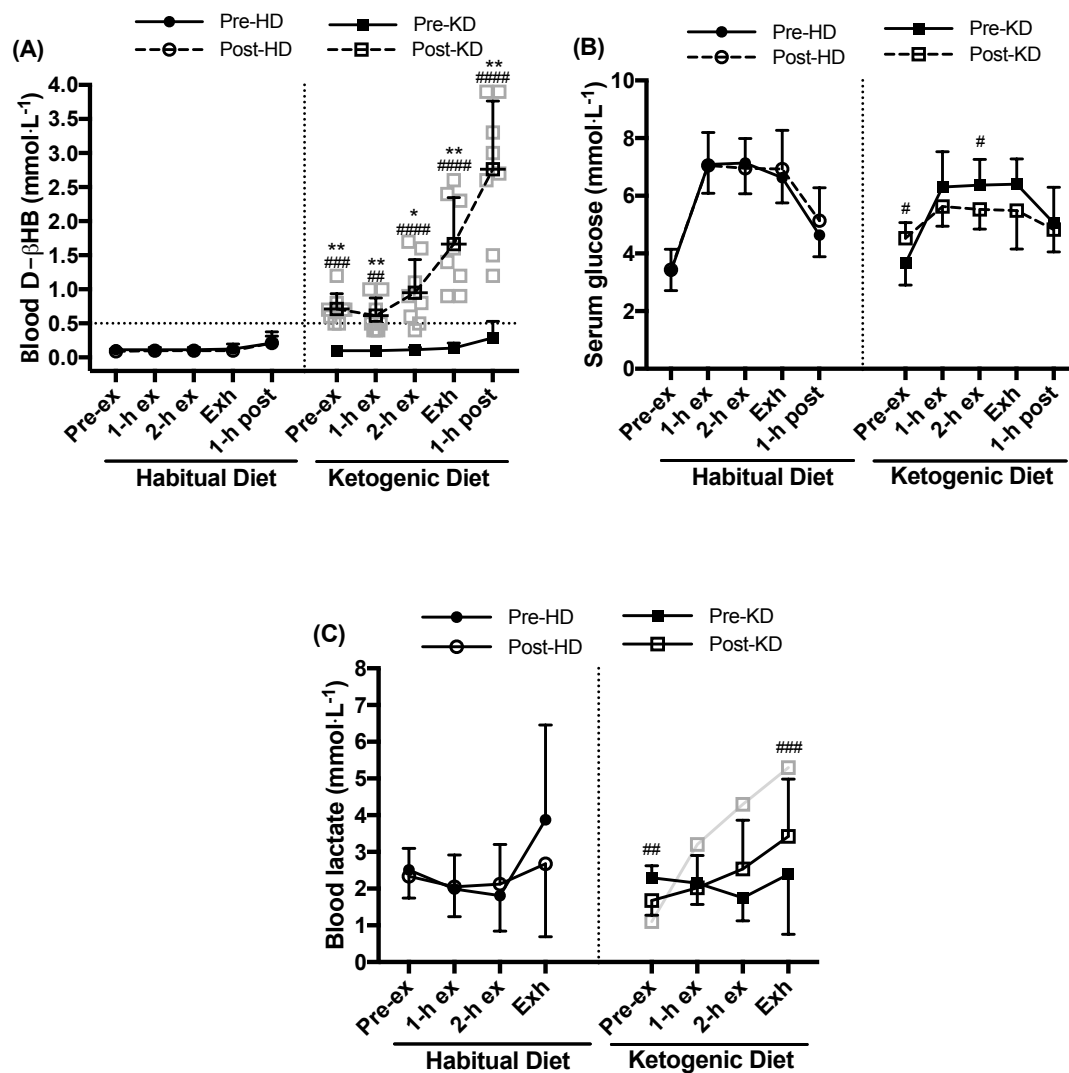


Figure 7.5. (A) capillary blood D-βHB, (B) serum glucose and (C) capillary blood lactate concentrations during the pre- and post-diet RTE trials. Values are mean ± SD. The individual grey responses in (A) are individual post-KD D-βHB values for participants and in (C) are post-KD lactate values for the participant who reduced TTE from 263 min to 145 min. HD *Habitual diet*; KD *Ketogenic diet*. Significantly different in the post-compared to pre-KD trial (* $p < 0.01$; ** $p < 0.001$). Effect size (d); #*moderate*, ##*large*, ###*very large*, and ####*extremely large*.

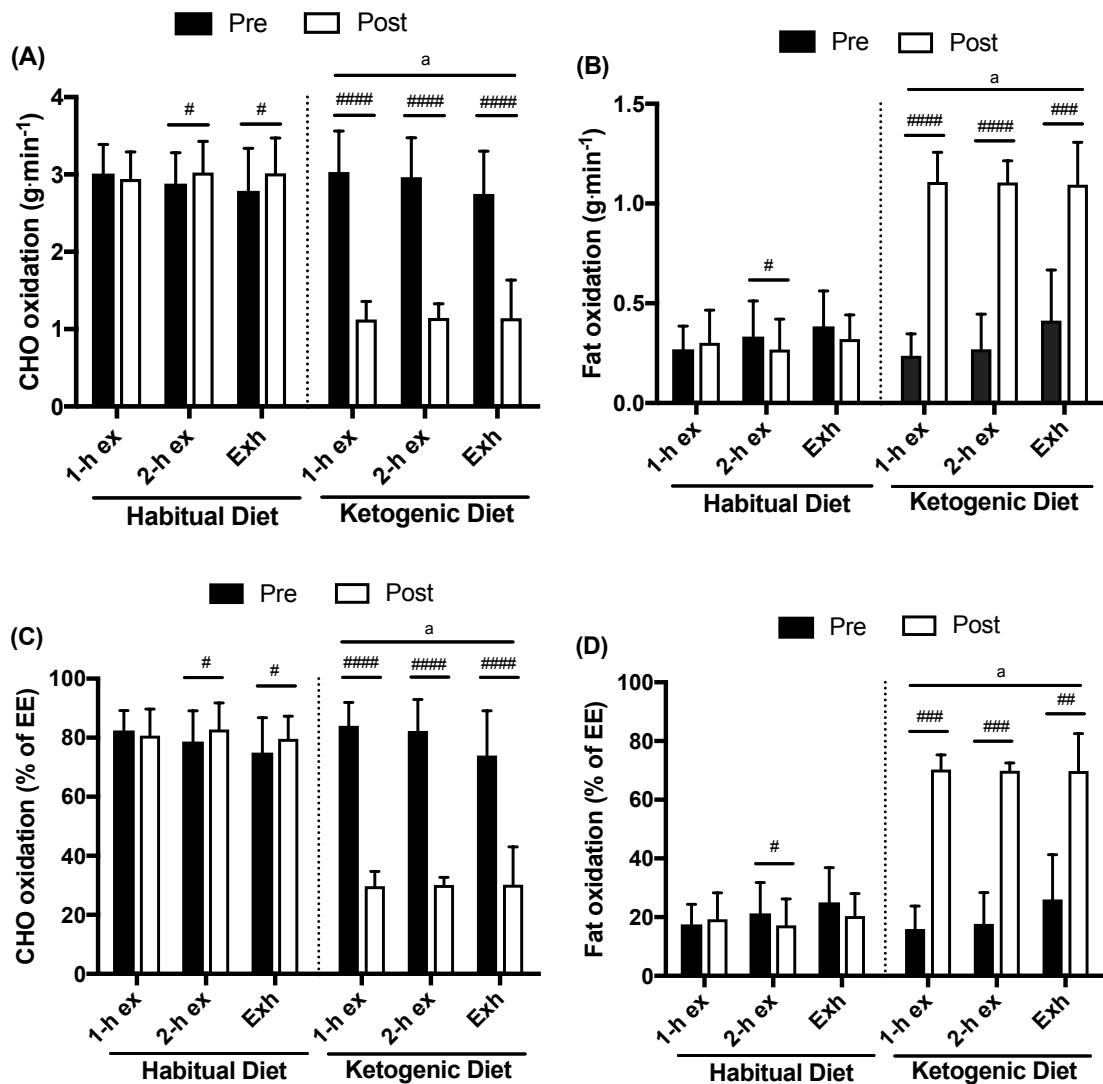


Figure 7.6. The contribution of substrate to energy expenditure during the pre- and post-diet RTE trials presented as (A) rate of carbohydrate oxidation and (C) percentage contribution of carbohydrate to total energy expenditure and (B) rate of fat oxidation and (D) percentage contribution to total energy expenditure. Significant effect of diet (^a $p < 0.001$). Effect size; #small, ##large, ###very large and ####extremely large effects.

7.4.9 Cardiorespiratory variables and perceived exertion during the run-to-exhaustion trial

Table 7.4 (p. 130) and Figure 7.7A-D (p. 131) summarise the cardiorespiratory variables and perceived exertion data during the RTE trials. There was a significant diet \times adaptation \times time interaction for absolute VO_2 ($p < 0.001$), with significant *small* increases in the post- compared to pre-KD trial during the first ($p = 0.025$; $d = 0.46 \pm 0.26$) and second hour ($p = 0.016$; $d = 0.48 \pm 0.23$) (Table 7.4). These effects increased when accounting for body mass, resulting in significant *moderate* increases in VO_2

occurring during the first ($p = 0.009$; $d = 0.83 \pm 0.37$) and second hour ($p = 0.005$; $d = 0.84 \pm 0.23$) and a significant *small* increase at exhaustion ($p = 0.046$; $d = 0.50 \pm 0.39$) (Figure 7.7A). No effects were observed between the pre- and post-HD trials for absolute and relative VO_2 (all $p > 0.05$, all $d = \text{trivial}$ or *unclear*). Despite a significant diet x adaptation x time interaction for exercise intensity relative to $\text{VO}_{2\text{max}}$, post hoc analysis could not locate specific differences between trials (all $p > 0.05$, all $d = \text{trivial}$ or *unclear*) (Figure 7.7B). There was a significant diet x adaptation interaction for HR ($p = 0.011$), with an increase in the post- compared to pre-KD trial ($p < 0.001$) (Table 7.4). Heart rate did not differ between the pre- and post-HD trials ($p = 0.85$).

Table 7.4. Summary of metabolic variables and perceived exertion during the pre- and post-diet run-to-exhaustion trials.

		Habitual Diet				Ketogenic Diet			
		Pre-exercise	1-h exercise	2-h exercise	Exhaustion	Pre-exercise	1-h exercise	2-h exercise	Exhaustion
Body mass (kg, adjusted) ES (\pm 90% CI)	Pre				-4.3 \pm 0.8				-4.4 \pm 0.5
	Post				-4.5 \pm 0.4				-3.9 \pm 1.1
					-0.15 \pm 0.58				0.87 \pm 1.23
VO₂^a (L·min⁻¹) ES (\pm 90% CI)	Pre		2.99 \pm 0.32	3.01 \pm 0.32	3.03 \pm 0.24		2.93 \pm 0.39	2.94 \pm 0.37	3.06 \pm 0.32
	Post		2.99 \pm 0.36	2.99 \pm 0.38	3.09 \pm 0.31		3.14 \pm 0.37 ^Ω	3.15 \pm 0.34*	3.12 \pm 0.29
			0.02 \pm 0.19	-0.04 \pm 0.19	0.21 \pm 0.31		0.46 \pm 0.26	0.48 \pm 0.23	0.18 \pm 0.28
RER^b ES (\pm 90% CI)	Pre		0.95 \pm 0.02	0.93 \pm 0.03	0.92 \pm 0.04		0.95 \pm 0.02	0.94 \pm 0.03	0.91 \pm 0.05
	Post		0.94 \pm 0.03	0.94 \pm 0.03	0.94 \pm 0.03		0.79 \pm 0.01	0.79 \pm 0.01	0.79 \pm 0.03
			-0.23 \pm 0.67	0.35 \pm 0.25	0.35 \pm 0.36		-6.57 \pm 0.93	-4.63 \pm 1.30	-2.70 \pm 0.46
EE (kJ·min⁻¹) ES (\pm 90% CI)	Pre		62.3 \pm 6.7	62.6 \pm 6.6	63.1 \pm 5.1		61.2 \pm 8.2	61.4 \pm 8.2	63.6 \pm 6.5
	Post		62.3 \pm 7.4	62.4 \pm 7.9	64.3 \pm 6.4		64.3 \pm 7.6	64.5 \pm 7.0	64.0 \pm 6.0
			0.01 \pm 0.18	-0.03 \pm -0.19	0.22 \pm 0.31		0.33 \pm 0.24	0.36 \pm 0.23	0.06 \pm 0.29
HR^c (beats·min⁻¹) ES (\pm 90% CI)	Pre	64 \pm 8	151 \pm 10	153 \pm 10	161 \pm 11	61 \pm 10	150 \pm 12	152 \pm 11	161 \pm 12
	Post	61 \pm 8	151 \pm 10	153 \pm 12	163 \pm 11	63 \pm 5	159 \pm 13*	161 \pm 12 ^Ω	168 \pm 15
		-0.40 \pm 0.45	0.02 \pm 0.24	0.07 \pm 0.26	0.15 \pm 0.16	0.21 \pm 0.46	0.72 \pm 0.22	0.72 \pm 0.37	0.54 \pm 0.36
RPE^c ES (\pm 90% CI)	Pre		11.7 \pm 0.8	12.8 \pm 0.9	19.38 \pm 0.52		11.4 \pm 0.9	12.1 \pm 1.4	19.38 \pm 0.52
	Post		11.8 \pm 0.8	12.6 \pm 1.5	19.50 \pm 0.53		11.6 \pm 1.1	12.5 \pm 1.4	19.38 \pm 0.52
			0.07 \pm 0.41	-0.23 \pm 0.56	0.21 \pm 0.74		0.17 \pm 0.88	0.28 \pm 0.49	0.00 \pm 0.61

Values are presented as mean \pm SD. ES = Cohen's *d* effect size as interpreted according to <0.2, >0.2, >0.6, >1.2, >2.0 and >4.0 for *trivial*, *small*, *moderate*, *large*, *very large* and *extremely large*, respectively. Body mass values are adjusted for fluid intake. VO₂ *oxygen uptake*; RER *respiratory exchange ratio*; EE *energy expenditure*; HR *heart rate*; RPE *rate of perceived exertion*. Significantly different in the post compared to pre KD (**p* < 0.05). Trending towards significantly different in the post-treatment trial compared to pre-treatment in ketogenic diet (^Ω*p* = 0.06). Main effect of diet; higher in the post-diet trial compared to pre-diet (^a*p* < 0.05). Main effect of diet; lower in the post-diet trial compared to pre-diet (^b*p* < 0.001). Main effect of time; incremental increase across each time point (^c*p* < 0.001).

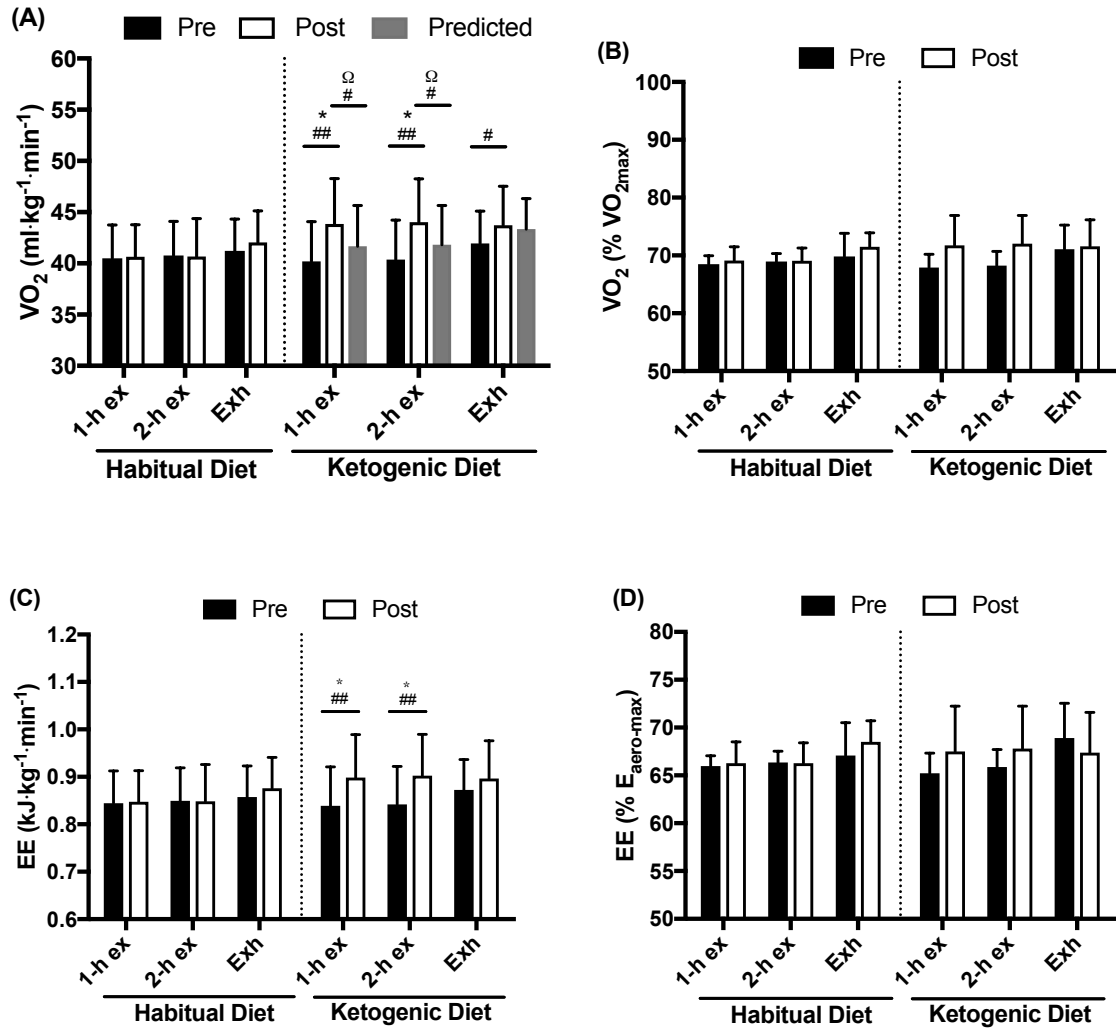


Figure 7.7. Exercise efficiency during the pre- and post-diet RTE trials presented as (A) oxygen uptake relative to body mass alongside predicted values for the post-KD trial, (B) oxygen uptake relative to VO_{2max} , (C) energy expenditure relative to body mass and (D) energy expenditure relative to $EE_{aero-max}$. Significantly different in the post- compared to pre-KD trial (* $p \leq 0.05$). Trending towards significantly different in the post- compared to pre-KD trial ($^{\Omega}p = 0.06$). Effect size (d); #*small* and ##*moderate*.

7.4.10 Exercise efficiency during run-to-exhaustion trial

In the post-KD trial, there was a significant adaptation x time interaction for predicted compared to measured VO_2 ($p = 0.001$) (Figure 7.7A), with trends for *small* increases in measured VO_2 during the first hour ($\Delta = 2.2 \pm 1.4 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $p = 0.06$; $d = 0.46 \pm 0.33$) and second hour ($\Delta = 2.2 \pm 1.2 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $p = 0.06$; $d = 0.48 \pm 0.30$). The shift in RER from the pre- to post-KD trial explained $55 \pm 32\%$ of the increase in VO_2 during the first 2 h. There was a significant diet x adaptation x time interaction for absolute EE ($p =$

0.001), with trends for *small* increases the first hour ($\Delta = 3.0 \pm 2.0 \text{ kJ}\cdot\text{min}^{-1}$; $p = 0.076$; $d = 0.33 \pm 0.25$) and second hour ($\Delta = 3.1 \pm 1.7 \text{ kJ}\cdot\text{min}^{-1}$; $p = 0.056$; $d = 0.36 \pm 0.23$) in the post- compared to pre-KD trial (Table 7.4, p. 130). When accounting for body mass, there was also a significant diet x adaptation x time interaction ($p < 0.001$), with significant *moderately* higher rates of EE during the first hour ($\Delta = 0.06 \pm 0.03 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.022$; $d = 0.65 \pm 0.36$) and second hour ($\Delta = 0.06 \pm 0.02 \text{ kJ}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.014$; $d = 0.67 \pm 0.31$) in the post- compared to pre-KD trial (Figure 7.7C, p. 131). Despite a significant diet x adaptation x time interaction for the intensity relative to $\text{EE}_{\text{aero-max}}$ ($p = 0.001$), post hoc analysis could not locate specific differences between trials (all $p > 0.05$) (Figure 7.7D, p. 131).

7.4.11 Exercise capacity and lactate accumulation based on RER at $\text{VO}_{2\text{max}}$

Comparisons for exercise capacity and blood lactate concentrations were made between pre- and post-KD trials based on RER at $\text{VO}_{2\text{max}}$ (i.e. <1.0 , $n = 4$ and >1.0 , $n = 4$). In the group with $\text{RER} < 1.0$ at $\text{VO}_{2\text{max}}$, TTE significantly declined from pre- to post-KD ($237 \pm 31 \text{ min}$ vs. $174 \pm 24 \text{ min}$; $p = 0.04$; $d = 1.49 \pm 1.04$). In contrast, in the group with $\text{RER} > 1.0$ at $\text{VO}_{2\text{max}}$, there was no effect of the KD on TTE ($241 \pm 27 \text{ min}$ vs. $265 \pm 21 \text{ min}$; $p = 0.15$; $d = 0.67 \pm 0.83$) (Figure 7.8A, p. 133). Using an unadjusted Student's unpaired t -test, mean change in TTE from pre- to post-diet was significant *large* reduction in the group with $\text{RER} < 1.0$ compared to > 1.0 at $\text{VO}_{2\text{max}}$ ($-63 \pm 38 \text{ min}$ vs. $24 \pm 21 \text{ min}$; $p = 0.009$, $d = 1.22 \pm 0.86$). Furthermore, using an unadjusted Student's unpaired t -test for each time point, the group with $\text{RER} < 1.0$ at $\text{VO}_{2\text{max}}$ exhibited a significant *large* increase in the difference for mean lactate concentration at exhaustion from pre- to post-diet compared to the group with $\text{RER} > 1.0$ at $\text{VO}_{2\text{max}}$ ($p = 0.015$, $d = 1.45 \pm 0.86$) (Figure 7.8B, p. 133). There were no differences in exercise intensity relative to VT_2 , $\text{VO}_{2\text{max}}$ or $v\text{VO}_{2\text{max}}$, nor rates of substrate oxidation or blood D- β HB and serum glucose concentrations between the two groups (data not shown).

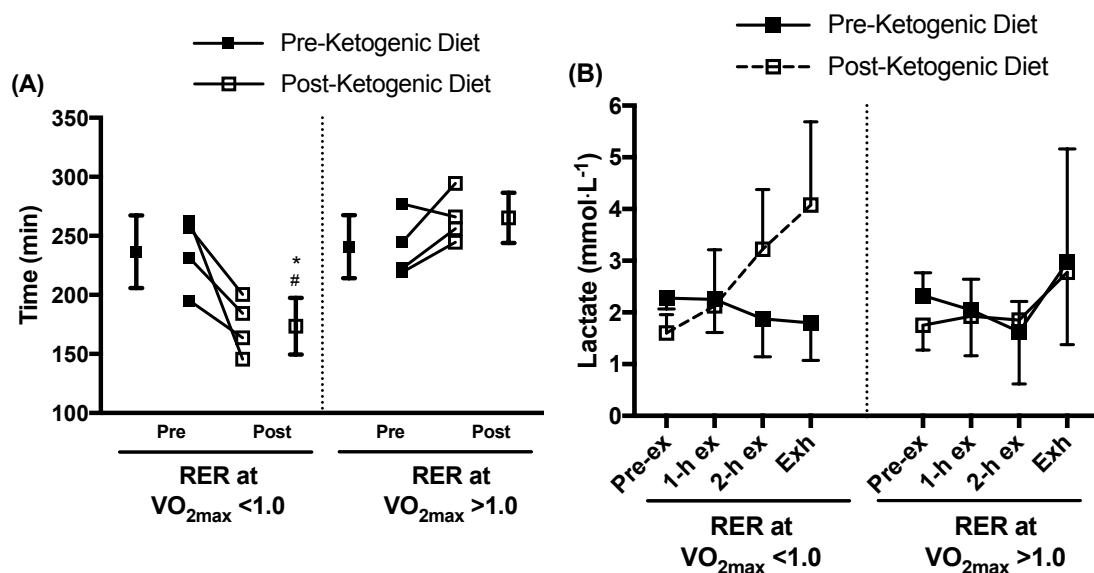


Figure 7.8. Performance variables for the pre- and post-KD RTE trials split into groups based on RER < 1.0 and > 1.0 at VO_{2max} in the post-KD metabolic test presented as (A) mean \pm SD and individual time-to-exhaustion and (B) mean \pm SD blood lactate concentration. Significantly different in the post- compared to pre-KD trial (* $p = 0.04$). Effect size (d); #large.

7.5 Discussion

The present study investigated the effect of a 31-day KD on submaximal exercise capacity without the interference of intensified training in endurance trained runners. The main findings were: 1) a 31-day KD preserved mean submaximal exercise capacity without the requirement of CHO restoration or supplementation; 2) exercise efficiency was impaired at intensities above 70% VO_{2max} as evidenced by oxygen uptake that could not be explained by shifts in RER alone and increased EE; 3) RER at VO_{2max} may have implications as a performance surrogate following keto-adaptation and; 4) keto-adaptation increased submaximal endurance variability as evidenced by a two-fold increase in the 90% CI for TTE and DTE in the post- compared to pre-KD trial, which coincided with reduced endurance capacity for 5 of 8 runners.

The aim of the dietary interventions were to polarise metabolic states as it has been suggested that increased fat oxidation rates and blood D- β HB concentrations following keto-adaptation can overcome the necessity of acute CHO fuelling strategies during continuous exercise lasting for several hours [9]. As previous studies providing meals, snacks and fluid to participants during the adaptation period could not be replicated [14,15], a novel approach to measure and verify prescribed dietary intakes was

implemented. This included an image-assisted (alongside a fiducial marker) weighed dietary record for 40% of the dietary adaptation days to reduce the potential of under- and mis-reporting [350], coding errors and daily dietary variation [351]. When combined with urinary AcAc (daily) and blood D- β HB (days 3, 7, 14, 21, 28 and post-KD tests) measures, all participants were extremely compliant to the KD. Whilst the HD was lower in CHO compared to recommendations for moderate training loads ($4.6 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$ vs. $5\text{--}7 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$) [5], this difference is negligible and did not appear to effect training adaptation or exercise capacity. Moreover, studies employing intensified training protocols during dietary-adaptation periods, such as the aforementioned investigation of keto-adaptation in elite race walkers [14], may limit direct comparison to the present study due to interferences from a training response.

Acute fuelling strategies before and during the RTE trials would have, in part, compensated for sub-optimal CHO intakes in the days prior. This required participants to ingest either a high- or low-CHO meal 2 h before the RTE trials and either CHO or fat whilst running according to the trial allocation. Arguably, the rate of CHO intake in the present study ($\sim 55 \text{ g}\cdot\text{h}^{-1}$) was below recommendations for optimal performance of these durations ($\sim 90 \text{ g}\cdot\text{h}^{-1}$) [173]. Nevertheless, $55 \text{ g}\cdot\text{h}^{-1}$ is comparable to recent publications of real world dietary behaviours in ultra-endurance athletes [352], with even small rates of CHO supplementation (i.e. $10 \text{ g}\cdot\text{h}^{-1}$) providing a performance benefit [173]. In turn, fat oxidation was ~ 4 -fold lower and CHO oxidation ~ 2.5 -fold higher in the high-CHO trials compared to keto-adapted trial. This was likely underpinned by a combination of; 1) elevated muscle [353] and hepatic glycogen content [354]; 2) elevated blood glucose uptake into the muscle [355]; 3) maintenance of blood glucose concentration [121] and; 4) reduction in hepatic glycogen utilisation [120]. However, if CHO was acutely ingested in the post-KD trial, this would oppose adaptations to the KD and suppress hepatic ketogenesis, thus compromising rates of fat oxidation and ketolysis [10].

To investigate exercise efficiency following keto-adaptation, oxygen uptake that could not be explained by shifts in RER from pre- to post-KD conditions, assuming no change to EE, was estimated. At lower intensities (particularly $<60\% \text{ VO}_{2\text{max}}$), the shift in RER could fully explain the increase in oxygen uptake. This coincides with a previous study demonstrating similar oxygen uptake at $62\text{--}64\% \text{ VO}_{2\text{max}}$ [15], which suggested that keto-adaptation improved exercise efficiency at lower intensities. Nevertheless, RER could

only account for 14% of the increase in oxygen uptake at 13.5 km·h⁻¹ during the metabolic test (~77% VO_{2max} when keto-adapted) and 55% of the increase in oxygen uptake during the first 2 h of the RTE trial (~72% VO_{2max} when keto-adapted). These effects appeared to underpin the increase in EE observed between the pre- and post-KD trials, and has previously been demonstrated in sedentary individuals [157], but was thought to be abrogated by endurance training [158]. However, this effect persisted with keto-adaptation regardless of training status in the present study. The unaccounted oxygen uptake may be due to elevated PPARα. In addition to PPARα's upregulation of fat oxidative genes, it also regulates the expression of mitochondrial uncoupling proteins [156]. Nevertheless, the effect of a ketogenic or LCHF diet on mitochondrial uncoupling in human skeletal muscle is unclear [157-159]; therefore, this remains speculative.

These findings also indicate keto-adaptation impairs high-intensity, endurance performance. Similar to previous studies [14,15], CHO oxidation was truncated at near maximal exercise intensities following keto-adaptation, which manifested as a *throttling* of RER at VO_{2max}. This may be due to an attenuation of glycogenolysis and pyruvate dehydrogenase (PDH) activity [147], and could have underpinned the 1 km·h⁻¹ reduction in velocity at VO_{2max}. Furthermore, RPE was nonsignificantly higher at >65% VO_{2max} during the metabolic test following keto-adaptation, which is in line with an earlier study investigating a 3 week KD intervention in elite race walkers [14]. However, during the submaximal RTE, RPE did not differ despite heart rate being 7-9 beats·min⁻¹ higher. This could be due to increased SNS activity [165], with potential neural effects following keto-adaptation compensating for the increase in metabolic stress. However, a decrement in high-intensity exercise performance does not necessarily negate ultra-endurance performance. Therefore, with anecdotal reports of (ultra-) endurance athletes successfully employing LCHF diets and improved performance [167,168] or exercise capacity [15] in select individuals, including in the present study, further research remains warranted to understand the individual response to LCHF and KDs for athletes competing at submaximal exercise intensities

A potential surrogate to identify the individual response to keto-adaptation is RER at VO_{2max}. In the present study, when RER was >1.0 compared to <1.0 (n = 4) at VO_{2max} (n = 4) following keto-adaptation, between diet comparisons of mean change from pre- to post-diet demonstrated a higher endurance capacity and lower lactate concentration at

exhaustion. This response occurred despite no difference in exercise intensity relative to VT_2 , VO_{2max} and vVO_{2max} . It is important to note, however, that 3-5 min stages during a graded exercise test may not be reflective of steady-state pulmonary VCO_2 , particularly at high-intensities, which can overestimate RER [124]. Although corrective models have been proposed, these have not been validated, but do highlight a potential limitation of the current analysis. Nonetheless, it is known that a reduction in RER at VO_{2max} is associated with impaired high-intensity performance due to a reduction in maximal CHO utilisation [14,15]. However, the present findings suggest there is also a relationship with endurance capacity at submaximal intensities.

Differences in endurance capacity between groups with RER <1.0 compared to >1.0 at VO_{2max} were also unrelated to rates of fat oxidation and serum glucose and blood D- β HB concentrations. As MFO can increase to $>1 \text{ g}\cdot\text{min}^{-1}$ within three days of adaptation to a LCHF diet [356] and blood KB concentrations increase rapidly (hours to days) in response to low-CHO availability (e.g. starvation, exhaustive exercise and a KD) [10], this emphasises they are unreliable markers for *optimal* keto-adaptation. Potentially, differences in endurance capacity were due to lower rates of lactate oxidation [150] and gluconeogenesis via lactate [35] in the group with RER <1.0 , thus resulting in the accumulation of blood lactate. Lactate production may also be higher due to increased shuttling of pyruvate to lactate dehydrogenase because of the inhibition in PDH activity [147]. Elevated blood lactate concentrations at submaximal exercise intensities also appear in endurance athletes self-reporting chronic (>8 months) keto-adaptation [35,48]; therefore, it is uncertain whether this impairment in CHO metabolism resolves or persists with long-term adherence to a KD.

7.6 Conclusion

These findings suggest 31 days of keto-adaptation can preserve mean submaximal exercise capacity. However, exercise efficiency was impaired, endurance variability increased and there was a greater risk of an endurance decrement at an individual level following keto-adaptation. Moreover, the suggestion that longer adaptation periods to a KD are necessary to enhance endurance performance is currently unsubstantiated, although shifts in RER at VO_{2max} may provide a time-course adaptation or performance surrogate to monitor such changes.

The effect of a 31-day ketogenic diet on T-cell cytokine gene expression within stimulated peripheral blood mononuclear cells and salivary secretory immunoglobulin A following submaximal-intensity, exhaustive exercise

8.1 Abstract

This study investigated the effect of a 31-day KD on T-cell related cytokine gene expression within multi-antigen-stimulated PBMCs and SIgA. A repeated-measures, crossover study was conducted in eight trained, male, endurance athletes ($\text{VO}_{2\text{max}}$, $59.4 \pm 5.2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Participants ingested their HD (13.1 MJ, 43% [$4.6 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 38% [$1.8 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) or an iso-energetic KD (13.7 MJ, 4% [$0.5 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] CHO and 78% [$4 \text{ g}\cdot\text{kg}\cdot\text{day}^{-1}$] fat) from day 0 to 31 ($p < 0.001$). On days 0 and 31, participants completed a RTE trial at 70% $\text{VO}_{2\text{max}}$ ($\sim 12.9 \text{ km}\cdot\text{h}^{-1}$) following the ingestion of a high-CHO meal ($2 \text{ g CHO}\cdot\text{kg}^{-1}$) or an iso-energetic low-CHO, high-fat meal ($<10 \text{ g CHO}$), with CHO ($\sim 55 \text{ g}\cdot\text{h}^{-1}$) or iso-energetic fat (coconut oil) supplementation ($0 \text{ g CHO}\cdot\text{h}^{-1}$) during exercise. Capillary and venous blood, and unstimulated saliva samples were collected at pre-exercise, exhaustion and 1-h post exhaustion. Whole blood cultures were stimulated with a multi-antigen vaccine for 24 h to determine T-cell related IL-4, IL-10 and IFN- γ mRNA expression within isolated PBMCs *in vitro*. Training load did not differ between trials ($p > 0.05$). T-cell related IFN- γ mRNA expression was higher at exhaustion in the post- compared to pre-KD trial ($p = 0.01$), which coincided with an increase in the IFN- γ /IL-4 mRNA ratio ($p = 0.006$); however, there were no mRNA effects for IL-4 and IL-10 expression, nor for each cytokine mRNA expression from the pre- to post-HD trial (all $p > 0.05$). Across all trials, T-cell related IL-10 mRNA expression increased at exhaustion and 1-h post-exhaustion compared to pre-exercise (all $p < 0.001$); whereas, IL-4 mRNA expression was lower at exhaustion ($p < 0.001$), then increased but remained lower than pre-exercise levels at 1-h post-exhaustion (all $p < 0.01$). Although there was no effect of diet on SIgA concentration or secretion rate (all $p > 0.05$), both increased following adaptation to each diet (all $p < 0.05$). In conclusion, a KD does not affect mucosal immunity as evidenced by no changes to SIgA markers; however, adaptation to a KD appears to transiently amplify the initiation of T-cell related IFN- γ response to an immune challenge *in vitro* immediately following submaximal, exhaustive exercise, suggesting enhanced pro-inflammatory type-1 T-cell immunity. Nonetheless, more research is warranted to confirm these findings and identify the potential impact on illness and infection risk.

8.2 Introduction

The impact of very low-CHO, KDs (<50 g·day⁻¹ or <5% of energy intake) on endurance performance is increasingly researched [14,15,174]. However, only a single published study has examined the effect of KDs on immune function [37]. This is concerning given the interaction of exercise and CHO availability can alter specific aspects of the immune system. For example, perturbations to *in vitro* immune markers are exacerbated following prolonged, strenuous exercise undertaken with low- compared to high-CHO availability [288], potentially exacerbating illness and infection risk *in vivo* [1]. Therefore, it seems plausible to hypothesise that the KD may elicit greater immunodepression compared to exercise undertaken following a higher-CHO diet. A recent study demonstrated no effect of a 3 week KD on SIgA following an intensified training load in elite race walkers [37]; however, the increased training stress potentially overrode any subtle effect of diet and only mucosal immunity was examined [37]. Considering endurance athletes frequently attain physical exhaustion during training and competition resulting in transient states of immunodepression [1], and the importance of immune function for optimal performance [3,4,187], further research to isolate the effect of KDs on immunity and illness risk is warranted.

T-cells of the adaptive immune system comprise 60-80% of the lymphocyte population and are critical for orchestrating an immune response to invading incumbent pathogens [18,19]. T-cells can be divided into type-1, type-2 and regulatory subsets, which elicit distinct cytokine profiles. Type-1 T-cells primarily produce IFN- γ , a pro-inflammatory cytokine that protects against intracellular pathogens (e.g. viruses) [20]. *In vitro* mitogen- and multi-antigen-stimulated circulating T-cell IFN- γ production has been shown to decline following prolonged, strenuous exercise [24,25] and in fatigued or over-trained athletes [16], respectively. Whereas, type-2 T-cells produce the anti-inflammatory and cross-regulatory cytokine to IFN- γ and IL-4; and TReg cells produce IL-10, a potent anti-inflammatory and immunosuppressive cytokine [212]. The *in vitro* mitogen- and multi-antigen-stimulated T-cell production of both IL-4 and IL-10 appears unaffected by acute exercise [27-29,205]. However, following periods of heavy training, the multi-antigen-stimulated whole blood production of IL-4 and IL-10 tends to increase [26,30], which may limit the immune system from mounting a pro-inflammatory response to a pathogenic challenge, thus increasing illness and infection risk.

Salivary secretory IgA is the principle antibody in mucosal fluids acting as the first line of defence within the oral and nasal mucosa [357]. Produced locally by plasma cells residing in the submucosa, SIgA is transported across the epithelial cell by the polymeric immunoglobulin receptor [357]. Immunoglobulin A appears to function by: 1) preventing antigens and microbes adhering to and penetrating the mucosal epithelium (immune exclusion); 2) interrupting replication of intracellular pathogens during transcytosis through epithelial cells (intracellular neutralisation) and; 3) binding to antigens on the lamina propria to facilitate their excretion through the epithelium back to the luminal surface (immune excretion) [358]. The SIgA response to acute exercise, particularly at submaximal intensities, is largely trivial [357]; however, reductions in SIgA concentration and/or secretion rate can occur when physically exhausted, such as following marathon running [359,360] and periods of heavy training [361-364]. SIgA concentration [365,366] and secretion rate [17,361] have been inversely associated with increased illness risk, and is currently considered an immune marker with high clinical relevance [240].

Maintaining CHO availability acts on immune function indirectly by attenuating the exercise-induced rise in stress hormones, such as cortisol, and directly by providing an energy source for immune cells [1]. For example, CHO ingestion (36 and 72 g·h⁻¹) during 150 min of cycling at ~65% VO_{2max} mitigated the exercise-induced rise in circulating cortisol concentration and post-exercise reduction in mitogen-stimulated T-cell IFN-γ production *in vitro* compared to a placebo [25]. However, no effects were observed for single-antigen-stimulated (influenza or tetanus toxoid) T-cell IFN-γ and IL-4 gene expression *in vitro* or plasma cortisol concentration following the ingestion of ~40 g CHO·h⁻¹ during 90 min of intermittent high-intensity running compared to placebo [32]. As such, more research is required to understand the effect of exercise and CHO availability on T-cell cytokine gene expression. In regards to mucosal immunity, cortisol also appears to inhibit the transepithelial transport of SIgA into the mucosal fluid [367] and tends to be inversely associated with SIgA concentration [368]. As such, SIgA concentration was shown to decline following a 3 week training camp with periodised CHO intake in trained triathletes [369]. However, it remains inconclusive if this effect persists with adaptation to a KD [37].

Adaptation to a KD shifts substrate preference from CHO to fat and can maintain blood glucose concentration throughout submaximal exercise without necessitating CHO ingestion [14,15,35,48]; therefore, it may exert unique immunomodulating properties. As it stands, however, athletes are typically recommended to ingest a high-CHO diet (>50% EI) and 30-60 g CHO·h⁻¹ during exercise to support immune function [1,7,286]. In concordance, the aim of this study was to investigate the effect of an isoenergetic 31-day KD on SIgA and *in vitro* T-cell related IL-4, IL-10 and IFN- γ gene expression within multi-antigen-stimulated PBMCs.

8.3 Methods

8.3.1 Study design

The study design is detailed in Chapter 7, section 7.3.1.

8.3.2 Participants

Participant characteristics are detailed in Chapter 7, sections 7.3.2 and 7.4.1.

8.3.3 Experimental trials

All exercise protocols are detailed in Chapter 7, section 7.3.3.

8.3.4 Dietary intervention and monitoring

Dietary intervention and monitoring are detailed in Chapter 7, section 7.3.4.

8.3.5 Training monitoring

Training monitoring is detailed in Chapter 7, section 7.3.5.

8.3.6 Calculation of whole body of substrate oxidation rates and energy expenditure

All indirect calorimetry calculations for whole body substrate oxidation rates and EE are detailed in Chapter 7, section 7.3.7.

8.3.7 Blood sampling and analysis

Venous blood samples were collected at pre-exercise, exhaustion and 1-h post-exhaustion into an 8 ml serum and two 6 ml dipotassium ethylenediamine tetra-acetic acid (K₂EDTA) vacutainers (Becton Dickinson and Co, USA) with the participants seated in an upright position. Due to the rapid exercise-induced lymphocyte kinetics [338], all blood samples were collected within 1 min of the specified time points. The cannula was flushed with 3-4 ml of saline every 30 min to maintain patency. Each serum vacutainer was left to clot for 30 min prior to centrifugation at 1500 g for 10 min at 4 °C. After which, samples were separated into two 1.5 ml aliquots to be stored at -80 °C prior to the analysis of glucose and cortisol concentration (Cobas Modular P800 Analyser, Roche Diagnostics, New Zealand). Capillary blood D-βHB (Freestyle Optium Neo, Abbott Diabetes Care, Victoria, Australia) concentration was measured at pre-exercise, exhaustion and 1-h post-exhaustion from a fingertip blood sample using standardised techniques.

8.3.8 Haematological analysis

K₂EDTA treated whole blood was used to determine total circulating leukocyte and differential cell concentration (Sysmex XT-2000i Automated Haematology Analyzer, Sysmex Corporation, Auckland, New Zealand). All cell concentrations were adjusted for plasma volume changes from the initial (pre-supplement) blood sample, with changes being estimated according to Dill and Costill (1974) [339].

8.3.9 Stimulated whole blood cultures

Blood samples in the K₂EDTA vacutainer were used immediately for stimulated whole blood cultures in a nontreated, sterile tissue culture plate (Guangzhou Jet Bio-Filtration Co. Ltd, China) as follows: for each sample, 3600 µL of whole blood was added to 400

μL of RPMI medium (Thermo Fischer Scientific, USA) with stimulant at a dilution of 1:100, giving a final stimulant dilution of 1:1000. The whole blood culture was incubated for 24 h at 37 °C and 5% CO₂ (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The stimulant was a commercially available multi-antigen vaccine designed to elicit a specific T-cell recall immune response. The 1:1000 stimulant dilution was chosen based on previously described dose response curves for whole blood multi-antigen-stimulated IFN-γ production [17].

8.3.10 Isolation of peripheral blood mononuclear cells

Isolation of PBMCs is detailed in Chapter 6, section 6.3.8.

8.3.11 Analysis of mRNA expression in peripheral blood mononuclear cells

The procedures for extracting and amplifying PBMC RNA are detailed in Chapter 4, section 4.3.5. However, in the present study, the housekeeping gene for mRNA expression analysis was the *T-cell receptor constant region of β-chain* (Cβ) [370], which was used to correct for αβ T-cell abundance within the PBMC population (Table 8.1). Further, mRNA for all target genes were normalised to the reference gene (Cβ) within the same participant, condition and timepoint, and to a calibrator of pre-exercise Ct values of the *pre-diet condition* within the same participant.

Table 8.1. Primer sequence for T-cell receptor constant region of β-chain used for PBMC gene expression.

Gene Name		5'-3' primer sequence
Cβ	FW	TCCAGTTCTACGGGCTCTCG
	RW	GACGATCTGGGTGACGGGT

FW *forward primer*, RW *reverse primer*, Cβ *T-cell receptor constant region of β-chain*. Primer sequence was sourced for Cβ [370].

8.3.12 Salivary IgA analysis

Unstimulated, 5 min saliva samples were collected at pre-exercise, exhaustion and 1-h post-exhaustion. Participants were in a seated position and asked to empty their mouth of any residual saliva prior, then leaning forward with their head tilted down and minimal

orofacial movement, passively drooled into pre-weighed sterile plastic containers. Participants did not receive fluid for the 10 min prior to each saliva collection. Each plastic container was subsequently weighed to measure saliva flow rate by dividing volume by time ($\text{g}\cdot\text{min}^{-1}$) based on an estimated saliva density of $1.00\text{ g}\cdot\text{ml}^{-1}$. Saliva was transferred into two eppendorfs to be stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Salivary IgA concentration was measured based on a previously published in-house sandwich enzyme-linked immunosorbent assay (ELISA) technique [364,371]. Briefly, flat-bottomed microtitration plates (Nunc Immunoplate, Life Technologies, USA) were coated with a buffer containing $1.0\text{ }\mu\text{l}\cdot\text{ml}^{-1}$ rabbit anti-human IgA secretory component capture antibody (Dako, Australia) in a $0.05\text{ mmol}\cdot\text{L}^{-1}$ carbonate/bicarbonate solution (i.e. PBS) at a pH of 9.6, covered, and incubated overnight at $4\text{ }^{\circ}\text{C}$. Plates were washed four times ($300\text{ }\mu\text{l}\cdot\text{well}^{-1}$) with a wash buffer (PBS, $0.03\text{ mmol}\cdot\text{L}^{-1}$ NaCl, 0.1% Tween) and blocked with 2.0% bovine serum albumin in PBS ($100\text{ }\mu\text{l}\cdot\text{well}^{-1}$) (Fraction V, Sigma-Aldrich, St. Louis, Missouri, USA), covered, and incubated at room temperature for 60 min. Thawed saliva samples were spun for 2 min at 13,400 rpm and the supernatant was diluted by 1:1000 with PBS. A top working standard of $1.0\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ IgA (IgA from Bovine Colostrum, Sigma-Aldrich, St. Louis, Missouri, USA) was prepared in PBS and serially diluted serially with PBS to $0\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ (PBS only). Plates were washed four times ($300\text{ }\mu\text{l}\cdot\text{well}^{-1}$) with a wash buffer. Standards and samples were loaded in duplicate, covered, and incubated at $4\text{ }^{\circ}\text{C}$ overnight. Plates were washed four times ($300\text{ }\mu\text{l}\cdot\text{well}^{-1}$) with a wash buffer and loaded with $50\text{ }\mu\text{l}\cdot\text{well}^{-1}$ of HRP conjugated polyclonal anti-IgA/PBS solution (1:2000 dilution of Polyclonal Rabbit Anti-Human IgA/HRP (Dako, Australia) in PBS), covered, and incubated at room temperature for 90 min. Plates were washed four times ($300\text{ }\mu\text{l}\cdot\text{well}^{-1}$) with a wash buffer and loaded with $50\text{ }\mu\text{l}\cdot\text{well}^{-1}$ of a chromogenic substance (TMB + Substrate-Chromogen, Dako, Australia), then incubated in the dark at room temperature for 8 min. $50\text{ }\mu\text{l}$ of a stopping solution ($1.0\text{ mmol}\cdot\text{L}^{-1}\text{ H}_2\text{SO}_4$) was added to each well and the absorbance of individual samples was determined spectrophotometrically at 490 nm on an automated absorbance plate reader (Multiskan Go, Thermo Fisher Scientific, USA). A graph was then plotted with target SIgA concentrations of the standards plotted on the χ axis and the mean optical density readings of the standards on the γ axis. A polynomial standard curve was fitted and the SIgA concentration of the samples calculated. The correct SIgA concentration of samples was calculated by multiplying sample concentration by the dilution factor (1000) to give a final concentration ($\mu\text{g}\cdot\text{ml}^{-1}$). All saliva samples from each participant were analysed in

duplicate on one microplate. The CV of this method based on analysis of these duplicate samples was $6.3 \pm 5.7\%$. SIgA secretion rate ($\mu\text{l}\cdot\text{min}^{-1}$) was calculated by multiplying SIgA concentration by the saliva flow rate.

8.3.13 Illness symptom log

To monitor illness, participants self-reported illness-related symptoms at weekly intervals. The questionnaire was adapted from published sources [17,372] and included the following symptoms; sore throat, catarrh in throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains, weakness, headache and loss of sleep. The presence of symptoms was recorded according to severity: light (no change in training load), moderate (modified training load) and severe (training cessation), and coded as 1, 2 or 3, respectively. Weekly total symptom score was calculated by multiplying symptom duration (i.e. days) by severity. When weekly total symptom score summed to ≥ 12 , which has previously been used to categorise discrete illnesses [17], participants were contacted to confirm the presence of illness, illness duration and impact on training load.

8.3.14 Data analysis

All data are expressed as mean (\pm SD) unless otherwise stated. Data were checked for normal distribution using the Shapiro-Wilk test and, where appropriate, statistical analysis was performed on the logarithmic transformation of the data (i.e. glucose and cortisol concentrations; WBC, lymphocyte, granulocyte and monocytes concentrations; IL-4, IL-10, IFN- γ mRNA expression and the IFN- γ /IL-4 mRNA expression ratio). A three-way (diet x adaptation x time) (D- β HB, glucose and cortisol concentration and immune variables) or two-way (diet x adaptation) (net energy balance and weekly total symptom score) repeated-measured ANOVA was performed. If Mauchly's test of sphericity was violated, adjustments to the degrees of freedom were made for the ANOVA using Greenhouse-Geisser ϵ (IBM SPSS Statistics software, version 21, IBM Corp., Chicago IL, USA). Where a significant effect was observed, post hoc analysis was conducted using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons applied to the unadjusted *p* value to locate specific differences. Within-group changes from pre- to post-intervention were examined using adjusted Student's

paired *t*-tests for dependant variables. Significance level was accepted at an alpha of $p < 0.05$. To interpret the magnitude of effect and to identify trends between trials within nonsignificant data, Cohen's *d* ES ($\pm 90\%$ CL) were estimated using a purpose-built spreadsheet [333], with ES thresholds set at <0.2 , >0.2 , >0.6 , >1.2 , >2.0 and >4.0 for *trivial*, *small*, *moderate*, *large*, *very large* and *extremely large* effects, respectively [334]. If the 90% CLs overlapped 0, the magnitude of effect was deemed *unclear*.

8.4 Results

8.4.1 Dietary intake and training load

Dietary intake and training load are detailed in Chapter 7, section 7.4.2.

8.4.2 Time-to-exhaustion, substrate utilisation, cardiorespiratory parameters and perceived exertion

Time-to-exhaustion, substrate utilisation, cardiorespiratory parameters and ratings of perceived exertion are detailed in Chapter 7, sections 7.4.4, 7.4.6 and 7.4.7. There were no diet x adaptation interaction or main effects for net energy balance.

8.4.3 Changes in body mass and plasma volume

There were no interaction or main effects for exercise-induced changes in body mass (corrected for fluid intake) (pre-HD, -4.32 ± 0.75 kg vs. post-HD, -4.45 ± 0.43 kg and pre-KD, -4.39 ± 0.54 kg vs. post-KD, -3.86 ± 1.05 kg; all $p > 0.05$). Nor were there interaction or main effects for percentage plasma volume change compared to the initial blood sample (all $p > 0.05$).

8.4.4 D- β HB, glucose and cortisol

There was a significant diet x adaptation x time interaction for blood D- β HB concentration ($p < 0.001$), with significant *very large* to *extremely large* increases in the post- compared to pre-KD trial across all time points (all $p < 0.001$) (Figure 8.1A, p. 148). No differences in blood D- β HB concentration were observed between the pre- and post-HD trials (all $p > 0.05$; all $d = \text{trivial}$ or *unclear*). There was a significant diet x adaptation

x time interaction for serum glucose concentration ($p = 0.028$); however, post hoc analysis could only locate a *moderate* trend towards an increase in serum glucose concentration at pre-exercise in the post- compared to pre-KD trial ($p = 0.08$; $d = 0.92 \pm 0.62$) (Figure 8.1B, p. 148). Serum glucose concentration was elevated at exhaustion and 1-h post exhaustion in all trials (all $p < 0.05$), except for the post-KD trial, which did not change from pre-ex concentrations (all $p > 0.05$). There were no three-way or two-way interactions for serum cortisol concentration (all $p > 0.05$) (Figure 8.1C, p. 148). However, there was a main effect of time on serum cortisol concentration, which increased at exhaustion from pre-exercise ($p < 0.001$), and then declined to 1-h post-exhaustion ($p < 0.001$), but remained elevated compared to pre-exercise ($p = 0.05$). There was also a diet x adaptation interaction for serum cortisol concentration ($p = 0.019$), with a significant increase in the post- compared to pre-KD trial ($p < 0.001$).

8.4.5 Circulating leukocytes, total lymphocytes, monocytes and granulocytes

There was no difference between pre-diet trials for leukocyte, total lymphocyte, monocyte or granulocyte cell concentrations, or the relative contribution of total lymphocytes to the total leukocyte population (all $p > 0.05$). There were no three-way or two-way interactions for circulating leukocyte, lymphocyte, monocyte or granulocyte concentrations and the contribution of lymphocytes to the leukocyte population (all $p > 0.05$). A main effect of time was observed for circulating leukocyte and differential cell concentrations (all $p < 0.001$). At the onset of exercise, there was an ingress of all leukocyte populations into the circulation, resulting in elevated cell concentrations at exhaustion compared to pre-exercise (all $p < 0.001$). All circulating differential leukocyte concentrations remained higher at 1-h post-exhaustion compared to pre-exercise (all $p < 0.001$), except for total lymphocytes, which declined to below pre-exercise concentrations ($p < 0.001$). Despite the increase in lymphocyte concentration at exhaustion, there was a reduction in their relative contribution to the leukocyte population ($p < 0.001$), which remained below pre-exercise values at 1-h post-exhaustion ($p < 0.001$).

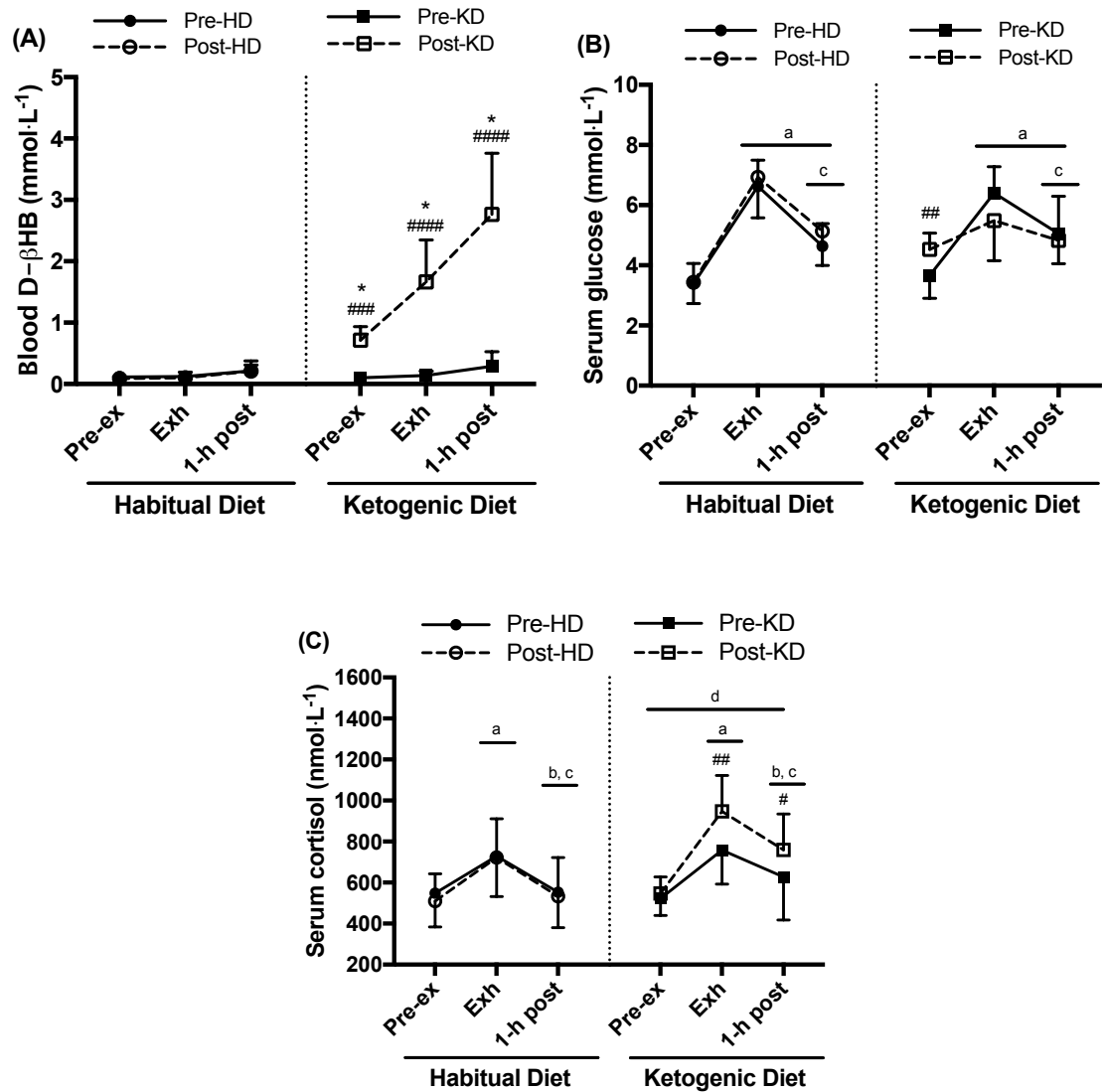


Figure 8.1. (A) capillary blood D-βHB, (B) serum glucose and (C) serum cortisol concentrations during the pre- and post-diet RTE trials. Values are presented as mean ± SD. Significantly higher in the post-KD compared to pre-KD trial for time point (* $p < 0.001$). Effect size (d); #small, ##moderate, ###very large and ####extremely large effects for the post- compared to pre-diet trial at time point. Main effect for time; significantly higher compared to pre-exercise (^a $p < 0.001$; ^b $p = 0.05$). Main effect for time; significantly lower compared to exhaustion (^c $p < 0.001$). Main effect for trial: significantly higher in post- compared to pre-KD (^d $p < 0.001$).

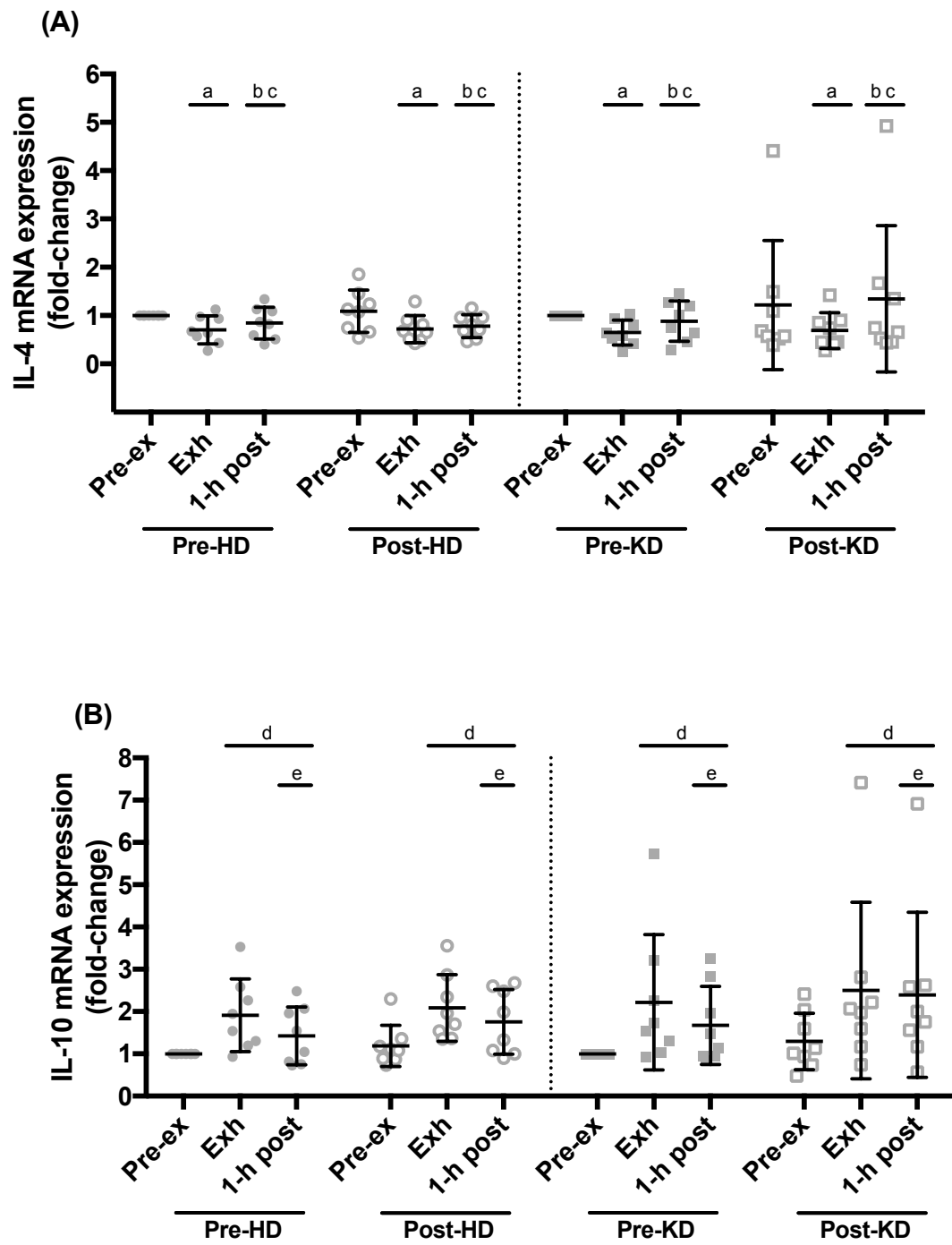
Table 8.2. Circulating leukocytes during pre- and post-diet run-to-exhaustion trials.

	Habitual Diet			Ketogenic Diet		
	Pre-exercise	Exhaustion	1-h post-exhaustion	Pre-exercise	Exhaustion	1-h post-exhaustion
<i>Leukocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i> ^{a,b}						
Pre	4.97 \pm 0.88	11.41 \pm 2.07	10.90 \pm 1.49	4.84 \pm 0.79	12.33 \pm 2.41	10.98 \pm 1.45
Post	4.69 \pm 0.59	10.92 \pm 2.27	10.13 \pm 2.51	4.42 \pm 0.65	12.03 \pm 2.13	10.61 \pm 1.43
ES (\pm 90% CI)	-0.29 \pm 0.34	-0.21 \pm 0.38	-0.54 \pm 0.68	-0.52 \pm 0.51	-0.09 \pm 0.55	-0.22 \pm 0.83
<i>Total lymphocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i> ^{a,c}						
Pre	1.71 \pm 0.19	1.96 \pm 0.53	1.53 \pm 0.44	1.53 \pm 0.25	1.76 \pm 0.34	1.21 \pm 0.31
Post	1.60 \pm 0.29	1.89 \pm 0.42	1.39 \pm 0.28	1.49 \pm 0.32	2.07 \pm 0.78	1.20 \pm 0.70
ES (\pm 90% CI)	-0.60 \pm 0.86	-0.08 \pm 0.37	-0.26 \pm 0.36	-0.18 \pm 0.49	0.54 \pm 0.58	-0.33 \pm 0.78
<i>Monocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i> ^{a,b}						
Pre	0.39 \pm 0.09	0.65 \pm 0.12	0.71 \pm 0.22	0.44 \pm 0.10	0.83 \pm 0.28	0.83 \pm 0.23
Post	0.34 \pm 0.13	0.64 \pm 0.20	0.67 \pm 0.21	0.36 \pm 0.11	0.79 \pm 0.27	0.70 \pm 0.21
ES (\pm 90% CI)	-0.54 \pm 0.63	-0.16 \pm 0.83	-0.07 \pm 0.68	-0.74 \pm 0.44	-0.07 \pm 0.66	-0.43 \pm 0.36
<i>Granulocytes ($\times 10^9 \text{ cells}\cdot\text{L}^{-1}$)</i> ^{a,b}						
Pre	2.96 \pm 0.78	8.76 \pm 1.52	8.86 \pm 1.31	2.85 \pm 0.68	9.57 \pm 2.00	8.95 \pm 1.56
Post	2.74 \pm 0.51	8.83 \pm 1.94	8.33 \pm 2.14	2.56 \pm 0.47	9.15 \pm 1.47	8.74 \pm 1.04
ES (\pm 90% CI)	-0.25 \pm 0.40	0.00 \pm 0.28	-0.23 \pm 0.64	-0.39 \pm 0.42	-0.16 \pm 0.49	-0.08 \pm 0.60

Values are presented as mean \pm SD. Main effect for time; significantly higher at exhaustion compared to pre-exercise (^a $p < 0.001$). Main effect for time; significantly higher at 1-h post-exhaustion compared to pre-exercise (^b $p < 0.001$). Main effect for time; significantly lower at 1-h post-exhaustion compared to pre-exercise (^c $p < 0.001$).

8.4.6 T-cell related cytokine mRNA expression within multi-antigen-stimulated PBMCs

Quantifiable mRNA expressions of PBMC C β , IL-4, IL-10 and IFN- γ were detected in all multi-antigen-stimulated whole blood samples (Figures 8.2A-D, pp. 151-152). There were no three-way or two-way interactions for IL-4 and IL-10 mRNA expression (all $p > 0.05$). However, there was an effect of time for IL-4 ($p = 0.004$) and IL-10 ($p < 0.001$). Compared to pre-exercise, IL-4 mRNA expression was lower at exhaustion ($p < 0.001$) and 1-h post-exhaustion ($p = 0.02$), but remained higher at 1-h post-exhaustion compared to exhaustion ($p = 0.005$) (Figure 8.2A, p. 151). Conversely, IL-10 mRNA expression was higher at exhaustion ($p < 0.001$) and 1-h post-exhaustion ($p < 0.001$) compared to pre-exercise, but declined from exhaustion to 1-h post-exhaustion ($p < 0.001$) (Figure 8.2B, p. 151). There was a diet x adaptation x time interaction for IFN- γ mRNA expression ($p = 0.002$), with a significant *moderate* increase at exhaustion in the post- compared to pre-KD trial ($p = 0.02$; $d = 0.89 \pm 0.39$) (Figure 8.3C, p. 152). No specific differences were observed between the pre- and post-HD trials (all $p > 0.05$; all $d = \text{unclear}$). There was a diet x adaptation x time interaction for the IFN- γ /IL-4 ratio ($p = 0.001$), with a significant *moderate* increase at exhaustion in the post- compared to pre-KD trial ($p = 0.01$; $d = 0.84 \pm 0.23$) and a trend towards a *moderate* reduction at exhaustion in the post- compared to pre-HD trial ($p = 0.49$; $d = -0.65 \pm 0.64$) (Figure 8.2D, p. 152).



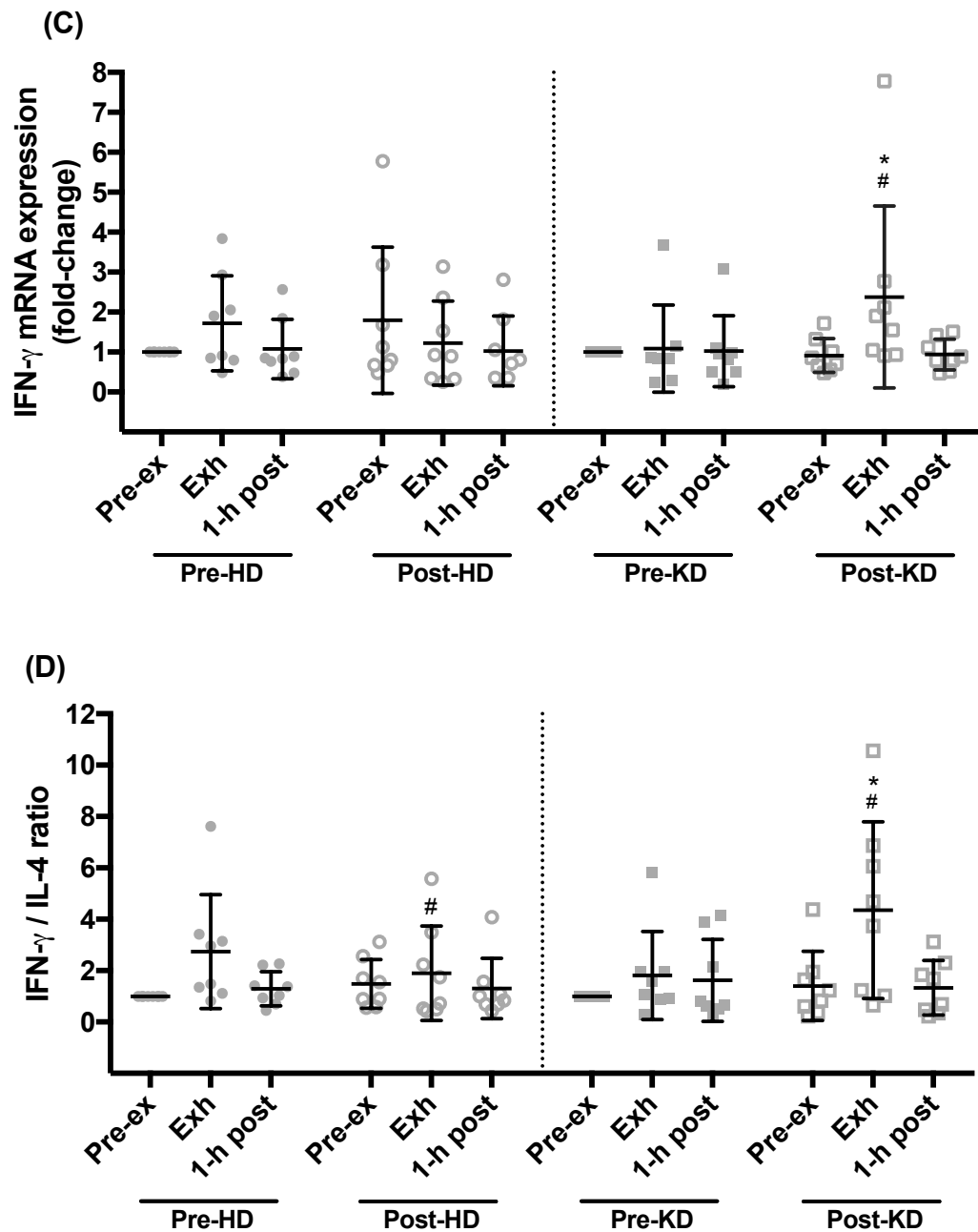
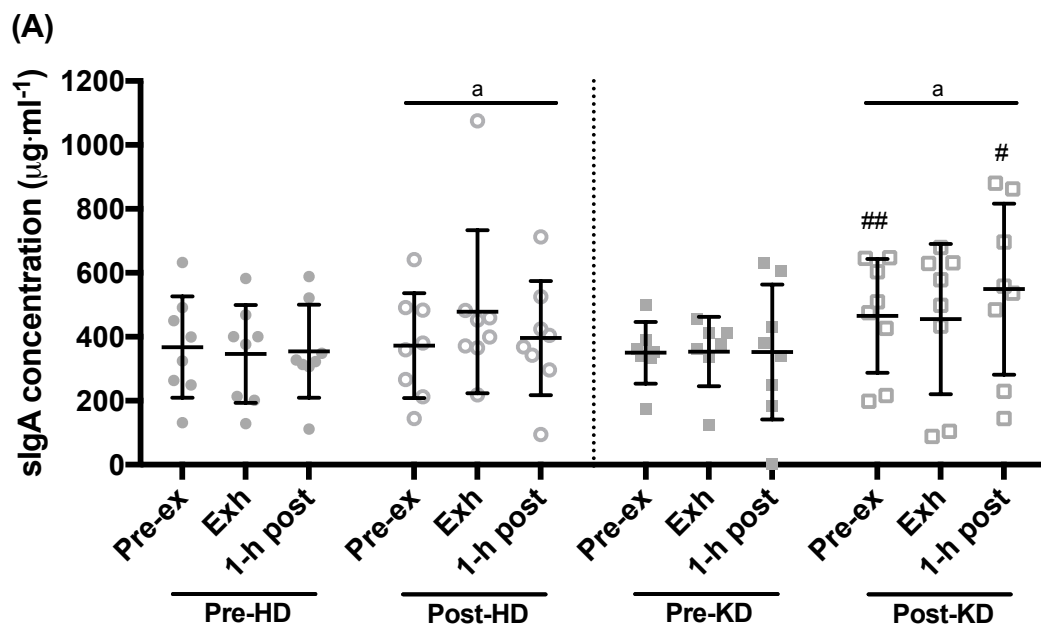


Figure 8.2. T-cell related cytokine mRNA expression following 24 h whole blood multi-antigen stimulation for (A) IL-4, (B) IL-10, (C) IFN- γ and (D) IFN- γ /IL-4 ratio during the pre- and post-diet RTE trials. HD *habitual diet*, KD *ketogenic diet*, IL-4 interleukin-4, IL-10 interleukin-10 and IFN- γ interferon- γ . Values are presented as mean \pm SD and individual responses. Significantly higher in post-KD compared to pre-KD trial for time point (* $p < 0.05$). Effect size (d); #*moderate* effect for the post- compared to pre-diet trial at time point. Main effect for time; significantly lower compared to pre-exercise (^a $p < 0.001$; ^b $p < 0.05$). Main effect for time; significantly higher compared to exhaustion (^c $p < 0.01$). Main effect for time; significantly higher compared to pre-exercise (^d $p < 0.001$). Main effect for time; significantly lower compared to exhaustion (^e $p < 0.001$).

8.4.7 Salivary immunoglobulin A

There were no diet x adaptation x time interactions for SIgA concentration, saliva flow rate or SIgA secretion rate (all $p > 0.05$) (Figures 8.3A-C, pp. 153-154). There was an effect of adaptation on SIgA concentration ($p = 0.019$) and secretion rate ($p = 0.002$), with an increase in the post-HD and KD trials, compared to the pre-HD and KD trials, respectively. There was an effect of time on saliva flow rate ($p = 0.04$) and SIgA secretion rate ($p = 0.048$). Saliva flow rate significantly increased from pre-exercise to exhaustion ($p = 0.006$), then declined from exhaustion to 1-h post-exhaustion ($p = 0.008$). Whereas, for SIgA secretion rate, there was only a trend for an increase from exhaustion to 1-h post-exhaustion ($p = 0.068$).



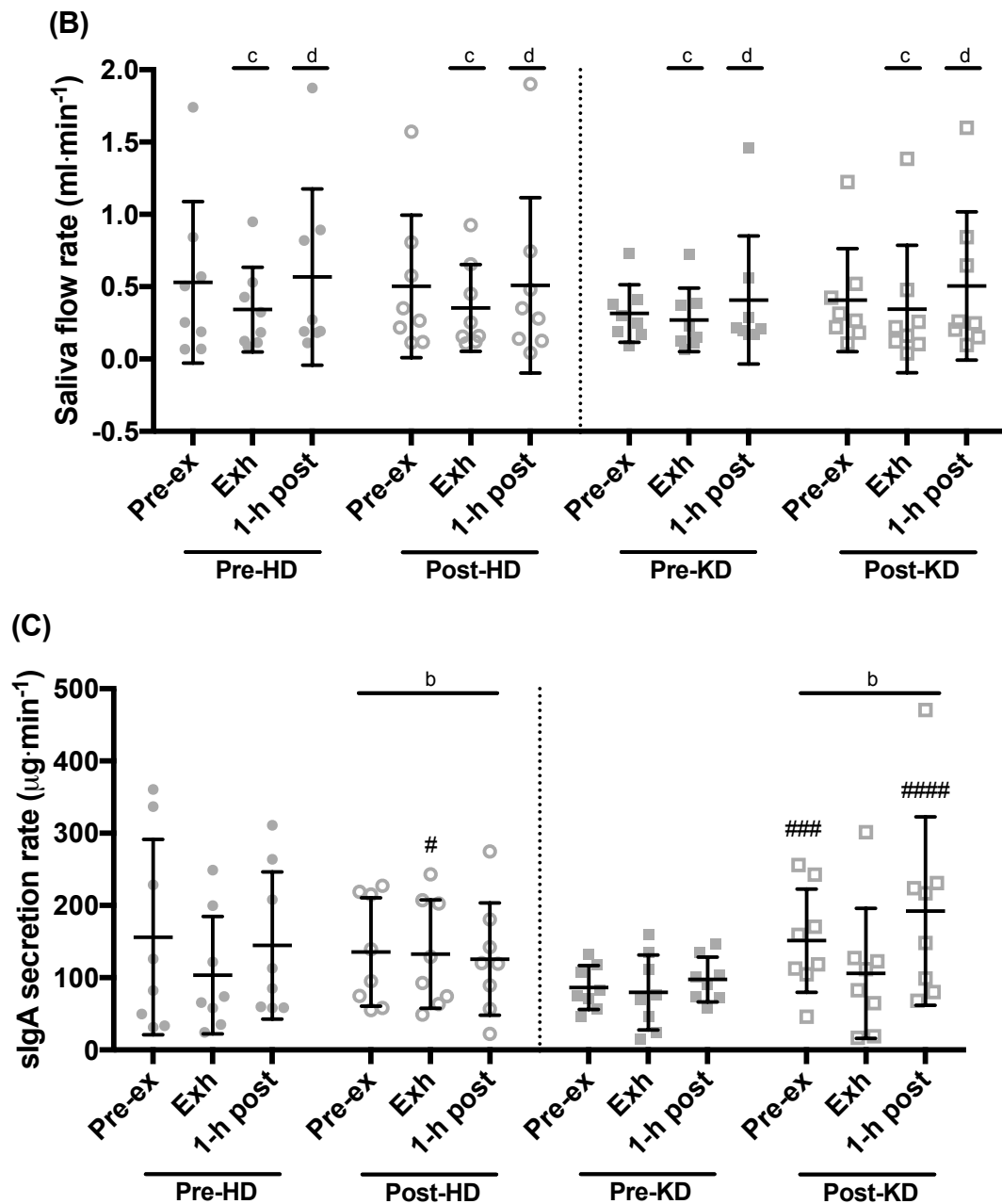


Figure 8.3. (A) salivary secretory immunoglobulin A concentration, (B) saliva flow rate and (C) salivary secretory immunoglobulin A secretion rate during the pre- and post-diet RTE trials. Values are presented as mean \pm SD and individual responses. Effect size (^d); [#]*small*, ^{###}*moderate*, ^{####}*large* and ^{#####}*very large* effects for the post- compared to pre-diet trial at time point. Main effect for adaptation; significantly higher post- compared to pre-adaptation trial (^a $p < 0.05$; ^b $p < 0.01$). Main effect for time; significantly lower compared to pre-exercise (^c $p < 0.01$). Main effect for time; significantly higher compared to exhaustion (^d $p < 0.01$).

8.4.8 Weekly total symptom score

Weekly TTS are depicted in Figure 8.4 (p. 155). There was no interaction ($p = 0.145$) or main effects for diet ($p = 0.167$) or time ($p = 0.34$) on weekly TSS. However, there was

a *small* trend towards an increase in TSS during the first week in the KD compared to the HD ($d = 0.51 \pm 0.50$). Despite a weekly TSS ≥ 12 on 27 occasions (HD, 12 vs. KD, 15), only one participant confirmed the presence of an illness. This occurred on day 22 (i.e. beginning of week 4) in the KD for a duration of four days and resulted in reduced training load. However, it resolved three days prior to the post-KD trial, with the participant displaying normal haematological values on arrival. No medications or supplements were used to prevent or treat symptoms for all participants during the study.

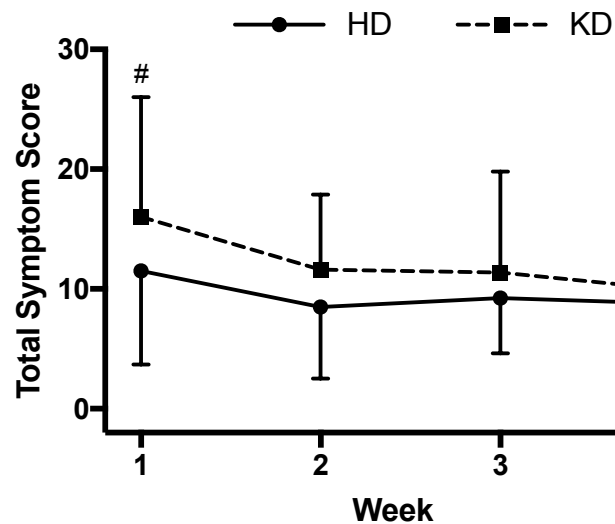


Figure 8.4. Weekly total symptom score during the habitual diet and ketogenic diet conditions. Values are presented as mean \pm SD. Effect size (d); #*small* effect between the HD and KD at time point.

8.5 Discussion

This study examined the effect of a 31-day KD on markers of mucosal and adaptive immune function in response to exhaustive moderate-intensity exercise. The main findings were adaptation to a 31-day KD: 1) increased T-cell related IFN- γ mRNA expression within multi-antigen-stimulated PBMCs and the IFN- γ /IL-4 mRNA ratio at exhaustion, which normalised 1 h into recovery; 2) had no effect on T-cell related IL-4 and IL-10 mRNA expression within multi-antigen-stimulated PBMCs; 3) had no effect on SIgA concentration and secretion rate and; 4) had no effect on illness prevalence. Therefore, adaptation to a KD may not impair immune function; rather, it transiently amplify the pro-inflammatory type-1 T-cell cytokine response to an immune challenge at the gene level immediately following exhaustive exercise.

A 31-day KD adaptation period was employed as at least 3–4 weeks is necessary to restore endurance performance and capacity [14,15]. Indeed, mean TTE was preserved despite CHO-restriction (detailed in Chapter 7, section 7.6.1), supporting participants were *keto-adapted*. In contrast, earlier studies investigating dietary CHO and fat manipulation on exercise-induced immune perturbations have typically implemented short-term interventions (i.e. <14 days) or have failed to control energy balance [288] and, therefore, may not reflect optimal dietary adaptation. In order to validate dietary adherence, participants' dietary intake and level of hyperketonaemia were rigorously monitored by a RD as described in Chapter 7, section 7.3.4, with nutrient intakes reported in Chapter 7, section 7.4.2. Moreover, the purpose of acute feeding strategies (i.e. low- or high-CHO pre-trial meal and CHO or fat supplementation during the RTE) was to further polarise substrate availability and metabolism (detailed in Chapter 7, sections 7.6.2 and 7.6.3). Whereas, if CHO was ingested in the post-KD trial, this would oppose adaptations to the KD and suppress hepatic ketogenesis, thus compromising rates of fat and KB oxidation [10]. It was also expected that stark differences in CHO availability would lead to greater exercise-induced rises in cortisol concentration in the post-KD trial. However, the TTE compared to matched-workload protocols are more likely to lead to a similar state of physiological exhaustion, including depleted skeletal muscle glycogen stores, thus minimising potential differences in cortisol concentration.

Unexpectedly, SIgA concentration and secretion rate increased following adaptation to both diets. Since exercise-induced serum cortisol concentration was higher at exhaustion in the post-KD trial, it is likely that elevated cortisol concentration was exacerbated during the adaptation period to the KD; theoretically, reducing SIgA transport and secretion [367]. Nonetheless, cortisol may not be an important modulator of SIgA [357,373], as mechanisms other than cortisol, such as increased SNS innervation of the salivary glands stimulating the production of concentrated SIgA [357], potentially being responsible for the observed effects on SIgA in the present study. Low training loads (average running distance of ~63 km·week⁻¹) in the present study could also have been an insufficient stressor for differences in SIgA to manifest between dietary conditions. In contrast, an earlier study demonstrated 3 weeks of *intensified* training can also increase SIgA concentration irrespective of high-CHO or KD interventions in elite race walkers [37]; supporting the notion that mucosal immunity can adapt, rather than weaken, in the face of intensified training [374].

An unanticipated finding was the transient increase in T-cell related IFN- γ gene expression within multi-antigen-stimulated PBMCs at exhaustion following adaptation to the KD. Since T-cell related IL-4 gene expression did not differ, there was a shift in the IFN- γ /IL-4 ratio towards IFN- γ predominance at exhaustion. However, these effects were short-lived and normalised 1 h into recovery. This suggests keto-adaptation can enhance resistance against intracellular pathogens, such as viruses, by promoting the initiation of a type-1 T-cell cytokine response. As these effects coincided with higher serum cortisol concentrations, impaired T-cell related IFN- γ gene expression would have been expected; however, the effect of cortisol on antigen-stimulated T-cell cytokine gene expression is not clear [32] and may not be an important suppressor of immune cell function following exercise, as previously suggested within *in vitro* [341] and *in vivo* models [197,342]. Alternatively, elevated blood KBs could act as an additional energy source for immune cells, with lymphocytes both producing [282] and oxidising KBs [51-53]; however, this is highly speculative as mechanisms demonstrating that KB oxidation favours a type-1 T-cell response are yet to be elucidated.

Irrespective of diet, T-cell related IL-10 gene expression within multi-antigen-stimulated PBMCs increased at exhaustion and subsequently declined but remained elevated 1 h into recovery. Therefore, an anti-inflammatory effect opposing IFN- γ gene expression would have been expected; however, no changes were observed. Furthermore, the current findings contrast a previous study demonstrating lower circulatory TReg cell concentration 1 h following a marathon [31]. However, as *in vitro* multi-antigen stimulated whole blood IL-10 production appears unaffected following prolonged, strenuous exercise [27-29], it is possible that increased IL-10 gene expression does not follow similar patterns. Speculatively, substrate availability may have influenced TReg cell function *in vitro*. Circulating FFA concentration was likely higher at exhaustion in all trials [375], which is preferentially used by TReg cells for energy, and can inhibit the activity of type-1 and type-2 T-cells [277], which may have promoted T-cell related IL-10 gene expression.

The whole blood culture in the present study aimed to model an *in vivo* pathogenic challenge. Multi-antigen stimulation was also chosen as it activates up to 2-5% of T-cells that recognises the antigen [241], thus allowing the detection of subtle perturbations to immune function typically occurring *in vivo*. Whereas, mitogens, superantigens and antibodies, nonspecifically activate a large proportion of T-cells. Although shifts in CD4⁺

and CD8⁺ T-cell subsets were not measured in the present study, expressing cytokine gene expression relative to the C β gene accounted for the exercise-induced shifts in T-cell concentration within the PBMC population. For example, C β gene expression would have increased in conjunction with the ingress of T-cells following the onset of exercise and vice versa following exercise cessation. Nonetheless, it is difficult to associate alterations in immune function to illness and infection susceptibility as little to no difference in illness rate and total symptom score was observed between dietary conditions. As athletes on average have been shown to experience about three URS/URTI or gastrointestinal infections per year [4,376], the current study was also unlikely to be of sufficient duration and power to elucidate differences in illness rate.

8.6 Conclusion

Adaptation to a KD maintains mucosal immunity, specifically SIgA concentration and secretion rate; whereas, for adaptive immunity, T-cell related IFN- γ gene expression within multi-antigen-stimulated PBMCs and the IFN- γ /IL-4 gene expression ratio is elevated at exhaustion, suggesting an enhanced initiation of a type-1 T-cell cytokine response to an immune challenge. As IFN- γ gene expression and the IFN- γ /IL-4 gene expression ratio normalises 1 h into recovery, it is possible that type-1 T-cell response is enhanced only transiently or, as immune cells egress from the blood, there is increased type-1 T-cell immunity in peripheral tissues. These effects occurred despite the immunosuppressive effect of elevated cortisol concentration, suggesting alternative mechanisms have an overriding influence on immunomodulation following exhaustive exercise, which may include the autonomic nervous system, substrate availability and/or trafficking patterns of circulatory immune cell subsets. In contrast, adaptation to a KD did not alter T-cell related IL-4 and IL-10 gene expression within multi-antigen-stimulated PBMCs. Altogether, it is uncertain whether this reflects illness risk *in vivo*, but it does suggest KDs may be used without exacerbating exercise-induced immunodepression. Nevertheless, further research into the effects of KDs on immune function among athletes is warranted.

General Discussion

This chapter summarises the main findings of the thesis in relation to the original aims outlined in Chapter 1. The thesis will be discussed as a cohesive whole under key themes, followed by the limitations, recommendations for future research and practical implications.

9.1 Main findings

In this thesis, nutritional ketosis was achieved via acute supplementation with a ketogenic supplement, BD (Chapters 4, 5 and 6) and endogenous hepatic ketogenesis via chronic adherence to a KD (Chapters 7 and 8). As these strategies exert two distinct metabolic profiles, they were evaluated in different exercise contexts for which they were deemed to possess greater ergogenic potential (i.e. high-intensity vs. submaximal intensity exercise). Therefore, it is difficult to directly compare performance/endurance and immune outcomes; however, similar themes will be identified and discussed in relation to previous research.

The main performance-related findings from this thesis were:

1. BD ingestion increased blood D- β HB concentration to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$ without altering oxygen uptake, RER or blood glucose and lactate concentrations during 85 min of SS exercise at 85% VT_2 ($\sim 73\% \text{VO}_{2\text{max}}$) (Chapter 5).
2. BD ingestion had no effect on pre-loaded cycling TT (~ 30 min) performance (Chapter 5).
3. 31-days adaptation to a KD impaired high-intensity endurance performance as evidenced by a lower RER and running velocity at $\text{VO}_{2\text{max}}$ (Chapter 7).
4. 31-days adaptation to a KD shifted fuel preference towards fat oxidation, as evidenced by reduced RER at all exercise intensities, but impaired exercise efficiency at intensities $\geq 70\% \text{VO}_{2\text{max}}$ as evidenced by increased unaccounted oxygen uptake and EE (Chapter 7).

5. 31-days adaptation to a KD preserved mean submaximal exercise capacity without necessitating CHO ingestion during a RTE at 70% $\text{VO}_{2\text{max}}$ (Chapter 7).

The main immune-related findings from this thesis were:

- 1) At rest, BD ingestion increased blood D- β HB concentration to $\sim 1 \text{ mmol}\cdot\text{L}^{-1}$ without altering serum glucose and cortisol concentrations or *in vitro* T-cell related IL-4, IL-10 and IFN- γ gene expression within SEB-stimulated PBMCs (Chapter 4).
- 2) BD ingestion increased *in vitro* T-cell related IFN- γ gene expression within SEB-stimulated PBMCs following 85 min of cycling at 85% VT_2 ($\sim 73\% \text{VO}_{2\text{max}}$), the proceeding ~ 30 min TT and 1 h into recovery. At 1 h in recovery, IFN- γ gene expression appeared to be below pre-exercise values, suggesting a transient effect. However, BD ingestion did not alter serum cortisol concentration or *in vitro* T-cell related IL-4 and IL-10 gene expression within SEB-stimulated PBMCs in response to exercise (Chapter 6).
- 3) 31-days adaptation to a KD transiently increased T-cell related IFN- γ gene expression within multi-antigen-stimulated PBMCs *in vitro* immediately following a RTE at 70% $\text{VO}_{2\text{max}}$, despite elevated serum cortisol concentration; however, adaptation to a KD did not alter T-cell related IL-4 and IL-10 gene expression within multi-antigen-stimulated PBMCs (Chapter 8).
- 4) 31-days adaptation to a KD did not alter SIgA concentration or secretion rate at rest or following a RTE at 70% $\text{VO}_{2\text{max}}$ (Chapter 8).

9.2 Key themes

9.2.1 Defining keto-adaptation for endurance athletes: What is optimal?

A primary conundrum of the present thesis was to establish and implement a dietary-training intervention to reliably examine the performance and immune effects of *keto-adaptation*. Keto-adaptation refers to the acute and chronic physiological adaptations exerted by conformity to a KD (see Table 9.1, p. 163, for comparison with ketone

supplementation). However, there is currently no clear definition or understanding of what constitutes *keto-adaptation* compared to fat-adaptation for endurance athletes or strategies for its optimisation. Only recently have studies investigated the effects of keto-adaptation beyond performance [37,316,317,377-379]. The primary rationale for keto-adaptation is to; 1) increase fat oxidation [14,15,48]; 2) reduce blood glucose utilisation [15] and; 3) reduce muscle [15,35] and hepatic [35] glycogen utilisation, thus preserving finite supplies of endogenous CHO to delay fatigue. As such, keto-adaptation is an extension of fat-adaptation (see Chapter 2, section 2.7), whereby there is chronic exposure to hyperketonaemia. Nonetheless, keto-adaptation is not necessarily a binary physiological state categorised by continual blood KB concentrations greater or less than 0.5 mmol·L⁻¹; rather, the ability to rapidly and efficiently increase ketogenesis and ketolysis relative to CHO availability and energy demands.

Several months of conforming to a KD has been suggested to be necessary to exert metabolic benefits. However, studies of this duration are cross-sectional, based on self-reported dietary intakes and did not examine performance [35,48] or failed to rigorously monitor dietary intake and training load [174]. Further, a scarcity of successful endurance athletes employing keto- and/or fat-adaptation techniques, despite earnest attempts to improve endurance performance following chronic adherence [177], emphasise that shorter time frames are likely to exert maximal adaptations – either ergogenic or ergolytic. As such, 3-4 weeks may be sufficient to restore (not improve) pre-adaptation endurance capacity at moderate intensities [15] and high-intensity endurance performance [14]; therefore, a 31-day keto-adaptation period was opted for in the present thesis. Based on the following criteria: 1) regular and reliable measurement of dietary intake; 2) daily pre-exercise measurement of blood and/or urinary KB concentrations; and 3) pre- and post-metabolic testing using a graded exercise protocol to determine cardiorespiratory parameters, RPE and substrate oxidation, for which were employed in the present thesis, the findings presented in Chapter 7 support the notion that participants were keto-adapted.

Table 9.1. Summary of metabolic responses during moderate intensity (60-80% $\text{VO}_{2\text{max}}$) exercise between ketone supplementation and keto-adaptation (3 week minimum) compared to nonketotic conditions.

Metabolic variable	Ketone supplementation	Keto-adaptation
Indirect estimation of whole body substrate oxidation	<ul style="list-style-type: none"> Indirect calorimetry is currently not validated to estimate CHO, fat or KB oxidation following ketone supplementation. 	<ul style="list-style-type: none"> CHO oxidation reduced by 2- to 3-fold [14,15,35,48] (Chapter 7). Fat oxidation (including fatty-acid-derived KBs) increases 2- to 3-fold [14,15,35,48] (Chapter 7). Maximal reported fat oxidation rates of $\sim 1.9 \text{ g}\cdot\text{min}^{-1}$ at $\sim 80\% \text{VO}_{2\text{max}}$ in elite racewalkers [14]. Indirect calorimetry is currently not validated to estimate KB oxidation.
Direct measurement of KB oxidation	<ul style="list-style-type: none"> No evidence available. 	<ul style="list-style-type: none"> No evidence available.
Blood FFA and glycerol concentration	<ul style="list-style-type: none"> Reduced blood FFA concentration following R-BD D-βHB monoester [73] and R,S-BD AcAc diester [67] ingestion; no evidence available for ketone salts or BD. Reduced blood glycerol concentrations following R-BD D-βHB monoester ingestion [73]; no evidence available for R,S-BD AcAc diester, ketone salts or BD. 	<ul style="list-style-type: none"> Increased blood FFA [15,35,48] and glycerol [35,48] concentrations.
IMTG stores and/or utilisation	<ul style="list-style-type: none"> Increased IMTG utilisation following R-BD D-βHB monoester ingestion [73]; no evidence available for R,S-BD AcAc diester, ketone salts or BD. 	<ul style="list-style-type: none"> No evidence available for keto-adaptation; however, IMTG stores are likely to increase following ingestion of a high-fat diet [380].
Glycogen stores and/or utilisation	<ul style="list-style-type: none"> Reduced skeletal muscle glycogen utilisation following R-BD D-βHB monoester ingestion [73]; no evidence available for R,S-BD AcAc diester, ketone salts or BD. No evidence available for hepatic glycogen utilisation during exercise. 	<ul style="list-style-type: none"> Reduced skeletal muscle glycogen stores ($\sim 50\%$) and utilisation [15,35]; however, a single study suggested no effect [48]. No measurement of hepatic glycogen stores; however, in most instances, depletion is obligatory to increase hepatic ketogenesis [10]. Reduced hepatic glycogen utilisation [35].
Blood glucose concentration and utilisation	<ul style="list-style-type: none"> Reduced blood glucose concentration following ingestion of R-BD D-βHB monoester (with and without CHO coingestion) [73] and R,S-BD AcAc diester [67]; effects of racemic ketone salts and BD are unclear [61-63,90] (Chapter 5). No evidence available for blood glucose utilisation. 	<ul style="list-style-type: none"> Blood glucose concentrations unchanged [15,35,48] (Chapter 7), but may remain lower compared to CHO ingestion during exercise [14]. Reduced blood glucose oxidation [15].

Blood lactate concentration	<ul style="list-style-type: none"> • Reduced blood lactate concentration following R-BD D-βHB monoester [73,74] and R,S-BD AcAc diester ingestion [67]; no effect for racemic ketone salts and BD [61-63,90] (Chapter 5). 	<ul style="list-style-type: none"> • Blood lactate concentration unchanged [14,15] (Chapter 7) or increased [35,48].
Acid-base balance	<ul style="list-style-type: none"> • Reduced blood pH and increased H^+ ion and reduced HCO_3^- concentrations following R-BD D-βHB monoester ingestion [79]; no evidence available for R,S-BD AcAc diester, ketone salts and BD. 	<ul style="list-style-type: none"> • Blood pH, H^+ ion and HCO_3^- concentrations unchanged [379].
Oxygen uptake and energy expenditure	<ul style="list-style-type: none"> • Oxygen uptake unchanged [61-63,67,73,90] (Chapter 5). 	<ul style="list-style-type: none"> • Increased oxygen uptake and energy expenditure at exercise intensities $>70\% VO_{2max}$ [14] (Chapter 7).
Heart rate	<ul style="list-style-type: none"> • Heart rate unchanged [61-63,67,73,90] (Chapter 5). 	<ul style="list-style-type: none"> • Increased heart rate [14] (Chapter 7).
Stress hormones	<ul style="list-style-type: none"> • Blood cortisol concentrations unchanged following coingestion of R-BD D-βHB monoester and CHO (compared to iso-energetic CHO ingestion) [73] and BD (Chapter 5); no evidence for R,S-BD AcAc diester and ketone salts. • No evidence available for blood catecholamines. 	<ul style="list-style-type: none"> • Increased blood cortisol concentration [312] (Chapter 8). • No evidence available for the effect of keto-adaptation on blood catecholamines; however, fat-adaptation can increase blood noradrenaline concentration [381].

The chapters within the present thesis pertinent to each metabolic variable are noted in brackets alongside citations. CHO *carbohydrate*, KB *ketone body*, VO_{2max} *maximum oxygen uptake*, FFA *free fatty acid*, β HB *β -hydroxybutyrate*, BD *R,S-1,3-butanediol*, AcAc *acetoacetate*, IMTG *intramuscular triglyceride*.

9.2.2 Nutritional ketosis and endurance performance

High-intensity ($>80\%VO_{2max}$) endurance performance up to ~ 3 hours is CHO-dependent [115], but not necessarily limited by CHO-availability [382]. Maximising energy production per unit of oxygen is important to performance; therefore, a shift towards fat oxidation may be ergolytic as the oxidation of fat compared with CHO results in a $\sim 5\%$ reduction in efficiency [108]. Whereas, at moderate intensities, depleting endogenous CHO-availability can result in fatigue [383]; therefore, strategies to reduce the rate of endogenous CHO utilisation at a given intensity is desirable. Ketone bodies downregulate skeletal muscle [73] and hepatic [33] glycogen utilisation and self-regulate their own production by suppressing adipocyte lipolysis [10]. Ketone bodies may also be a more efficient fuel source than CHO and fat, as demonstrated in isolated rodent tissue models [136,137,139], with increased energy yield per C_2 unit [137] and greater Gibbs free energy of ATP hydrolysis [65]. Therefore, nutritional ketosis is frequently implicated in

fuelling strategies for endurance performance; however, potential performance benefits and risks exist at both high and moderate exercise intensities. Discussed below are the effects of ketone supplementation and keto-adaptation on endurance performance for which there are tabulated summaries in Appendices A and B, including the findings of the current thesis.

9.2.2.1 Ketone supplementation and high-intensity endurance performance

Ketone and/or ketogenic supplements theoretically increase substrate provision for energy production in an attempt to enhance high-intensity exercise performance; however, BD ingestion in Chapter 5 did not improve performance during a ~30 min pre-loaded TT. Similarly, high-intensity endurance performance has been shown to be either unaffected [63,90] or impaired [62] following the ingestion of racemic ketone salts and BD. These findings may be due to acute increases in blood D- β HB concentrations up to ~1 mmol·L⁻¹ having no meaningful effect on oxygen uptake, substrate utilisation or RPE. Moreover, adverse gastrointestinal effects and, as observed in Chapter 5, dizziness, nausea and euphoria can arise with ketone supplementation. Whereas, the racemic R,S-BD AcAc diester, in conjunction with recommended CHO fuelling strategies, was shown to increase capillary blood D- β HB concentrations to 0.8-1.3 mmol·L⁻¹ (serum D- β HB ~0.4 mmol·L⁻¹) and serum AcAc concentration to ~0.5 mmol·L⁻¹ during a ~50 min cycling TT [67]. Blood glucose, fatty acid and lactate concentration was lower than the placebo condition, which was potentially due to the concomitant increase in serum AcAc concentration [107], which has a central role in KB metabolism (Figure 2.2, p. 14). Nonetheless, performance time was impaired by ~2% (~3.7% reduction in average power output [~339 vs. ~335 W]), which was attributed to severe gastrointestinal distress, with one participant failing to complete the test (who also had the highest serum AcAc concentration) [67]. Therefore, it seems racemic KSs should be avoided for high-intensity endurance performance, particularly if exercise duration does not deplete CHO-availability.

Nonracemic ketone supplements exert greater metabolic effect during exercise. For example, the coingestion of the R-BD D- β HB monoester and CHO compared to an isoenergetic CHO only solution increased blood D- β HB concentrations ~2.0-3.5 mmol·L⁻¹ whilst cycling at 70% $\text{VO}_{2\text{max}}$, which reduced skeletal muscle glycogen utilisation, PDHc activity, plasma lactate, blood glucose and FFA concentrations and increased

IMTG utilisation [73]. Within the same cohort of studies, the coingestion of R-BD D- β HB monoester and CHO after an overnight fast increased distance cycled by ~2% during a pre-loaded 30 min TT [73]. However, R-BD D- β HB monoester ingestion with CHO fuelling strategies in team-sport athletes, which increased blood D- β HB concentrations to 1.0-2.5 mmol·L⁻¹ and reduced blood glucose and lactate concentration, did not affect exercise capacity (~229 vs. ~267 s) in a RTE protocol preceded by 75 min of intermittent high-intensity running [74]. Similarly, maximal power output did not change following the ingestion of the R-BD D- β HB monoester compared to placebo prior to an incremental exercise test, despite increased leg discomfort and anxiety of breathing [78] and increased acid-base balance [79]. Therefore, it also appears that *nonracemic* ketone supplements do not provide an ergogenic benefit compared to *racemic* ketone supplements.

9.2.2.2 Keto-adaptation and high-intensity endurance performance

The deleterious effects of keto-adaptation on high-intensity endurance performance have been reviewed in Chapter 2, section 2.7. The primary concern is lowering of CHO availability and oxidation via suppression of glycogenolysis and PDHc activity [147]. In Chapter 7, this manifested as a reduction in RER at $\text{VO}_{2\text{max}}$, which has been previously demonstrated [14,15], and reduced running speed at $\text{VO}_{2\text{max}}$. Additionally, in Chapter 7, 31-days of keto-adaptation increased EE and oxygen uptake beyond what can be explained by reductions in RER for pre- to post-adaptation (i.e. unaccounted oxygen uptake) when exercising at >70% $\text{VO}_{2\text{max}}$, suggesting increased mitochondrial uncoupling. Furthermore, 3 weeks of keto-adaptation during intensified training in elite race walkers negated improvements in a 10 km TT performance compared to a high-CHO diet, despite blood D- β HB concentrations of 0.9-1.5 mmol·L⁻¹ [14]; the performance decrement can be attributed to a greater oxygen cost of exercise and unaccounted oxygen uptake (from re-analysis of reported RER values using customised equations in Chapter 7, section 7.3.7). Therefore, despite KBs bypassing PDHc for oxidation in exercising muscles, increased ketolysis appears insufficient to overcome the reduction in CHO oxidation rates following keto-adaptation, thus compromising high-intensity endurance performance.

9.2.2.3 *Ketone supplementation and prolonged, moderate-intensity endurance performance*

Ketone supplementation has been *tentatively* linked with performance effects for endurance competition lasting several hours [6]; however, no studies have examined their effect. Despite the potential for increased IMTG utilisation following R-BD D- β HB monoester ingestion [73], IMTG stores are depleted by ~50-70% during the initial 2-3 hours of (fasted) exercise, which places greater reliance on adipose tissue lipolysis to maintain fat oxidation [384-387]. Since ketone ester ingestion also suppresses adipose tissue lipolysis [67,73], an important fuel source for prolonged events [388], CHO-oxidation rates could increase, thus accelerating fatigue. Moreover, endurance competition lasting several hours is highly influenced by gastrointestinal symptoms, which can be exacerbated by ketone supplementation [67,68,74] (including Chapter 5), thus preventing sufficient CHO intake. Clearly, further research investigating the effects of ketone supplementation on prolonged, moderate-intensity endurance performance is required.

9.2.2.4 *Keto-adaptation and prolonged, moderate-intensity endurance performance*

Despite plausible ergogenic effects of keto-adaptation on submaximal intensity exercise, there is a scarcity of research examining its effects on endurance performance lasting >3 h. Chapter 7 aimed to address the limitations of the only study having investigated the effects of keto-adaptation on submaximal intensity exercise capacity [15]; to do this, the methodology involved 31-days of keto-adaptation using a randomised, repeated-measures, cross-over design and acute fuelling strategies during the RTE trials according to the trial allocation (see Chapter 7, section 7.3). Mean TTE was preserved at 70% $\text{VO}_{2\text{max}}$; however, only three of eight participants experienced an endurance improvement (see Figure 7.4), suggesting some athletes may be metabolically, or genetically, endowed to benefit from keto-adaptation. This increased endurance variability seems may be a hallmark of keto-adaptation, as previously demonstrated [15]; therefore, it may not be able to support high training loads and rates of EE for some athletes. This means that CHO ingestion before and/or during competition is obligatory to promote endurance performance during competition lasting several hours [6].

In Chapter 7, fat oxidation (including fatty-acid-derived KBs) contributed to >70% EE (> 1 g·min⁻¹) during the keto-adapted RTE trial (i.e. 70% VO_{2max}), which is similar to other studies examining substrate oxidation during exercise at 70-80% VO_{2max} [14,35]. However, even in trained endurance athletes ingesting a mixed diet (49 % EI from CHO), fat oxidation rates can exceed 1 g·min⁻¹ when approaching completion of a 60 min distance TT preceded by 2 h at 60 % VO_{2max}, despite ingesting 90 g CHO·h⁻¹ [389]; this highlights that factors other than diet are influential modulators of substrate oxidation. Furthermore, the contribution of KBs to EE during exercise is uncertain; but they can be considered an intermediate metabolite of fat oxidation during keto-adaptation due to hepatic ketogenesis. As blood D-βHB concentration steadily rose throughout the RTE in Chapter 7 (i.e. >1.5 mmol·L⁻¹), the rate of ketogenesis likely exceeded ketolysis; similarly, blood D-βHB concentrations have tended to exceed ~1.5 mmol·L⁻¹ when exercising ≥2 h without CHO ingestion [14,35,48]. As such, the suggested ergogenic benefits of elevated fat and KB oxidation do not appear to outweigh recommended CHO fuelling strategies.

9.2.3 Is there an optimal blood ketone body concentration for endurance performance?

Optimal implies an ergogenic effect either via the regulatory or contributory role of KBs to substrate provision for energy production; however, there is currently no clear benefit of nutritional ketosis for endurance performance following either ketone supplementation or keto-adaptation (Appendices A and B, pp. 210 and 213, respectively). Published studies also contain small sample sizes (~8-12) and lack rigor to identify positive and negative responders due to the underlying noise of measurement error [390]. Blood KB concentrations also reflect the rates of ketogenesis (or KB appearance following KS ingestion) and KB uptake by peripheral tissues, thus making interpretation difficult. Considering blood KB concentrations during keto-adaptation are metabolically regulated depending on energy demand and CHO-availability [10], it may not be possible to identify an optimal range for endurance performance. In contrast, ketone supplementation can acutely manipulate blood KB concentration, which has prompted suggestion of an optimal blood D-βHB range of 1-3 mmol·L⁻¹ [11]; however, 1 mmol·L⁻¹ appears too low (Appendix B, p. 213). Therefore, for KSs, further research exploring specific dosing strategies should consider the following: 1) blood KB concentration measurement

technique; 2) blood D- β HB/L- β HB/AcAc concentration and ratio; 3) substrate availability and metabolism (e.g. fasted vs. fed or acute CHO fuelling strategies); 4) previous exposure to hyperketonaemia; 5) athlete training status; 6) physiological demands of competition; and 7) tolerability.

9.2.4 Nutritional ketosis and immune function

Dietary macronutrient manipulation and nutritional ketosis may exert unique effects on immune function and illness risk at rest and in response to exercise (as previously discussed in Chapter 3, section 3.8). Within Chapters 4, 6 and 8, immune function was examined using *in vitro* stimulated whole blood cultures to measure T-cell related IFN- γ , IL-4 and IL-10 gene expression within isolated PBMCs; these cytokine are primarily produced by type-1, type-2 and regulatory T-cells, respectively [20,212]. This subclinical immune marker was chosen due to its ability to characterise the initiation of an inflammatory systemic immune response to a pathogenic challenge. Moreover, models for *in vitro* antigen-stimulated whole blood cytokine production have been associated with increased illness and infection susceptibility in athletes [16,17]. However, the exploration of immune function at the gene level provides valuable mechanistic insight into the accumulative factors altering immune competency [337]. Additionally, in Chapter 8, SIgA was measured as a marker of mucosal immunity because it is the principle antibody within the mucosal fluids. Reductions in SIgA concentration [365,366] and secretion rate [17,361] have been inversely associated with increased illness risk in athletes during prospective cohort studies.

An initial objective of this thesis was to determine the effect of hyperketonaemia on T-cell related IFN- γ , IL-4 and IL-10 gene expression within stimulated PBMCs at rest. The most efficacious method to elevate blood KB concentration is via ingestion of ketone or ketogenic supplements [13]. In Chapter 4, BD was ingested in doses of 0.5 + 0.0, 0.7 + 0.0 and 0.35 + 0.35 g·kg⁻¹ (separated by 1.5 h), which resulted in blood D- β HB concentrations up to ~1 mmol·L⁻¹, with no significant differences between trials. There were no changes to serum glucose concentration, which contrasts previous studies acutely elevating blood KB concentration [13,72]; this could have been due to: 1) low blood D- β HB concentrations; 2) the interference of L- β HB on the suppressive action of D- β HB on CHO metabolism [92]; or 3) the interference of BD metabolism on β HB. Similarly,

there was no effect on T-cell related IFN- γ , IL-4 or IL-10 gene expression for the 3.5 h proceeding the initial BD bolus. As such, it was assumed that any effect of acute hyperketonaemia on metabolism and immune function during Chapters 6 and 8 was due to the additional influence of exercise and dietary interventions.

9.2.4.1 Exercise, nutritional ketosis and the pro-inflammatory T-cell cytokine gene expression to an immune challenge

Interferon- γ is a pro-inflammatory type-1 T-cell cytokine important for modulating the immune response against intracellular pathogens (e.g. viruses) [20]. This is pertinent to the athletic population as the majority of infectious-related illnesses are of viral origin [189,192]. In Chapter 6 and 8, T-cell related IFN- γ gene expression within stimulated PBMCs temporarily increased or was unaffected following exercise. This contrasts earlier studies demonstrating the number and capacity of mitogen-stimulated T-cells to produce IFN- γ declines immediately following exercise and during the initial hours of recovery, which may persist for up to 24 h [24,25,225]. However, gene expression may not mirror patterns of cytokine protein production, as it was previously demonstrated that single-antigen-stimulated (influenza or tetanus toxoid) T-cell IFN- γ gene expression remained unchanged from pre- to post-exercise [32]. The findings in Chapters 6 and 8 occurred despite increased serum cortisol concentrations, which is typically referred to as immunosuppressive and anti-inflammatory (see Chapter 3, section 3.7.1), may not suppress immune function within *in vitro* [341] and *in vivo* models [197,342]. Therefore, exercise may mobilise T-cells with a higher capacity to express IFN- γ . However, it is possible that shifts in blood substrate and stress hormones may disturb the translation of genes to proteins, which was not examined in the present thesis.

In Chapter 6 and 8, BD ingestion and adaptation to the KD transiently amplified T-cell related IFN- γ gene expression within stimulated PBMCs immediately after exercise. By 1 h of recovery, IFN- γ gene expression had normalised to pre-exercise levels; albeit they were marginally elevated in Chapter 6 compared to placebo. These findings were unexpected as the BD and KD trials resulted in different metabolic milieus, yet produced similar effects on stimulated T-cell related IFN- γ gene expression. BD ingestion reduced serum glucose concentrations following the TT ($p = 0.60$; $d = -0.48 \pm 0.46$); however, this was insufficient to influence serum cortisol concentration. Whereas, adaptation to the

KD increased serum cortisol concentration at exhaustion and 1 h into recovery, but did not alter serum glucose concentrations. The latter was probably due to lower muscle glycogen content [391] and increased eccentric loading (i.e. running vs. cycling) [392], which increased circulating IL-6, a primary driver of cortisol production [315]. These findings suggest hyperketonaemia may favour a type-1 T-cell cytokine response to an immune challenge and further highlight the unclear relationship between cortisol, immunosuppression and inflammation following exercise.

To explain the effects of hyperketonaemia on T-cell related IFN- γ gene expression, it was speculated in Chapters 6 and 8 that differences in substrate availability within the stimulated culture could alter the T-cell response. By using stimulated whole blood cultures, circulating substrate is captured within an *in vitro* medium that can exert influence over the period of stimulation. Therefore, as serum glucose concentrations did not differ and do not necessarily influence stimulated T-cell IFN- γ expression at physiological concentrations [32], increased blood KB concentrations may have provided additional substrate for oxidation by lymphocytes [51-53], which favoured IFN- γ gene expression. However, as T-cell related IFN- γ gene expression within stimulated PBMCs was markedly elevated immediately after exercise and not 1 h into recovery, T-cell subset trafficking patterns may have also had an effect. Therefore, hyperketonaemia may only exert a pro-inflammatory effect on mobilised T-cells prior to their extravasation from the blood to peripheral tissues.

9.2.4.2 Exercise, nutritional ketosis and the anti-inflammatory T-cell cytokine gene expression to an immune challenge

Interleukin-4 is an anti-inflammatory, type-2 T-cell cytokine, which cross-regulates IFN- γ and is important for orchestrating the immune response against extracellular pathogens (e.g. bacteria and fungi) [20]. In Chapters 6 and 8, T-cell related IL-4 gene expression within stimulated PBMCs declined following exercise and remained below pre-exercise levels 1 h into recovery. This contrasts with previous studies demonstrating no effect of exercise on the number and capacity of mitogen-stimulated T-cells to produce IL-4 [24,25,225] and single-antigen-stimulated T-cell IL-4 gene expression [32]. BD ingestion and adaptation to a KD also appeared to have no effect on T-cell related IL-4 gene expression, indicating that the mechanisms underpinning hyperketonaemia favouring IFN- γ do not necessarily oppose IL-4 following stimulation.

To the author's knowledge, no studies had investigated the effect of exercise on stimulated T-cell IL-10 gene expression prior to the present thesis. IL-10 is a potent anti-inflammatory cytokine primarily produced by TReg cells, which suppresses the action of both IL-4 and IFN- γ [212]. Interestingly, T-cell related IL-10 gene expression within stimulated PBMCs differed between Chapters 6 and 8. In Chapter 6, there was a decline 1 h into recovery; whereas in Chapter 8, an increase at exhaustion and 1 h into recovery was observed. It is possible divergent effects on stimulated T-cell related IL-10 gene expression may be due to a combination of differences in cell trafficking patterns, cortisol concentration and substrate availability. It has previously been suggested that TReg cells decline in the hours following strenuous exercise [31,234], which aligns with the findings in Chapter 6, but not Chapter 8. However, as described in Chapter 3, section 3.4.3, circulatory TReg cell concentrations do not appear to elicit classical exercise-induced mobilisation patterns, like type-1 T-cells; therefore, differences in IL-10 gene expression patterns between studies were not surprising.

Compared to Chapter 6, Chapter 8's submaximal exercise to exhaustion protocol led to higher serum cortisol concentrations (~ 641 vs. ~ 789 nmol·L⁻¹) and, theoretically, higher FFA concentration; albeit the latter was not measured. TReg cells preferentially use FFA compared to glucose for fuel [277] and cortisol has known anti-inflammatory effects, which has been shown to preferentially increase CD4⁺ T-cell IL-10 gene expression [263]. Nevertheless, if higher FFA and cortisol concentrations favoured T-cell IL-10 gene expression within stimulated PBMCs, adaptation to a KD would have been expected to amplify this response; however, it did not. As TReg cells appear to have a significant role in exercise-induced immunodepression, further research is required to confirm their exercise-induced trafficking patterns and capacity to produce IL-10 to an immune challenge following exercise under a variety of dietary conditions.

9.3 Limitations

9.3.1 Indirect measurement of substrate oxidation

The contribution of KBs to EE during exercise was unable to be measured in the present thesis. As previously discussed in Chapter 2, section 2.4, there are several confounding factors when estimating KB oxidation using indirect methods currently proposed [73,105] and, whilst it could have been possible to replicate these in Chapter 5, it was not

performed in order to avoid creating a precedence for future research. Furthermore, skeletal muscle and hepatic glycogen, IMTG, adipose triglyceride, and blood glucose and lactate could not be measured. Indeed, ketone supplementation can modulate CHO and fat metabolism during exercise [73]; however, it could not be confirmed that BD ingestion exerted similar effects in Chapter 6. Keto-adaptation also reduces skeletal muscle glycogen content and utilisation during exercise [15,35], hepatic glycogen utilisation [35] and blood glucose oxidation [15]. Nonetheless, these effects were also unable to be confirmed in Chapter 8.

9.3.2 Staphylococcal enterotoxin B vs. multi-antigen stimulation

Two different immunogenic agents were used for the stimulation of whole blood cultures in the present thesis. These were SEB from *Staphylococcus aureus* (Chapter 6) and a multi-antigen vaccine (Chapter 8). As such, differences in immunogenicity (explained in Chapter 3, section 3.5) may preferentially increase the expression of some cytokines over others; meaning findings between Chapters 6 and 8 for T-cell cytokine gene expression within stimulated PBMCs may not be directly comparable. However, as gene expression was relative to a calibrator gene within the same individual for each trial, this may have attenuated potential differences in cytokine gene expression between studies.

9.3.3 Measurement of exercise-induced stress

Exercise can regulate immune function, particularly T-cells and SIgA, via cortisol and increased in SNS innervation [268,357]; in addition to numerous other factors. However, only cortisol was measured in the present thesis, limiting the understanding of the stress response to exercise and nutritional ketosis. It is possible that during periods of nutritional ketosis, elevated blood β HB concentration may increase SNS activity by acting as an agonist for the G-protein-coupled receptor [393]; although, not all studies are in agreement [394]. Furthermore, LCHF diets have been shown to increase SNS innervation during exercise, irrespective of CHO restoration strategies, as evidenced by increased blood noradrenaline concentration [381] and heart rate variability [165]. Importantly, catecholamines and glucocorticoids may elicit a synergistic effect on the suppression of T-cell IFN- γ production [266]; whereas, a heightened SNS activity can preferentially mobilise cytotoxic (IFN- γ^+) T-cells into circulation [219,343]. It is, therefore, probable

that SNS innervation was a key modulator of circulatory T-cell cytokine gene expression within the present thesis.

9.3.4 Measurement of T-cell phenotype

T-cells elicit distinct mobilisation and extravasation patterns depending on their differentiation and capacity to respond to immune challenges. As we only measured T-cell cytokine gene expression, it is possible that differences in the T-cell subset contribution to the PBMC population altered cytokine gene expression (e.g. increased IFN- γ phenotype), in addition to changes in the capacity of individual T-cells to respond to an immune challenge. For example, previous studies have characterised type-1 T-cells as IFN- γ^+ /IL-4 $^-$ and vice versa for type-2 T-cells, whilst measuring their intracellular cytokine protein production [24,25], thus isolating their findings to specific T-cell subsets.

9.3.5 Sampling time points during exercise

T-cells exhibit unique mobilisation and extravasation patterns in response to exercise, as discussed in Chapter 3. As such, it is possible the sample time points for blood collection in the present thesis were suboptimal to assess immune competency and illness risk during recovery. For example, the 1 h post exercise time point was chosen based on previous studies demonstrating a reduction in T-cell type-1 cytokine production and to coincide with the exercise-induced lymphopenia [18]. However, this does not necessarily suggest immune cell function is impaired at this point; rather, there is a relocation of immune cells to peripheral tissues where contact with a pathogen is more likely to occur [217]. Ideally, blood samples would be collected at multiple time points during the hypothetical *window of opportunistic infection*, which could provide greater insight into the alterations of T-cell cytokine responses to an immune challenge [29]. Furthermore, as exercise was performed across several hours in Chapter 8, additional blood sampling points during exercise could have provided greater insight into alterations of immune cell function prior to their relocation to peripheral tissues.

9.3.6 Immune cell concentration adjustment

Immune cell counts in Chapters 6 and 8 were adjusted based on changes in plasma volume, which were estimated from differences in haematocrit (Hct) and haemoglobin from pre-exercise using equations from Dill and Costill (1974) [339]. However, automated haematology analysers have been suggested to corrupt true Hct estimates, compared to manually spun Hct, by using hyperosmotic reagents; thus reducing red blood cell volume and erroneously underestimating changes in Hct [395]. Nonetheless, these suggestions by Watson and Maughan (2014) have been criticised [396] as they demonstrated an increase in Hct following the onset of exercise, which should, theoretically, have been a reduction. Therefore, more research is required to identify the effect of automated haematology analysers on estimating changes in plasma volume.

9.3.7 Clinical relevance of immune markers

In vivo immune markers are arguably more clinically relevant compared to the immune markers in the present thesis [240]. For example, cutaneous measures of *in vivo* immunity, such as delayed-type hypersensitivity responses to intradermal injection of antigens or contact hypersensitivity responses to epicutaneous application of antigens, have been used within recent exercise and nutrition investigations [197,342,397]. Whilst it may not be an exact replicate of an infection, *in vivo* immune models do, in part, compensate for the cooperation, complementation and compensation within the immune system [22]. However, T-cell cytokine gene expression and SIgA provide valuable mechanistic insight into the accumulative effects to immunodepression that can increase illness and infection susceptibility. Further, T-cell cytokine production and SIgA are considered to have medium- and high-suitability within immunonutrition investigations, respectively [240]. Albeit changes in cytokine gene expression do not necessarily predict changes in protein production, it does characterise the initiation of cytokine production during immune activation. Therefore, when multiple immune markers are measured simultaneously, such as in Chapter 8, effects on immune competency can be determined with more confidence.

9.4 Directions for future research

9.4.1 Direct calorimetry

The importance of KBs to EE during exercise following ketone supplementation and keto-adaptation is unknown. Therefore, future studies should consider direct calorimetry, such as stable isotope tracer methodologies, to evaluate KB oxidation rates during exercise of different intensities and durations and following a variety of nutritional interventions. The currently proposed indirect methods to measure KB oxidation also warrants validation [105]. Identifying a maximal KB oxidation rate in a variety of contexts will help gauge whether the suppression of CHO metabolism (via hyperketonaemia and/or low-CHO diets) can be compensated for, particularly during high-intensity exercise, to meet the energy demands of exercise. For ketone supplementation, this may help to establish optimal blood D- β HB concentration ranges and dosing strategies. Furthermore, confirmation of alterations to substrate (e.g. skeletal muscle glycogen, IMTG, adipose tissue triglyceride, blood glucose and lactate) metabolism following the ingestion of the R-BD D- β HB monoester [73] needs to be confirmed and if this translates across different athletic populations.

9.4.2 Monitoring keto-adaptation

As previously discussed in Chapter 9, section 9.2.1, *keto-adaptation* is difficult to define due to a scarcity of research. Currently, a major challenge is elucidating what constitutes *optimal* keto-adaptation; therefore, evaluating the metabolic characteristics within and between individuals relative to performance outcomes should be explored. For example, in Chapter 7, RER at $\text{VO}_{2\text{max}}$ was identified as a potential performance surrogate, as a reduction in RER appeared to negate submaximal endurance capacity and increase lactate accumulation and, therefore, should be included in future studies to assess its utility.

9.4.3 Keto-adaptation and intermittent carbohydrate ingestion

An area of interest is the effect of intermittent CHO ingestion on performance within keto-adapted athletes. Anecdotally, keto-adapted athletes can ingest >50 g CHO-day⁻¹ during intensified training, without dramatically compromising blood KB concentrations. This may be due to a low insulin response to CHO ingestion during exercise [120] and

the rapid oxidation of ingested CHO to support EE [398]. Furthermore, as keto-adaptation tends to impair high-intensity endurance performance, it is uncertain whether intermittent CHO restoration periods prior to high-intensity training can, in part, counteract suppressive effects of nutritional ketosis on CHO metabolism.

9.4.4 Gastrointestinal tolerance and trainability

Ketone supplementation often causes gastrointestinal distress. Currently, it is unknown how different forms of ketone supplements, dosing strategies, and coingestion of CHO (and other nutrients) influence gastrointestinal symptoms. It is possible that repeated exposure to ketone supplements may result in a trainability effect within the gut, thus improving tolerance over time, similar to CHO [399]. This may be important when aiming to maximise blood KB concentrations, potentially during high-intensity endurance exercise, or when gradually ingesting ketone supplements over several hours, such as during ultra-endurance competition.

9.4.5 T-cell trafficking patterns

T-cell trafficking patterns influence overall immune competency. Blood contains only ~2% of immune cells and transports T-cells and other immune cells between lymphoid and nonlymphoid tissue. In mice, T-cells are predominantly released from the spleen and accumulate in the lungs and Peyer's patches in response to exercise [271]; however, additional homing sites may also exist for humans. As such, the type-1/type-2/regulatory T-cell balance within peripheral tissues can shift, which may influence immune surveillance and cytotoxicity [217]. Future studies should, therefore, aim to examine exercise- and dietary-induced changes in T-cell function within peripheral tissues in humans.

9.4.6 *In vitro* analysis of hyperketonaemia on stimulated T-cell cytokine production

The transient increase in T-cell IFN- γ gene expression within stimulated PBMCs following exercise in the present thesis was difficult to explain. A possible mechanism was KB availability within the *in vitro* culture; however, this was highly speculative. As such, future studies should examine the effect of β HB and AcAc at physiological

concentrations on T-cell function to an immune challenge *in vitro*, whilst excluding for potential confounders (e.g. glucose availability and cortisol).

9.5 Conclusions and practical recommendations

The findings from this thesis further current understanding of the impact of ketone supplementation and keto-adaptation on endurance performance and immune function. It is likely that racemic ketone supplements provide no performance benefit to endurance athletes and fail to increase blood D- β HB to concentrations that have a meaningful effect on substrate metabolism and exercise efficiency. Similarly, keto-adaptation does not delay fatigue at submaximal exercise intensities compared to recommended CHO intakes, but may offer a metabolic advantage during events that are CHO limiting. Nutritional ketosis also appears to act as an immunological adjuvant for T-cells by promoting the initiation (i.e. gene expression) of a IFN- γ response to an immune challenge *in vitro* following strenuous exercise, which could promote protection against viral pathogens. However, this may not be associated with T-cell IFN- γ protein production or *in vivo* immune competency.

Therefore, based on the findings of the present thesis, the following recommendations are provided to endurance athletes, dietitians, nutritionists, sport scientists and coaches.

- Avoid racemic ketone supplements to induce nutritional ketosis, as these typically fail to increase blood D- β HB concentration to $>1 \text{ mmol}\cdot\text{L}^{-1}$ and exert little to no effect on substrate metabolism and risk a performance detriment.
- Trial ketone supplementation in conjunction with additional fuelling strategies during training to evaluate tolerance and effects on performance specific to the physiological demands of competition.
- If employing keto-adaptation, performance effects may be experienced within 3-4 weeks of adaptation to a KD. If performance is not normalised or training load cannot be maintained, consider re-introducing dietary CHO to support fuelling requirements.
- Strategies to induce nutritional ketosis should not be utilised to augment immune function based on the present findings. Similarly, the avoidance of ketone supplementation and keto-adaptation due to the potential of increased illness and infection risk is also unsubstantiated.

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Appendices

Appendix A: The effects of ketone and ketogenic supplementation on endurance performance and capacity

Reference	Study design and participant characteristics	Ketone supplement protocol	Dietary protocol	Performance protocol	Blood KB concentration range (pre- to post-exercise)	Performance outcome
Cox et al. (2016) [73]	Crossover design; 8 (6 M, 2 F) highly-trained cyclists VO _{2max} 5.37 ± 0.3 (M) and 3.30 ± 0.01 (F) L·min ⁻¹ (sex-specific relative VO _{2max} and body mass not reported)	Two doses of 0.287 g·kg ⁻¹ R-BD D-βHB monoester (40 % E) and CHO (60 % E) ingested at 30 min pre-exercise and pre-TT (vs. energy-matched CHO only placebo)	Overnight fast; replication of preceding night's meal; caffeine avoidance 24 h pre-exercise	Cycling 30 min TT preceded by 60 min at 75 % W _{max}	Plasma D-βHB 1.7-2.5 mmol·L ⁻¹ (laboratory analysis)	R-BD D-βHB monoester significantly improved TT performance compared placebo (~411 m increase distance cycled)
Rodger et al. (2017) [63]	Crossover design; 12 highly-trained male cyclists; VO _{2peak} 68 ± 6.7 ml·kg ⁻¹ ·min ⁻¹	Two doses of 11.7 g D,L-βHB salt ingested at 20 min pre-exercise and 45 min during ex (vs. placebo)	Replication of 48 h (unspecified) dietary intake pre-exercise; caffeine avoidance 24 h pre-exercise; fast 2.5 h pre-exercise	Cycling 4 min TT preceded by 90 min at 80% VT ₂	Capillary blood D-βHB 0.3-0.6 mmol·L ⁻¹ (point-of-care device)	No significant effect of racemic ketone salt on TT performance compared to placebo (average power output ~364 vs. ~355 W)
O'Malley et al. (2017) [62]	Crossover design; 10 recreationally active males; VO _{2peak} 45 ± 10 ml·kg ⁻¹ ·min ⁻¹	Single dose of 0.3 g D,L-βHB·kg ⁻¹ salt (~24.9 g) ingested at 30 min pre-exercise (vs. placebo)	Overnight fast; replication of 24 h dietary intake; nutrient composition not specified	Cycling 150 kJ TT preceded by 5 min at 30, 60 and 90% VT ₂	Capillary blood D-βHB 0.7–0.9 mmol·L ⁻¹ (point-of-care device)	Racemic ketone salt significantly impaired TT performance compared to placebo (~711 vs. ~665 s)

Leckey et al. (2017)[67]	Crossover design; 11 internationally competitive male cyclists; VO_{2peak} 71.4 ± 5.6 ml·kg ⁻¹ ·min ⁻¹	Two doses of 0.25 g·kg ⁻¹ R,S-BD AcAc diester ingested at 50 and 30 min pre-TT (vs. placebo)	High-CHO meal and snack (total of 3 g CHO·kg ⁻¹) the previous night; breakfast 2 h pre-TT (2 g CHO·kg ⁻¹); 200 mg caffeine 2 h pre-TT; 27 g CHO and 50 mg caffeine 5 min pre-TT	Cycling 31.17 km simulated World Championship TT course with 250 ml 6% CHO sports drink mid-TT	Serum D-βHB ~0.4 mmol·L ⁻¹ (laboratory analysis); serum AcAc ~0.5 mmol·L ⁻¹ (laboratory analysis); capillary blood D-βHB 0.8-1.3 mmol·L ⁻¹ (point-of-care device)	Racemic R,S-BD AcAc diester significantly impaired TT performance compared to placebo (average power output ~339 vs ~352 W)
Evans et al. (2018) [61]	Crossover design; 11 trained male team sport athletes; estimated VO_{2max} 53.9 ± 2.2 ml·kg ⁻¹ ·min ⁻¹	Three doses of R-BD D-βHB monoester (0.375, 188 and 188 g·kg ⁻¹) ingested at 20 min pre-exercise, 30 and 60 min during ex, respectively	~6 g CHO·kg ⁻¹ previous day; two high-CHO meals (total of 6 g CHO·kg ⁻¹) ingested at least 3 h pre-exercise; caffeine avoidance 24 h pre-exercise	Running to exhaustion alternating between 20 m at 55 and 95 % VO_{2max} preceded by 5 x 15 min intermittent high-intensity exercise with 1.2 g·min ⁻¹ CHO during exercise	Plasma D-βHB 1.0-2.6 mmol·L ⁻¹ (laboratory analysis)	No significant effect of R-BD D-βHB monoester on exercise capacity compared to placebo (~229 vs. 268 s)
Scott et al. (2018) [90]	Crossover design; 11 trained male runners; VO_{2peak} 64.2 ± 5.0 ml·kg ⁻¹ ·min ⁻¹	Three doses of R,S-BD (0.25, 0.125 and 0.125 g·kg ⁻¹) and 60 g CHO ingested at 30 min and immediately pre-exercise and pre-TT, respectively (vs. energy matched placebo of ~110 g CHO)	Overnight fast; replication of 48 h unspecified dietary intake pre-exercise	Running 5 km TT preceded by 60 min at 75% VO_{2peak}	Plasma D-βHB 0.8–1.0 mmol·L ⁻¹ (laboratory analysis)	No significant effect of racemic R,S-BD on TT performance compared to placebo (~21.0 vs. 21.1 min)

Faull et al. and Dearlove et al. (2019) [78,79]	Crossover design; 12 (9 male, 3 female) trained endurance athletes (rowing, cycling, running or swimming); $\text{VO}_{2\text{max}}$ $56.6 \pm 2.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Single dose of $0.33 \text{ g}\cdot\text{kg}^{-1}$ R-BD D- β HB monoester ingested at 30 min pre-exercise (vs. placebo)	Overnight fast; additional dietary requirements not specified	Cycling incremental exercise test to exhaustion commencing at 100 W and increasing by 25 W every 3 min until exhaustion	Capillary blood D- β HB $2.0\text{-}3.8 \text{ mmol}\cdot\text{L}^{-1}$ (point-of-care device)	No significant effect of R-BD D- β HB monoester on W_{max} compared to placebo (~ 393 vs. ~ 389 W)
Chapter 5	Crossover design; 9 trained male cyclists; $63.9 \pm 2.5 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	Two doses of $0.25 \text{ g}\cdot\text{kg}^{-1}$ R,S-BD ingested at 30 min pre-exercise and 30 min pre-TT	Overnight fast; $6 \text{ g CHO}\cdot\text{kg}^{-1}$ day prior to trial; caffeine avoidance 24 h pre-exercise	Cycling $7 \text{ kJ}\cdot\text{kg}^{-1}$ TT preceded by 85 min at 85% VT_2	Capillary blood D- β HB $0.4\text{-}0.8 \text{ mmol}\cdot\text{L}^{-1}$ (point-of-care device)	No significant effect of racemic BD on TT performance compared to placebo (~ 28.7 vs. ~ 28.5 min)

M male, F female, $\text{VO}_{2\text{max}}$ maximal oxygen uptake, BD R,S-1,3-butanediol, β HB β -hydroxybutyrate, E energy, TT time-trial, CHO carbohydrate, W_{max} maximal power output, kJ kilojoules, VT_2 second ventilatory threshold, $\text{VO}_{2\text{peak}}$ peak oxygen uptake, AcAc acetoacetate.

Appendix B: The effects of chronic (3 week minimum) keto-adaptation on endurance performance and capacity

Reference	Study design and participant characteristics	Keto-adaptation protocol	Monitoring of dietary compliance	Performance protocol and acute fuelling requirements	Blood KB range concentration (pre- to post-exercise)	Performance outcome
Phinney et al. (1983) [15]	Crossover design, order effect (CHO-trial first); 5 trained male cyclists; $\text{VO}_{2\text{max}} \sim 69.0 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	28 days; $\sim 2\%$ EI CHO and $\sim 85\%$ EI fat; normal training	Clinical ward study with all meals provided, with participants allowed 1 meal away from the clinic per day; twice daily measurement of urinary acetone; twice weekly measurement of plasma D- β HB; daily weight	Cycling to exhaustion at $62\text{--}64\% \text{VO}_{2\text{max}}$; overnight fast	Plasma D- β HB $1.28\text{--}1.45 \text{ mmol}\cdot\text{L}^{-1}$ (laboratory analysis)	No significant effect of keto-adaptation on exercise capacity compared to CHO-trial (~ 151 vs. ~ 147 min)
Burke et al. (2016) [14]	Unbalanced design (crossover of some participants); 21 elite male race walkers ($n = 9$ high-CHO, $n = 8$ periodised-CHO, $n = 9$ KD); average pre-diet $\text{VO}_{2\text{peak}}$ for all groups $\sim 64.2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	21 days; $\sim 4\%$ EI CHO and $\sim 78\%$ EI fat; intensified training	Training camp study with all meals provided; dietary intake recorded by trained researchers, however, designated recording frequency not reported; no reported daily monitoring of urinary or blood KB concentration	Racewalking 10 km TT with individualised (unspecified) pre-competition fuelling strategies according to dietary allocation (i.e. fat fed in keto-adapted trial)	Capillary blood D- β HB $0.3\text{--}0.7 \text{ mmol}\cdot\text{L}^{-1}$ (point-of-care device)	Keto-adaptation significantly impaired TT performance compared to the high-CHO and periodised-CHO diets (~ 23 sec slower vs. ~ 190 and ~ 124 sec faster, respectively)
Zinn et al. (2017) [166]	Crossover design, order effect (CHO-trial first); 5 recreationally trained athletes (1 M runner, 1 F runner, 3 F cyclists); $\text{VO}_{2\text{peak}} 44\text{--}54 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (visually extrapolated from results)	10 weeks; $\sim 5\%$ EI CHO and 66% EI fat; normal training	Free-living athletes; weekly dietary reporting; dietary counselling by registered dietitian only following participant-reported diet nonconformity; capillary blood D- β HB concentration, albeit designated testing frequency not specified;	Cycling incremental exercise test to exhaustion commencing at 30 W and increasing by 30 W every 3 min until exhaustion	Not reported	Keto-adaptation significantly impaired time-to-exhaustion (~ 2 min less) and nonsignificantly reduced W_{max} (~ 18 W less) compared to CHO-trial

			single written 1-day diet record in week 5			
McSwiney et al. (2018) [174]	Parallel design; 20 trained male athletes (multiple endurance sports); average pre-diet $\text{VO}_{2\text{max}}$ for both groups $\sim 53.1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	12 weeks; $\sim 5\%$ EI CHO and $\sim 77\%$ EI fat in conjunction with normal training	Free-living athletes; weekly dietary counselling by researcher (qualification not specified); single written 3-day diet record in final week; no daily monitoring of urinary or blood KB concentration	Cycling 100 km TT with individualised (unspecified) pre-competition fuelling strategies according to dietary allocation; during exercise CHO $0 \text{ g}\cdot\text{h}^{-1}$ in keto-adapted trial and $30\text{-}60 \text{ g}\cdot\text{h}^{-1}$ in CHO trials	Fasting plasma D- β HB $0.5 \pm 0.4 \text{ mmol}\cdot\text{L}^{-1}$ (laboratory analysis); pre- and post-TT circulating KB concentrations not measured	No significant effect of keto-adaptation on TT performance compared to CHO-diet ($\sim 4.07 \text{ min}$ faster vs. $\sim 1.13 \text{ min}$ faster)
Chapter 7	Crossover design; 8 trained male runners; $\text{VO}_{2\text{max}}$ $59.4 \pm 5.2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$	31 days; $\sim 4\%$ EI CHO and $\sim 78\%$ EI fat in conjunction with normal training	Free-living athletes; compulsory daily contact and counselling with a registered dietitian; image-assisted, written diet record for 2 days in weeks 1-3 and final 5 days; daily urinary AcAc; weekly capillary blood D- β HB	Running to exhaustion at $70\% \text{ VO}_{2\text{max}}$ (3-strike method) with pre-competition fuelling strategies in preceding days according to dietary allocation; high-CHO (2 g CHO or high-fat ($<10 \text{ g CHO}$) $\sim 2 \text{ h}$ pre-trial; during exercise CHO $0 \text{ g}\cdot\text{h}^{-1}$ in keto-adapted trial and $\sim 55 \text{ g}\cdot\text{h}^{-1}$ in CHO trials	Capillary blood D- β HB $0.6\text{-}1.7 \text{ mmol}\cdot\text{L}^{-1}$ (point-of-care device)	No significant effect of keto-adaptation on exercise capacity compared to CHO-diet ($\sim 19.3 \text{ min}$ less vs. $\sim 6.3 \text{ min}$ less)

CHO *carbohydrate*, $\text{VO}_{2\text{max}}$ *maximal oxygen uptake*, EI *energy intake*, β HB *β -hydroxybutyrate*, KD *ketogenic diet*, $\text{VO}_{2\text{peak}}$ *peak oxygen uptake*, KB *ketone body*, TT *time-trial*, W_{max} *maximum power output*.

Participant Information Sheet

Study title: **The effect of dietary ketones on immunity and tolerability in healthy, male recreational athletes at rest**

Locality: **Auckland University of Technology**

Lead investigator: **David Shaw**

Project supervisor: **Deborah Dulson**

Ethics committee ref: **17/NTB/39**

ANZCTR ref: **ACTRN12617000731392**

Phone number: **021 0827 6137**

Email: **nutrition@daveshaw.co.nz**

My name is David Shaw, a PhD student at Auckland University of Technology (AUT). Along with Senior Lecturer, Dr Deborah Dulson, we invite you to take part in a study investigating the effects of dietary ketogenic supplement, 1,3-butanediol (BD), on immunity and tolerability whilst at rest. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is eight pages long, including the Consent Form. Please make sure you have read and understood all the pages. If you have any queries, please contact David Shaw (lead investigator).

What is the purpose of this study?

Athletes are constantly searching for novel dietary strategies to enhance performance and health. The use of BD, a dietary ketogenic supplement, has been suggested to influence endurance performance, with both positive and negative effects. The consumption of BD can increase blood ketone concentration to levels similar of a low carbohydrate, ketogenic diet, without the burden of drastic dietary change. The presence of ketones in the blood provides an additional energy source to muscle and the brain, and influences how the body uses its carbohydrate and fat stores. In some athlete circles, BD is frequently being used, however, its effect on immunity and tolerability is not known. Considering athletes can be at a higher risk of illness, this study is important to provide insight into the effect of different doses of BD on immune markers at rest.

This study will be undertaken by the lead investigator, David Shaw, at Auckland University of Technology and will contribute to the fulfilment of a PhD thesis. The results will help to inform a subsequent exercise trial, which altogether, will provide a comprehensive evaluation of the safety and performance effects of BD in endurance athletes. Additionally, the results will aim to be published in academic journals and presented at scientific conferences to inform the wider community. David Shaw has received no external funding for this study nor has been influenced to undertake this research by third parties. The study has been provided ethical approval by the Northern B Health and Disability Ethics Committee, New Zealand.

Thank you for considering to participant in this research study.

How was I identified and why am I being invited to participate in this research?

You were originally identified due to being male, aged between 18-35 years. There will be a total of 6 participants in this study, with the selection occurring on a first in, first served policy. Due to the complex nature of the immune system, there is several criteria that you will need to meet to be eligible. Please carefully consider your fulfilment of each (see below).

- ✓ You are male.
- ✓ You are active.
- ✓ You are between 18-35 years of age.
- ✓ You are without cardiovascular, metabolic, neurological, immunological or autoimmune disorders.
- ✓ You have normal baseline haematological (red and white blood cell) values (this will be tested for during your initial appointment).
- ✓ You have been free of illness (e.g. cough, cold, runny nose, sore throat, fever, weakness and headaches, muscle pain, repetitive sneezing, persistent muscle soreness, joint aches and pains) for more than 4 weeks.
- ✓ You are not experiencing any current gut symptoms.
- ✓ You have not consumed any medications or supplements known to effect immunity for more than 2 weeks.
- ✓ You are a nonsmoker.
- ✓ Your weight has been stable for more than 1 month.
- ✓ You have been consuming a mixed diet for more than 1 year.
- ✓ No family member has died before the age of 50 with a heart condition.
- ✓ You are comfortable with providing blood samples, and having your body composition measured.

What will my participating in this study involve?

If you would like to participate, you will be asked to attend the Sport Performance Research Institute of New Zealand (SPRINZ) at the AUT Millennium campus in Mairangi Bay for an initial meeting with David Shaw. During this time, the study will be clearly explained, your eligibility will be confirmed, the consent form will be signed, you will be asked to complete an eligibility and health screening questionnaire and a form acknowledging the unintentional doping risk associated with BD consumption. Following this, you will be required to visit the SPRINZ physiology laboratory, based at the AUT Millennium campus for 4 trials, with each separated by 3-10 days.

Your participation in each trial will consist of the following,

06:00 - Initial 30 minutes

- Arrive at the SPRINZ physiology laboratory at 06:00 h having fasted from 23:00 h the previous day and having abstained from caffeine, alcohol and strenuous exercise for the previous 24 h.
- Provide David Shaw with a diet record of everything you have consumed (food and fluid) within the previous 24 h. You will be asked to consume this during the 24 h prior to the second, third and fourth trials.
- You will then have a cannula inserted into your antecubital vein.
- After ~10 min of resting quietly, initial blood samples, blood ketone levels and tolerability scores will be collected.
- You will then be asked to void your bladder before your body mass (shorts only), height and sum of 8 skinfolds are measured.
- Precisely on 06:30 h, you will consume 200 ml of a citrus drink containing 0 (placebo) or a designated amount of BD. You will then consume another 200 ml at 08:00 h containing either 0 or a designated amount of BD. The total amount of BD will be either 0, 0.5 or 0.7 g·kg⁻¹. You will be blinded to the amount of BD in your drink.

06:30 - Next 210 minutes

- Provide blood samples 30, 120, 150 and 210 min following the initial intake of BD.
- Complete a tolerability questionnaire following collection of the final blood sample.
- Finger prick capillary blood ketone level check 15, 30, 45, 60, 90, 120, 150 and 210 min following the initial intake of BD.
- All samples will be collected in the SPRINZ laboratory. It is essential that you remain rested during this time and do not eat or drink anything, except for water which you can consume at desired.

10:00 – Next 15 to 30 minutes

- You will be provided with 400 ml of a carbohydrate drink.
- You will be able to ask any questions you were unable to ask during the experiment.
- You will be reminded about the date and requirements of your next trial.

Sample collection

- All blood samples will be collected by a trained individual.
- Blood samples will be ascertained via cannulation to the antecubital vein (arm) into two vacutainers.
- Ketone levels will be measured via a finger prick.
- All samples will de-identified and coded according to your participant number.
- Your samples will be discarded once analysed, unless it is your wish for them to be returned to you.

Why is the study designed this way?

This study is a single-blinded randomised, crossover trial. This is so you act as your own control, which significantly increases the power of the study. You are blinded to the inclusion and dose of BD to ensure this does not influence your responses during the study.

What will happen to my samples after they have been collected?

- All blood samples will be analysed in the AUT Millennium Immunology Laboratory and the AUT City Campus Roche Laboratory.
- No samples will be sent overseas.
- You have the right to have all your samples returned to you at the end of the study. If this is you wish, please inform the researchers. If not, all samples will be suitably disposed of using standardised procedures.

What are the costs of participating in this research?

There is no financial commitment required of participants, except for their transportation costs to and from the SPRINZ laboratory and for the replication of their dietary intake during the 24 h prior to each trial. Participants will be expected to commit about 18 h over the course of the study. This will be divided into the following areas:

- Initial appointment: 1 h
- Trials (1-4): 17 h (i.e. 4.25 h hours each)

If you choose not to participant in the study or decide to not continue part way through the study, you will incur no costs.

What are the possible benefits and risk of participating in this study?

- There is the possibility that you may experience some risks from participating in this study. These include;
- Slight discomfort during cannula insertion and blood collection.
- Dislike the unpleasant taste of the drink.
- Experience some mild gut symptoms, such as pain or urgency.
- Light headedness.
- If you are tested for substances banned by the World Anti-Doping Agency (WADA) and Drug Free Sport New Zealand, it is paramount that you are aware there is the possibility that the BD product may produce a positive result if tested after consumption. This is due to BD not having been tested for contamination or banned substances by a third party and, therefore, its compliance to WADA regulations cannot be assured. If you are unsure what this means, please enquire with Dave Shaw.

All symptoms, side effects and abnormal red and white blood cell values will be immediately reported to a sports doctor to ensure participant safety.

- Alternatively, you will gain the following benefits from participation in this study. These include:

- Information regarding your individual immune response to the intake of BD. These results will also be interpreted by the lead researcher and communicated in an understandable manner.
- Reports of haematological values (e.g. haemoglobin, haematocrit, white blood cells and subsets) (if any are outside of normal range, you will be notified immediately).
- Access to a NZ registered dietitian and sports nutritionist after completion of data collection. Access will be limited to a period of 3 months following completion of participation and will include 1 initial consult (1 h) with a meal plan.
- Information on BD and how it may influence your performance. However, the product itself will not be provided to you.

Who pays for this study?

All funding for this study comes from the leading investigators PhD research budget provided by AUT and his own budget. Unfortunately, due to the limited funding, it is not possible for you to be reimbursed for travel costs, food purchases, time away from work etc. It is hoped that the personalised and advanced information received from participation in this study will be sufficient compensation.

What if something goes wrong?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

What are my rights and how will my privacy be protected?

Your participation in this study is voluntary and you are free to remove yourself from the study at any stage, without any costs, repercussions or disadvantages. You have the right to access all of your personal information at any stage during the study and ask for the results to be meaningfully communicated if you are uncertain. However, you will not own any intellectual property arising from this study or analysis of your samples.

All of the data produced from your participation in the study will remain de-identified and stored on the password protected AUT network to protect your confidentiality. Nevertheless, it is possible that you will present in the laboratory alongside 1-2 other participants. If you prefer to participate in isolation, please state this to David Shaw.

What happens after the study or if I change my mind?

If you are unsure about any aspect of the study protocol, you are able to contact David Shaw or his PhD supervisors (see contact details below).

Following your participation in the study, there will be no future requirement for your participation. However, you will be provided with an opportunity to be considered in the main experimental trial, which can be further explained to you on completion.

All of the data collected during the study will only be available to the researchers and yourself. Data storage will be de-identified and kept within a password protected AUT

network to protect your confidentiality for a period of up to 10 years. If the data is published in the public domain, your name and personal information will not be revealed.

Biological specimens will be analysed and then stored in a -80°C freezer for about 1 year. If you would like to have your samples returned, please state this to David Shaw. Following analysis, you will be contacted regarding your results within a time frame of about 6 months.

Who do I contact for more information or if I have concerns?

If you have any questions, concerns or complaints about the study at any stage, you can contact:

Name: **David Shaw, PhD student and leading investigator**
Telephone number: **021 08276137**
Email: **nutrition@daveshaw.co.nz**

Name: **Dr Deborah Dulson, PhD and study supervisor**
Telephone number: **09 921 9999 ext. 7417**
Email: **deborah.dulson@aut.ac.nz**

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

For Maori health support please contact :

Name: **Dr Isaac Warbrick, PhD and co-director of Taupua Waiora Centre for Māori Health Research**
Telephone number: **09 921 9999 ext. 7591**
Email: **Isaac.warbrick@aut.ac.nz**

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS
Email: hdec@moh.govt.nz

Consent form

Study title: **The effect of dietary ketones on immunity and tolerability in healthy, male recreational athletes at rest (pilot study).**

Lead investigator: **David Shaw**

Project supervisor: **Deborah Dulson**

Please tick to indicate your consent to the following

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	<input type="checkbox"/>
---	--------------------------

I have been given sufficient time to consider whether or not to participate in this study.	<input type="checkbox"/>
--	--------------------------

I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	<input type="checkbox"/>
---	--------------------------

I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	<input type="checkbox"/>
---	--------------------------

I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	<input type="checkbox"/>
---	--------------------------

If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes	No
	<input type="checkbox"/>	<input type="checkbox"/>

I consent to the research staff collecting and processing my information, including information about my health.	<input type="checkbox"/>
--	--------------------------

I consent to my GP or current provider being informed about my participation in the study and of any significant abnormal results obtained during the study.

Please state your GP's contact details (optional).

Name:	Yes	No
	<input type="checkbox"/>	<input type="checkbox"/>

Practice:	<input type="checkbox"/>	<input type="checkbox"/>
-----------	--------------------------	--------------------------

Address:

Phone:

Email:

I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	<input type="checkbox"/>
---	--------------------------

I have answered the questions regarding my health, training, competition, weight, diet, medication and supplement use to the best of my ability. ☐

I agree to comply with all the study's requirements and know my responsibilities as a study participant. ☐

I understand the compensation provisions in case of injury during the study. ☐

I know who to contact if I have any questions about the study in general. ☐

I wish to have my blood samples returned to me in accordance with right 7 (9) of the *Code of Health and Disability Services Consumers' Rights* ☐ Yes ☐ No

Declaration by participant:

I hereby consent to take part in this study.

Participant's name: _____

Signature: _____

Date: _____

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name: _____

Signature: _____

Date: _____

Appendix D: Health and Eligibility Screening Questionnaire

Eligibility & Health Screening Questionnaire

1. Date of birth:
2. What is your ethnicity?
3. Do have a history of competitive sport? Y / N
If yes, please state

What sport do you compete in?

How long have you been competing?

How long have you been training?

On average, how many hours do you train per week?
4. Have you been consuming a mixed diet for over 1 year? Y / N
5. Do you currently smoke? Y / N
6. Have you ever smoked? Y / N
7. Are you prepared to give blood samples and have your body composition measured? Y / N
8. Do you have any medical issues, allergies or intolerances? Y / N
If yes, please describe.
9. Are you currently experiencing any symptoms of illness? Y / N
If yes, please describe.
10. When was the last time you remember experiencing symptoms of illness?
Please describe.
11. Are currently experiencing any gut symptoms? Y / N
If yes, please describe.
12. When was the last time you remember experiencing gut symptoms?
Please describe.
13. What is your weight (estimate)?
14. Has your weight been stable for at least 1 month? Y / N
15. Do you have a cardiovascular condition or a have a family member who died below the age of 50 from a heart related condition? Y / N
If yes, please describe.

16. Are you currently injured or have a condition that may affect your ability to sense pain? Y / N

17. Are you consuming any medications or supplements? Y / N
If yes, please state.

18. Are you diabetic, pre-diabetic or have metabolic syndrome? Y / N

Answer only if applicable.

19. Has your doctor ever said you have a heart condition that you should only do physical activity recommended by a doctor? Y / N

20. Do you feel in pain in your chest when you do physical activity? Y / N

21. In the past month, have you had chest pain when you were not doing physical activity? Y / N

22. Do you lose your balance because of dizziness or do you ever lose consciousness?
Y / N

23. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change to your physical activity? Y / N

24. Do you know of any reason why you should not do physical activity? Y / N

Declaration by participant:

I have answered all questions to the best of my ability.

Participant's name: _____

Signature: _____

Date: _____

Participant Information Sheet

Project title: **The effect of dietary ketones on immunity, tolerability and performance in well-trained, male cyclists.**

Locality: **Auckland University of Technology**

Lead investigator: **David Shaw**

Project supervisor: **Deborah Dulson**

Ethics committee ref: **17/NTB/39**

Phone number: **021 0827 6137**

Email: **nutrition@daveshaw.co.nz**

My name is David Shaw, a PhD student at Auckland University of Technology (AUT). Along with Senior Lecturer Dr Deborah Dulson, we invite you to take part in a study investigating the effects of consuming the dietary ketogenic supplement, 1,3-butanediol (BD), on performance, immunity and tolerability during and following a strenuous cycling bout. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is nine pages long, including the Consent Form. Please make sure you have read and understood all the pages. If you have any queries, please contact David Shaw (lead investigator).

What is the purpose of this study?

Athletes are constantly searching for novel dietary strategies to enhance performance and health. The use of BD has been suggested to influence endurance performance, with both positive and negative effects. The consumption BD can increase blood ketone concentration to levels similar of a low carbohydrate, ketogenic diet, without the burden of drastic dietary change. The presence of ketones in the blood provides an additional energy source to muscle and the brain, and influences how the body uses its carbohydrate and fat stores. However, its effects on performance, immunity and tolerability in male endurance athletes is not known. Considering endurance athletes are at a higher risk of illness, this study is important to provide an initial insight into the effect of BD on these health markers.

This study will be undertaken by the lead investigator, David Shaw, at Auckland University of Technology and will contribute to the fulfilment of a PhD thesis. The results

will be published in academic journals and presented at scientific conferences to inform the wider community. David Shaw has received no external funding for this study nor has been influenced to undertake this research by third parties. The study has been provided ethical approval by the Northern B Health and Disability Ethics Committee, New Zealand.

How was I identified and why am I being invited to participate in this research?

Thank you for considering to participant in this research study.

You were originally identified due to being a male endurance athlete, aged between 18-35 years. There will be a total of 8-12 participants in this study, with selection occurring on a first in, first served policy. Due to the complex nature of the immune system, there is several criteria that you will need to meet to be eligible. Please carefully consider your fulfilment of each (see below).

- ✓ You are male.
- ✓ You have a recent history of competitive cycling.
- ✓ You are between 18-35 years of age.
- ✓ You have been training for more than two years.
- ✓ You train more than 6 hours a week.
- ✓ You are without cardiovascular, metabolic, neurological, immunological or autoimmune disorders.
- ✓ You have normal baseline haematological (red and white blood cell) values (this will be tested for during your initial appointment).
- ✓ You have been free of illness (e.g. cough, cold, runny nose, sore throat, fever, weakness and headaches, muscle pain, repetitive sneezing, persistent muscle soreness, joint aches and pains) for more than 4 weeks.
- ✓ You are not experiencing any current gut symptoms.
- ✓ You have not consumed any medications or supplements known to effect immunity for more than 2 weeks.
- ✓ You are a nonsmoker.
- ✓ Your weight has been stable for more than one month.
- ✓ You have been consuming a mixed diet for more than one year.
- ✓ No family member has died before the age of 50 with a heart condition.
- ✓ Are comfortable with providing blood samples and having body composition measured.
- ✓ Have a peak oxygen consumption (VO_{2peak}) over $60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (this will be ascertained during the pre-experimental tests).
- ✓ Do not possess an injury that will affect cycling performance.

What will my participating in this study involve?

If you would like to participate, you will be required to sign a consent form. Following this, you will be asked to attend the Sport Performance Research Institute of New Zealand (SPRINZ) laboratory at the AUT Millennium campus in Mairangi Bay for an initial meeting with the lead investigator. During this time, the study will be clearly explained, your eligibility will be confirmed, the consent form will be signed, you will be asked to complete an eligibility and health screening form and a form acknowledging the unintentional doping risk associated with BD consumption. Following this, you will be required to visit the SPRINZ physiology laboratory, based at the AUT Millennium campus for a pre-experimental test, a familiarisation trial and two experimental trials.

Your participation in each trial will consist of the following,

Pre-experimental test and time trial familiarisation

- Arrive at the SPRINZ physiology laboratory at a time of convenience having fasted for the previous 4 h and having abstained from caffeine, alcohol and strenuous exercise for the previous 24 h.
- You will be asked to void your bladder before your body mass (underwear only), height and sum of 8 skinfolds are measured.
- You will then complete a ~30 min incremental cycling test to exhaustion to estimate a variety of metabolic parameters. You will be required to bring your cycling shoes with cleats and preferred cycling clothing.
- If your $\text{VO}_{2\text{peak}}$ is over $60\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, you will be deemed eligible for the study. If eligible, you will be provided with 15 min to rest, then complete a cycling time trial equivalent to $7\text{kJ}\cdot\text{kg}^{-1}$ (~25-30 mins).
- If your $\text{VO}_{2\text{peak}}$ is below $60\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, you will be deemed ineligible for the study and unable to continue.
- You will be wearing a ventilatory mask for the duration of the incremental test, but not the time trial.
- Following completion and if deemed eligible, you will be reminded of the requirements for the full familiarisation protocol, which includes consuming a $6\text{g}\cdot\text{kg}^{-1}$ carbohydrate diet for the 24 h prior to arriving (in addition to other requirements). Nutritional guidelines will be provided and you will be asked to record your dietary intake during this time.

Familiarisation trial

- Three to seven days after the pre-experimental test and time trial familiarisation, you will return to the SPRINZ physiology laboratory between 07:00 and 08:00 h having fasted from 23:00 h the previous day.
- You will be asked to consume 500ml of water prior to arrival.
- Upon arrival, you will be asked to provide your diet record to the lead investigator. You will then be need to replicate this diet for both experimental trials.
- You will be asked to void your bladder before your body mass (underwear only) measured.
- You will then mount the cycle ergometer and complete 85 min of cycling at a power output eliciting 85% of your secondary ventilatory threshold (determined in your pre-experimental test). You will then be provided with 5 min of rest. Immediately following this, you will complete a time trial equivalent to $7\text{kJ}\cdot\text{kg}^{-1}$ (~25-35 min) as fast as possible.
- During the initial 85 min of the cycling test, you will be able to drink water ad-libitum and have a ventilatory mask fitted every 20 min for 3 min.
- Following the completion of the time trial, you will be asked to remove wet clothing (except shorts) and towel dry yourself. You will then be weighed to estimate fluid loss.
- If you cannot complete the full protocol, you will be provided with a second attempt. If unsuccessful on both attempts, you will not be able to proceed in the study.
- Following completion, you will be reminded of the requirements of the experimental procedure, which will include replicating your high carbohydrate

diet and consuming 500 ml of water before arriving (in addition to other requirements).

Experimental procedure

- Five to ten days after the familiarisation trial, you will arrive at the laboratory between 07:00 and 08:00 h having fasted from 23:00 h the previous day and having abstained from caffeine and alcohol for the previous 24 h and strenuous exercise for the previous 48 h.
- You will then have a cannula inserted into the antecubital vein (i.e. superficial arm vein).
- After ~10 min of resting quietly, initial blood samples, blood ketone and lactate levels and tolerability scores will be collected.
- You will then be asked to void your bladder before your body mass (shorts only) measured.
- You will consume a citrus drink ($2 \text{ ml}\cdot\text{kg}^{-1}$) containing either placebo or BD.
- Blood samples, blood ketone and lactate levels will be collected 25 min after consumption of the drink.
- You will then complete an identical cycling protocol as your familiarisation trial. Blood samples and ketone and lactate levels will be collected at 30 and 60 min of cycling and during the 5 min rest period.
- You will be restricted to $2 \text{ ml}\cdot\text{kg}^{-1}$ of water every 15 min during the trial.
- Before leaving the cycle ergometer, blood samples, blood ketone and lactate levels will be collected.
- Immediately afterwards, you will be asked to remove wet clothing and towel dry yourself. You will then be weighed (shorts only) to estimate fluid loss.
- Immediately following this, tolerability scores will be collected. You will then be provided with $5 \text{ ml}\cdot\text{kg}^{-1}$ of water.
- You will be required to remain resting in the laboratory for 1 hour. You will not be able to eat or drink other fluids.
- After 1 hour, blood samples, blood ketone levels and tolerability scores will be collected.
- After each test, you will be provided with a 400-600 ml of a carbohydrate drink and will be able to ask any questions. You will also be reminded of the requirements of your next trial.

Sample collection

- All blood samples will be collected by a trained individual.
- Blood samples will be ascertained via venepuncture to the antecubital vein into three vacutainers.
- Ketone and lactate levels will be measured via a finger prick.
- All samples will be de-identified and coded according to your participant number.
- Your samples will be discarded once analysed, unless it is your wish for them to be returned to you.

Why is the study designed this way?

This study is a single-blinded randomised, crossover trial. This is so you act as your own control, which significantly increases the power of the study. You are blinded to the inclusion and dose of BD to ensure this does not influence your responses during the study.

What will happen to my samples after they have been collected?

- All blood samples will be analysed in the AUT Millennium Immunology Laboratory and the AUT City Campus Roche Laboratory.
- No samples will be sent overseas.
- You have the right to have all your samples returned to you at the end of the study. If this is you wish, please inform the researchers. If not, all samples will be suitably disposed of via standardised procedures.

What are the costs of participating in this research?

There is no financial commitment required of participants, except for their transportation costs to and from the SPRINZ laboratory and for the replication of their dietary intake during the 24 h prior to each trial. Participants will be expected to commit about 13.5 h over the course of the study. This will be divided into the following areas:

- Initial appointment: 1 h
- Pre-experimental test: 2 h
- Familiarisation protocol: 2.5 h
- Experimental trials (1 and 2): 8 h (4 h each)

If you choose not to participant in the study or decide to not continue part way through the study, you will incur no costs.

What are the possible benefits and risks of this study?

There is the possibility that you may experience some risks from participation in this study. These include:

- Loss of breath and discomfort from strenuous exercise (this will not be unfamiliar to some of your typical training bouts)
- Slight discomfort during the insertion of the cannula.
- Dislike the unpleasant taste of the drink.
- Experience some mild gut and systemic symptoms.
- Light headedness.
- If you are tested for substances banned by the World Anti-Doping Agency (WADA) and Drug Free Sport New Zealand, it is paramount that you are aware there is the possibility that the BD product may produce a positive result if tested after consumption. This is due to BD not having been tested for contamination or banned substances by a third party and, therefore, its compliance to WADA regulations cannot be assured. If you are unsure what this means, please enquire with David Shaw.

All symptoms, side effects and abnormal cell values will be immediately reported to a sports doctor to ensure participant safety.

Alternatively, you will gain the following benefits from participation in this study. These include:

- Information regarding your individual immune response and tolerance to the intake of BD and strenuous exercise.
- Reports of haematological values (e.g. haemoglobin, haematocrit, white blood cells and subsets) (if any are outside of normal range, you will be notified immediately).
- Performance and metabolic testing (e.g. $\text{VO}_{2\text{max}}$ test, maximal fat burning capacity, ventilatory thresholds).
- Access to a NZ registered dietitian and sports nutritionist after completion of data collection. Access will be limited to a period of 3 months following completion of participation and will include 1 initial consult (1 h) with a meal plan.
- Information on BD and how it may influence your performance. However, the product itself will not be provided to you.

Who pays for this study?

All funding for this study comes from the leading investigators PhD research budget provided by AUT. Unfortunately, due to the limited funding, it is not possible for you to be reimbursed for travel costs, food purchases, time away from work etc. It is hoped that the personalised and advanced information received from participation in this study will be sufficient compensation.

What if something goes wrong?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

What are my rights and how will my privacy be protected?

Your participation in this study is voluntary and you are free to remove yourself from the study at any stage, without any costs, repercussions or disadvantages. You have the right to access all of your personal information at any stage during the study and ask for the results to be meaningfully communicated if you are uncertain. However, you will not own any intellectual property arising from this study or analysis of your samples.

All of the data produced from your participation in the study will remain de-identified and stored on the password protected AUT network to protect your confidentiality. Nevertheless, it is possible that you will present in the laboratory alongside one other participant. If you prefer to participate in isolation, please state this to the lead investigator.

If you are unsure about any aspect of the study protocol, you are able to contact the lead investigator, David Shaw, or his PhD supervisors (see contact details below).

What happens after the study of if I change my mind?

Following your participation in the study, there will be no future requirement for your participation. However, you will be provided with an opportunity to be considered in the main experimental trial, which can be further explained to you on completion.

All of the data collected during the study will only be available to the researchers and yourself. Data storage will be de-identified and kept within a password protected AUT network to protect your confidentiality for a period of up to 10 years. If the data is published in the public domain, your name and personal information will not be revealed.

Biological specimens will be analysed and then stored in a -80 °C freezer for about 1 year. If you would like to have your samples returned, please state the lead investigator. Following analysis, you will be contacted regarding your results within a time frame of about 6 months.

Who do I contact for my information of if I have concerns?

If you have any questions, concerns or complaints about the study at any stage, you can contact:

Name: **David Shaw, PhD student and leading investigator**
Telephone number: **021 08276137**
Email: **nutrition@daveshaw.co.nz**

Name: **Dr Deborah Dulson, PhD and study supervisor**
Telephone number: **09 921 9999 ext. 7417**
Email: **deborah.dulson@aut.ac.nz**

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

For Maori health support please contact :

Name: **Dr Isaac Warbrick, PhD and co-director of Taupua Waiora Centre for Māori Health Research**
Telephone number: **09 921 9999 ext. 7591**
Email: **Isaac.warbrick@aut.ac.nz**

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS
Email: hdec@moh.govt.nz

Consent form

Study title: **The effect of dietary ketones on immunity, tolerability and performance in well-trained, male cyclists.**

Lead investigator: **David Shaw**

Project supervisor: **Deborah Dulson**

Please tick to indicate you consent to the following

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	<input type="checkbox"/>
---	--------------------------

I have been given sufficient time to consider whether or not to participate in this study.	<input type="checkbox"/>
--	--------------------------

I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	<input type="checkbox"/>
---	--------------------------

I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	<input type="checkbox"/>
---	--------------------------

I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	<input type="checkbox"/>
---	--------------------------

If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes	No
	<input type="checkbox"/>	<input type="checkbox"/>

I consent to the research staff collecting and processing my information, including information about my health.	<input type="checkbox"/>
--	--------------------------

I consent to my GP or current provider being informed about my participation in the study and of any significant abnormal results obtained during the study

Please state your GP's contact details (optional).

Name:	Yes	No
Practice:	<input type="checkbox"/>	<input type="checkbox"/>
Address:		
Phone:		
Email:		

I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	<input type="checkbox"/>
---	--------------------------

I agree to comply with all the study's requirements and know my responsibilities as a study participant	<input type="checkbox"/>				
I understand the compensation provisions in case of injury during the study.	<input type="checkbox"/>				
I have answered the questions regarding my health, training, competition, weight, diet, medication and supplement use to the best of my ability	<input type="checkbox"/>				
I know who to contact if I have any questions about the study in general.	<input type="checkbox"/>				
I wish to have my blood samples returned to me in accordance 7 (9) of the <i>Code of Health and Disability Services Consumers' Rights</i>	<table border="1"> <tr> <td>Yes</td> <td>No</td> </tr> <tr> <td><input type="checkbox"/></td> <td><input type="checkbox"/></td> </tr> </table>	Yes	No	<input type="checkbox"/>	<input type="checkbox"/>
Yes	No				
<input type="checkbox"/>	<input type="checkbox"/>				

Declaration by participant:

I hereby consent to take part in this study.

Participant's name: _____

Signature: _____ Date: _____

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name: _____

Signature: _____ Date: _____

Participant Information Sheet

Date Information Sheet Produced:

31/1/18

Project Title

The effect of a ketogenic diet on immune function, gastrointestinal microbiota, stress and performance in healthy, male athletes.

An Invitation

My name is David Shaw, a PhD student at Auckland University of Technology (AUT). Along with Senior Lecturer Dr Deborah Dulson, we invite you to take part in a study investigating the effects of a ketogenic diet on performance and immunity. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is ten pages long, including the Consent Form. Please make sure you have read and understood all the pages. If you have any queries, please contact David Shaw (primary researcher).

What is the purpose of this research?

Athletes are constantly searching for novel dietary strategies to enhance performance and health. A ketogenic diet has been suggested to influence endurance performance, with both positive and negative effects. Commonly referred to as keto- or fat-adaptation, consuming a low-carbohydrate, ketogenic diet (<50 g CHO·day⁻¹ and fat ~80% energy intake) can enhance the body's ability to utilise fat and provides the additional energy source, ketones. In some athlete circles, a ketogenic diet is being used to promote performance, however, its effect on immunity, gastrointestinal microbiota and stress in male endurance athletes is not well known. Considering endurance athletes are at a higher risk of illness and gastrointestinal distress, this study is important to provide an insight into the effect of a ketogenic diet on these health markers.

This study will be undertaken by the primary researcher, David Shaw, at Auckland University of Technology and will contribute to the fulfilment of a PhD thesis. The results will be published in academic journals and presented at scientific conferences to inform the wider community. David Shaw has received no external funding for this study nor has been influenced to undertake this research by third parties.

How was I identified and why am I being invited to participate in this research?

Thank you for showing interest in this study. There will be a total of 10-12 participants in this study, with selection occurring on a first in, first served policy. Due to the complex nature of the immune system, there is several criteria that you will need to meet to be eligible. Please carefully consider your fulfilment of each (see below).

- ✓ You are male.
- ✓ You have completed a full marathon or ultra-endurance run within the previous 6 months.
- ✓ You are between 20 and 40 years of age.
- ✓ You have been training for more than two years.
- ✓ You run more than 50 km per week.
- ✓ You are without cardiovascular, metabolic, neurological, immunological or autoimmune disorders.
- ✓ You have normal baseline haematological (red and white blood cell) values (this will be tested for during your initial appointment).
- ✓ You have been free of illness (e.g. cough, cold, runny nose, sore throat, fever, weakness and headaches, muscle pain, repetitive sneezing, persistent muscle soreness, joint aches and pains) for more than 4 weeks.
- ✓ You are not experiencing any current gut symptoms.
- ✓ You have not consumed any medications or supplements known to effect immunity for more than 2 weeks.
- ✓ You are a nonsmoker.
- ✓ Your weight has been stable for more than one month.
- ✓ You have been consuming a mixed diet and haven't restricted high carbohydrate foods for more than one year.
- ✓ No family member has died before the age of 50 with a heart condition.
- ✓ Are comfortable with providing blood, saliva and stool samples and having body composition measured.
- ✓ Have a peak oxygen consumption ($\text{VO}_{2\text{max}}$) over $50 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (this will be ascertained during the preliminary tests).
- ✓ Do not possess an injury that will affect running performance.
- ✓ You are able and willing to consume a ketogenic diet for 4 weeks.
- ✓ You are able and willing to monitor and report your diet, training, stress and cold symptoms for a period of 2 ½ months.

Please note, participants initially deemed eligible who do not comply with study requirements will be requested to discontinue and excluded from the study.

How do I agree to participate in this research?

By signing the consent form, you are agreeing to part in this study. However, your participation in this research is voluntary (it is your choice) and whether or not you choose to participate will neither advantage nor disadvantage you. You are able to withdraw from the study at any time. If you choose to withdraw from the study, then you will be offered

the choice between having any data that is identifiable as belonging to you removed or allowing it to continue to be used. However, once the findings have been produced, removal of your data may not be possible.

What will happen in this research?

If you would like to participate, you will have a brief meeting with David Shaw (primary researcher) via phone / Skype / Facetime to explain the requirements of the study, potential burden of participating, benefits of participating and to answer any queries. If you would like to continue with the study, you will be required to complete the following which will occur over a period of approximately 3 months.

Screening and consent

Initially, you will meet with the primary researcher at AUT Millennium. Here, you will be screened for eligibility and asked to complete sign a consent form and health and eligibility questionnaire. An initial blood draw will be collected by a trained individual via venepuncture to the antecubital vein to ensure red blood cell, white blood cell and differential cell counts are normal as part of screening. Following this, you will be asked to complete the Athlete Diet Index (~20 min).

Days -5 to -1

You will be required to complete an image-assisted weighed dietary record, which will be sent to the primary researcher in real time using WhatsApp mobile phone app.

Day -2

Preliminary test: You will arrive at the laboratory between 06:00 and 07:00 h following an overnight fast and having abstained from caffeine, alcohol and strenuous exercise for the previous 24 h. You will be asked to void your bladder before having your body mass, height and sum of 8 skinfolds measured. Then you will perform an incremental step test to volitional exhaustion on a motorised treadmill by walking for 3 min at 6.5 km·hr⁻¹, then running for 3 min stages at ~9, 10.5, 12, 13.5 and 15 km·hr⁻¹. You will have a ventilatory mask fitted to analyse expired gas and your heart rate will be continuously measured. Following the completion of the 15 km·hr⁻¹ stage, treadmill speed will be reduced to 11 km·hr⁻¹ and subsequently increased of 0.5 km·hr⁻¹ every 30 sec until you can no longer run.

You will be required to attain a VO_{2max} of >55 ml·kg⁻¹·min⁻¹ to continue with the study. If eligible, you will be asked to refrain from strenuous exercise until your experimental trial on day 0 (i.e. 48 h). In order to assess differences in VO_{2max} and substrate oxidation, you will be required to perform the same test twice for each dietary allocation. For an overview of the study, see Figure 1 below.

Initial education session: Immediately following the initial preliminary test for each dietary condition, you will be required to undertake a detailed education session (1 h) covering the dietary, training and specimen collection requirements of the study. All of your queries will be answered. You will be informed of your dietary allocation to offer sufficient time to prepare as well as be provided with stool collection materials. Following this, you will be asked to follow your typical dietary intake prior to a main event for the following 2 days (i.e. day -2 and -1) and consume a standardised breakfast (i.e. 2 g·kg⁻¹

of carbohydrate at 06:00) (guidance will be provided) accompanied by a 500 ml of water on the morning of their experimental trial. You will be asked to replicate this diet between days -5 and 0 for the alternate dietary arm and days 26 and 30 of the habitual diet arm.

Day -1

You will be required to collect and freeze a stool specimen at home to bring to the lab the following day.

Day 0

Experimental trial: You will arrive at the laboratory at 07:00 h having consumed your prescribed breakfast (i.e. 2 g CHO·kg⁻¹) at 06:00 and refrained from caffeine and alcohol for the previous 24 h. Please note, for the final experimental trial on the ketogenic diet, you will be required to continue consuming a ketogenic diet following the second preliminary test and will be prescribed an isocaloric low-carbohydrate, high-fat breakfast accompanied by 500 ml of water to be consumed at 06:00. You will also collect your first morning void and bring this to the laboratory in a plastic container provided.

On arrival, you will have a cannula inserted into your antecubital vein by a trained individual for serial blood sampling during the trial. Baseline body mass, venous bloods and saliva samples, ketone and lactate levels will be collected. You will commence running at 07:30 h at a speed eliciting 70% of your VO_{2max} until exhaustion. You will be prescribed to 4 ml·kg⁻¹ every 20 min of either a carbohydrate-electrolyte drink or an electrolyte-only drink for the final run to exhaustion following the ketogenic diet. You will have a ventilatory mask fitted for 4 min to collect expired gas samples every 30 min and at exhaustion, with perceived exertion and heart rate obtained during the last 30 sec. A venous blood sample (serum vacutainer only) and capillary blood ketones and lactate will be collected at 60 and 120 min and the cannula will be flushed with 3-4 ml of saline every 30 min.

On attainment of volitional exhaustion, treadmill speed will be reduced to 4.4 km·h⁻¹ for 2 min, then restored to the speed eliciting 70% VO_{2max} and you will again run to exhaustion. This process will be repeated so at the third attainment of volitional exhaustion the test will be terminated. Body mass, venous bloods and saliva samples, ketones and lactate levels will be collected immediately upon termination of the test. The same researcher will monitor each experimental trial. Standardised feedback will be provided and there will be a financial incentive (i.e. supermarket voucher) for the participant who accumulates the longest duration across the four experimental trials. You will not be notified of their results until the end of all experimental trials. You will be provided with 5 ml·kg⁻¹ of water during the 1 h of recovery, following which venous bloods and saliva samples, and capillary blood ketones will be collected.

Days 0 to 31

Dietary intervention: Following each initial experimental trial, you will be randomised to either; 1) maintain your habitual diet or; 2) ketogenic diet for the following 31 days. You will be advised to commence your dietary allocation immediately following the experimental trial.

The ketogenic diet will contain (approximately) ≤50 g·day⁻¹ CHO, 15-20% protein and 75-80% fat. You will be provided with a nutrition plan that is specific your taste

preference, budget and preparatory skills as a guide. Total energy intake will be matched to the food diaries provided during screening, however, adjustments will be made during the study to prevent weight fluctuations. No medications or supplements known to affect immune function may be consumed during the entirety of the study. You will have daily access (phone and email) the primary researcher, David Shaw, who is a NZ registered dietitian.

Dietary monitoring: You will be required to provide an image-assisted weighed dietary diary alongside a fiducial marker (provided) for two self-selected and nonconsecutive days during the periods; days 1-7, 8-14 and 15- 21, and reported to the primary researcher in real time using a phone app that can be viewed remotely to monitor compliance and provide immediate feedback. You will also self-monitor your weight daily during the 4 weeks (education will be provided for how to standardise weigh-ins) and report to the primary researcher. During the ketogenic diet intervention, you will be asked to self-measure your daily morning urine ketones (acetoacetate) using self-testing strips (Ketostix, Bayer) at a recommended time of 07:00 to 08:00 h and asked to send a picture of the strip to the primary researcher for confirmation.

Participants who do not comply with study requirements or fail to exhibit positive daily urinary ketones after 7 days or for more than 85% of the days (i.e. 6 of 7 days) during days 8-31 will be requested to discontinue and excluded from the study. Urinary ketones will not be monitored during the control diet.

Training monitoring: All training sessions will be self-monitored and reported using online forms. Training load will be quantified via each session's duration (h : min), average heart rate (HR), maximum HR (HRmax) and perceived exertion. You will be asked to replicate your training during the subsequent trial arm to minimise training effects. However, if you are initially randomised to your habitual diet, you will be asked to perform 80% of your typical load during the first 2 weeks due to the anticipated reduction in training load during the transition of a ketogenic diet.

Perceived stress, gastrointestinal symptoms and upper respiratory symptoms: You will complete a validated stress, gut symptom and illness questionnaire using online forms at baseline and the end of each week. Participants will be required to complete all of the forms.

Days 26 to 30

You will be required to complete an image-assisted weighed dietary record, which will be sent to the primary researcher in real time using WhatsApp mobile phone app. When consuming your habitual diet, you will aim to replicate your diet as per days -5 to -1.

Days 29 to 31

You will complete the procedures of days -2 to 0, with slightly alterations depending on the dietary arm. Following the ketogenic diet, you will complete a one-to-one interview (described below).

One-to-one interview: Following the completion of the ketogenic diet, you will be interviewed (~30 min) on (open-ended) questions relevant to their experience on a ketogenic diet, including their social, mental and physical experiences. Each interview will take place in a private room at AUT Millennium.

14-21 day washout period: You will be asked to maintain or return to your habitual dietary and training habits. You will be required to report any illness, upper respiratory symptoms or gastrointestinal symptoms to the primary researcher. No supplements or medications that affect immune function may be consumed during this period. At the end of the washout period, you will be required to complete the alternative dietary arm (i.e. habitual diet or ketogenic diet).

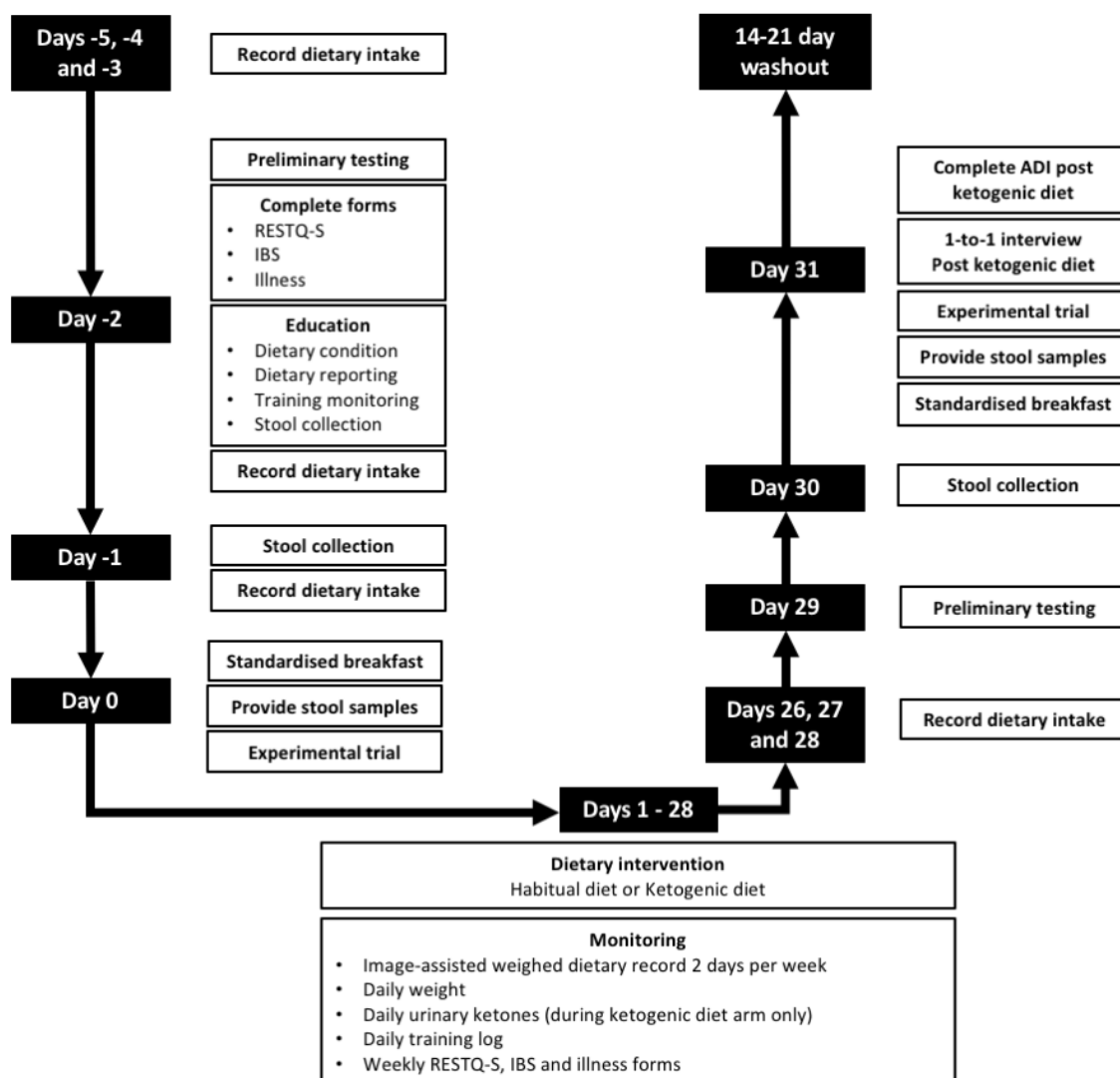


Figure 1. An overview of the study's requirements.

What will happen to my samples after they have been collected?

- All saliva and blood samples will be analysed in the AUT Millennium Immunology Laboratory and the AUT City Campus Roche Laboratory.
- All stool samples will be analysed at the University of Auckland.
- No samples will be sent overseas.
- You have the right to have all your samples returned to you at the end of the study. If this is you wish, please inform the researchers. If not, all samples will be suitably disposed of via standardised procedures.

What are the discomforts and risks?

- You may experience some discomfort and fatigue during each exercise test as each test is to exhaustion. However, it is likely that you will be familiar with the nature of each exercise protocol due to your habitual training.
- Although you are unlikely to experience major symptoms from consuming a ketogenic diet, you may experience early fatigue during exercise. Additionally, some 'fogginess' may be experienced, however, this can typically be well managed.
- You may experience some discomfort during blood collection, however, this will only be transient.
- You may experience some embarrassment or discomfort with providing stool samples.

How will these discomforts and risks be alleviated?

All of this study's protocols will be carried out in a professional and confidential manner. The lead researcher is trained in venepuncture and cannulation and a NZ registered dietitian.

What are the benefits?

- Information regarding your individual immune response to diet and exercise.
- Information regarding your gastrointestinal microbiota and how this responds to diet.
- Reports of haematological values (e.g. haemoglobin, haematocrit, white blood cells and subsets) (if any are outside of normal range, you will be notified immediately).
- Performance and metabolic testing (e.g. $\text{VO}_{2\text{max}}$ test, maximal fat burning capacity, ventilatory thresholds).
- \$100 Westfield voucher upon completion of the trial and the opportunity for a supermarket voucher depending on your performance during the trial.
- The potential to win a \$500 supermarket voucher. This will be provided to the participant who accumulates the most time running across the four experimental trials.
- \$35 of olive oil and \$20 of coconut oil.
- Discount card for Simply Fresh (fruit and vegetable store located in Northcote), which can be used for the duration of the study.
- Access to a NZRD and sports nutritionist after completion of data collection. Access will be limited to one free 1 h consultation within the three month period following completion of participation.

What compensation is available for injury or negligence?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

Your participation in this study is voluntary and you are free to remove yourself from the study at any stage, without any costs, repercussions or disadvantages. You have the right to access all of your personal information at any stage during the study and ask for the results to be meaningfully communicated if you are uncertain. However, you will not own any intellectual property arising from this study or analysis of your samples.

All of the data produced from your participation in the study will remain de-identified and stored on the password protected AUT network to protect your confidentiality. Only de-identified / aggregated data will be published. If you are unsure about any aspect of the study protocol, you are able to contact the primary researcher, David Shaw, or his PhD supervisors (see contact details below).

What are the costs of participating in this research?

There is no financial commitment required, except for your transportation costs to and from the AUT laboratory and for managing your habitual and ketogenic diet. You will be expected to commit about 45 hours over the course of the study (~2.5 months). This will be divided into the following areas:

- Preliminary test: (1 h x 4) 4 h
- Dietary education: (1 h x 2) 2 h
- Experimental trial: (5 h x 4) 20 h
- Dietary/training/stress/gut symptom reporting: 10 h
- Weekly saliva drools: 1 h
- Total: 37 h (across a period of about 2.5 months)
- If you choose not to participate in the study or decide to not continue part way through the study, you will incur no costs.

What opportunity do I have to consider this invitation?

You have 2 weeks to decide if you wish to participate in this study. If you decide to participate, you can withdraw at any time.

Will I receive feedback on the results of this research?

Yes, you will be provided with a 1-2 page summary of all your results following the completion of your participating in each dietary arm.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, Deborah Dulson, deborah.dulson@aut.ac.nz, +64 9 921 9999 x 7417

Concerns regarding the conduct of the research should be notified to the Executive Secretary of AUTECH, Kate O'Connor, ethics@aut.ac.nz, 921 9999 ext 6038.

Whom do I contact for further information about this research?

Please keep this Information Sheet and a copy of the Consent Form for your future reference. You are also able to contact the research team as follows:

Researcher Contact Details:

David Shaw, *nutrition@daveshaw.co.nz*, 021 0827 6137

Project Supervisor Contact Details:

Deborah Dulson, *deborah.dulson@aut.ac.nz*, +64 9 921 9999 x 7417

Consent form

Project Title

The effect of a ketogenic diet on immune function, gastrointestinal microbiota, stress and performance in healthy, male athletes.

Lead investigator: David Shaw

Project supervisor: Deborah Dulson

Please tick to indicate you consent to the following

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	<input type="checkbox"/>	
I have been given sufficient time to consider whether or not to participate in this study.	<input type="checkbox"/>	
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	<input type="checkbox"/>	
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	<input type="checkbox"/>	
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	<input type="checkbox"/>	
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes <input type="checkbox"/>	No <input type="checkbox"/>
I consent to the research staff collecting and processing my information, including information about my health.	<input type="checkbox"/>	
I consent to my GP or current provider being informed about my participation in the study and of any significant abnormal results obtained during the study		
Please state your GP's contact details (optional).		
Name:	Yes <input type="checkbox"/>	No <input type="checkbox"/>
Practice:		
Address:		
Phone:		
Email:		
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	<input type="checkbox"/>	
I agree to comply with all the study's requirements and know my responsibilities as a study participant	<input type="checkbox"/>	
I understand the compensation provisions in case of injury during the study.	<input type="checkbox"/>	

I have answered the questions regarding my health, training, competition, weight, diet, medication and supplement use to the best of my ability	<input type="checkbox"/>				
I know who to contact if I have any questions about the study in general.	<input type="checkbox"/>				
I wish to have my blood and saliva samples returned to me in accordance 7 (9) of the <i>Code of Health and Disability Services Consumers' Rights</i>	<table> <tr> <td>Yes</td> <td>No</td> </tr> <tr> <td><input type="checkbox"/></td> <td><input type="checkbox"/></td> </tr> </table>	Yes	No	<input type="checkbox"/>	<input type="checkbox"/>
Yes	No				
<input type="checkbox"/>	<input type="checkbox"/>				

Declaration by participant:

I hereby consent to take part in this study.

Participant's name: _____

Signature: _____

Date: _____

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name: _____

Signature: _____

Date: _____

Ketogenic Diet Handbook



Created by Dave Shaw
NZ Registered Dietitian

This handbook was created for participants in Dave's PhD study investigating the effects of a ketogenic diet on health and performance in athletes. It is not for use by the general public.

Introduction

Thank you for participating in this PhD research investigating the effects of a ketogenic diet on athlete health and performance. This handbook is designed to guide your dietary choices when on the ketogenic diet arm of the trial. Due to the restrictive nature and small margin of error when consuming a ketogenic diet, it is important that your compliance is near perfect throughout the next 31 days. Use this handbook, the personalised meal plan and Dave's expertise to help you implement your new way of eating.

Importantly, please refrain from using other resources (websites, books, magazines, phone apps, friends advice etc.) unless otherwise specified in this handbook as they may not be accurate or reliable sources of information. Additionally, avoid searching for the potential benefits or detriments of a ketogenic diet as these may influence your perception of the diet and bias your results.

Throughout your participation, you are able to contact the Dave with any questions and/or concerns at any time and he will respond as soon as possible. His details are below;

Mobile: 021 0827 6137 (either text, call or WhatsApp)

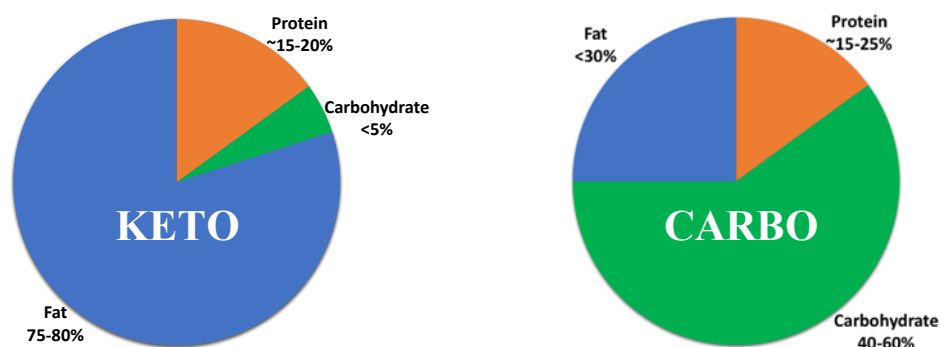
Email: nutrition@daveshaw.co.nz

The following sections will provide you with a detailed description of what ketogenic diet involves and your responsibilities.

What is a ketogenic diet?

A ketogenic diet is a very low-carbohydrate, protein restricted, high fat diet. Carbohydrate intake is reduced to below 30-50 g per day, whereas fat intake is increased to maintain energy balance, with protein intake remaining about the same as in your typical diet. Due to a depletion of carbohydrate in your body, your liver produces ketones predominantly from the breakdown of fat (either stored or dietary fat) that can be used as an additional energy source for the brain and muscles. It takes about 3-7 days to increase blood ketones to levels indicating a state of 'ketosis'.

Macronutrient profile of the Ketogenic Diet vs High Carbohydrate Diet



The figures above demonstrates the contribution of macronutrients to energy intake on a typical high(er) carbohydrate diet versus ketogenic diet. Recommendations for the general population suggests that 40-60% of energy intake should come from carbohydrate, 15-25% from protein and 30% or less from fat. For endurance athletes, carbohydrate recommendations are towards the higher end and possibly even higher in some situations. Please bear in mind that this doesn't mean a ketogenic diet is necessarily better or worse for you. This is what we're aiming to find out with your help.

By participating in this study, you are at the interface of the creating of new knowledge and nutritional recommendations. Indeed, you have very important part in this study and your results (as part of a de-identified, aggregated group set) will be read by many people, including the general public, scientists, practitioners and athletes. However, to achieve this, we need reliable and valid results.

Therefore, dietary compliance is crucial. This is because it's easy to revert back from a ketogenic state with a single high carbohydrate meal, snack or drink, even if you think you'll be burning it off during your next workout. It's an all or nothing diet. So, to confirm you are in ketosis and consuming sufficient calories (i.e. energy), you will be required to provide three things – two of which you will also be providing throughout your participation in the study.

1. Daily morning weight

To be measured pre-breakfast, after you have gone to the toilet and wearing similar or minimal clothing. The purpose of this is to monitor energy balance. Often people lose weight on a ketogenic diet, but we need to prevent this by consuming sufficient calories. The goal is weight maintenance.

2. Daily morning urinary ketosis test

Ideally, to be measured during 07:00 and 08:00am by urinating onto a ketone analysis stick. This is to confirm the presence of ketones in your body. You will be provided with ketostix for the duration of the ketogenic diet arm.

3. Photo-assisted weighed dietary diary via WhatsApp

You will be educated on how to accurately record and photograph your diet. You will be required to provide 2 x 24 h (nonconsecutive) dietary records during weeks 1, 2 and 3 of your dietary intervention, and for 5 day blocks (i.e. 3 days before preliminary test and first two days of your testing block) that will be coded for nutrient analysis.

Carbohydrate – How low do you need to go?

As a rule of thumb, you will need to reduce your daily carbohydrate intake to less than 30-50 g. Initially, this may be difficult to comprehend as you are changing many of your dietary habits. In this case, it's easier to start off by listing the foods you should avoid – see the list below. Keep in mind that you are basically going cold turkey on carbohydrate for the next 4 weeks. But don't worry, there are plenty of nutritious and delicious foods you can eat that we will discuss later on.

Do not eat

- **Grains and cereals**
Including wheat, corn, pasta, rice, oats, buckwheat, quinoa, rye, cereals, breakfast cereals, bread, baked products etc.
- **Sugar**
Honey, golden syrup, maple syrup, table sugar, chocolate, ice-cream, baked products, lollies etc.
- **Fruit**
Apples, bananas, plums, nectarines, oranges etc. (however, some berries can be consumed in moderation)
- **Starchy vegetables**
Potato, kumara, sweet potato, yams, taro, pumpkin, corn etc.
- **Legumes**
Lentils, chickpeas, kidney beans etc
- **Processed meat**
Sausages, flavoured / glazed ham and bacon etc.
- **Low fat foods**
These tend to be higher in carbohydrate and sugar than full-fat versions. You also need the additional fat to help you retain energy balance.

Do not drink

- **Alcohol**
Beer, wine, RTDs, liqueurs etc. (however, some spirits may be fine in very small quantities, but check with Dave first)
- **Soft drink**
Cola, lemonade etc. (however, you may have small servings of zero sugar and diet options)
- **Juice**
- **Sports drink and coconut water**
Powerade, Gatorade, Horleys Replace, Pure etc.
- **Energy drinks**
V, Mother, Redbull etc.
- **Flavoured milk and milk**
Plain (cows) milk contains 3-5 g of carbohydrate per 100 ml, therefore, very small portions are allowed.

What foods are low in carbohydrate?

- **Nonstarchy, colourful vegetables**

Fresh, raw	Carbohydrate per 100g	Carbohydrate per cup
Asparagus	1.4	2
Bamboo shoots	1.3	1.8
Broccoli	0.4	0.4
Brussel sprouts	2.1	1.9
Carrot	5	5.8
Celery	1.2	1.5
Courgette	1.7	3
Cucumber	1.5	3
Cauliflower	2	2.3
Cabbage	2.4	2.5
Capsicum	2.3	2
Eggplant	2.6	2.3
Green beans	2.4	3.3
Kale	3.4	3.9
Leek	3.7	3.5
Mushrooms	0	0
Lettuce, cos	1.8	0.9
Onions	4.3	6
Radish	1.9	3
Rhubarb	1.7	4
Spinach	0.6	0.3
Spring onion	4.6	4.8
Tomato	2.4	5.8



- **Meat, chicken, fish, liver, kidney and eggs**

These options tend to provide almost no carbohydrate when in their natural state. Be careful of flavoured or marinade options. Importantly, this doesn't mean you can eat unlimited amounts and you also need to meet your protein targets and stay below upper limits.

- **Nuts and seeds**

Nuts and seeds contain small amounts of carbohydrate. **Limit your intake to 60 g per day** (inclusive of both). Additionally, be careful of sweetened versions e.g. honey roasted)

- **Berries, tomato, avocado and olives**

Most fruits should be avoided, however, you can eat berries, tomatoes, avocado and olives in limited amounts. **Please consume less than 50 g per day of each**, however, if you do not eat one you may have slightly more of another.

Protein – Not too little and not too much

Protein-balance is an important part of the ketogenic diet because you need to consume enough to prevent muscle breakdown but not too much to prevent ketogenesis. The latter is due to protein's gluconeogenic (or carbohydrate producing) properties. Often, it's thought that a ketogenic diet is a high protein diet, similar to the Atkins diet, but it's not. Rather, it's moderate in protein, with the majority of energy coming from fat.

Raw	Protein per 100g	Protein per serve
Beef (with fat)	21.4g	38.3g / small steak
Lamb (with fat)	20.4g	16.3g / small chop
Pork (with fat)	21.5g	27.1g / small chop
Chicken (with skin)	20.1g	36g / small breast
Salmon	21.3g	25.2g / small fillet
Liver	20g	---
Kidney	18.2g	---
Eggs	12.6g	5.5g / regular egg



Many protein sources also provide a source of fat. It's fine to include fattier options or low grade cuts into your ketogenic diet. Protein should be consumed throughout the day in 3-6 small to moderate meals, rather than a large amount at once.

Fat – Your main energy source

Fat will make up the majority (75-80%) of your energy intake. Increasing your dietary fat is essential to maintain energy balance and prevent you losing weight. Remember, you can't consume both a low carbohydrate and low fat diet simultaneously. Typically, people transitioning to a ketogenic diet do not consume sufficient fat and overeat protein. By viewing your personalised meal plan, you will be able to better understand your new requirements.

- Fats and oils**

	Fat per 100g	Fat per serve
Butter	70.1g	13.3g / Tbs
Lard	100g	17g / Tbs
Cream, fluid	35.9g	6g / Tbs
Olive oil	100g	18.4g / Tbs
Coconut oil	99.9g	18.4g / Tbs
Coconut cream	18.9g	3.9g / Tbs



- **High fat foods**

	Fat per 100g	Fat per serve
Salmon	13.9g	16.4g / small fillet
Olives, green	20.5g	5.4g / ¼ cup
Avocado	21.6g	17.2g / ½ avocado
Almonds	55.1g	16.9g / ¼ cup
Seeds, mix	49.1g	18.7g / ¼ cup
Brie (cheese)	32g	1.6g / small slice
Peanut butter	54.3g	13.6g / Tbs



Please avoid

- Margarine
- Vegetable oils

Here are some suggestions to boost your fat intake

- Drizzle / pour olive oil over your meals and salads
- Consume ‘fat bombs’
- Eat the fat / skin / rind of meats
- Add cream to your hot drinks rather than milk

Fluid and electrolytes

A ketogenic diet may increase your loss of fluid and electrolytes. To combat this, ensure you are drinking plenty of water to stay hydrated and rehydrate after exercise. Your electrolyte (sodium, magnesium, potassium) levels may also decline, therefore, ensure you consume extra. If not, you may experience light headedness and fatigue. Extra doesn't mean a sprinkle of salt, it means an increase of 1-2 g of salt per day.

Here are some suggestions to boost your salt intake

- Season food with salt and add more at the table.
- Consume a (low carbohydrate) salty soup or broth once or twice daily.
- If you cook meat, don't discard the fluid, use it in other dishes, such soups.

What can you drink?

- Water
- Tea
- Coffee
- Salty soups
- Diet / zero sugar soft drink
- Vegetable juice
- Spirits (**only in small amounts**)

What to do when dining out

Eating out is still possible on a ketogenic diet as most cafes and restaurants have low-carbohydrate options. However, you will to pay close attention to the menu and come up with some strategies to ensure you can stick to the diet.

- View the menu before you go so you know what to ask for when you arrive.
- Avoid places where you may be tempted by high carbohydrate foods and drinks.
- Avoid fast food restaurants, in general, as they typically provide highly processed, high(er) carbohydrate options.
- Replace carbohydrate-based sides, such as chips and rice, with a green salad.
- Avoid milky coffees and instead, ask for milk or cream on the side.
- Avoid deep fried and battered options.
- Avoid gravy.
- Ask for the bread to be removed (e.g. eggs with salmon, avocado and mushrooms , rather than eggs on toast).
- Avoid sweet sauces and dressings or ask for the ingredient list (many sauces and dressings contain sugar, such as Thai curries and salad dressings).
- Avoid processed meat.

What options do you have?

- Salad with meat / chicken / fish with an oil based dressing or vinaigrette. These can be acquired from a variety of places, such as Pita Pit, Subway and Tank.
- Vegetable omelette.
- Steak / chicken breast or thighs / salmon or tuna fillet with salad.
- Bunless burger with premium mince or chicken breast and an unsweetened sauce.
- Add fat by asking for butter or olive oil for your meal or salad.
 - Consider carrying a small bottle of olive oil with you in case where you dine out doesn't have any in stock.

What can you snack on?

- Fat bombs (see example recipe below)
- Eggs (e.g. boiled)
- Cheese
- Cold cuts
- Avocado (limit to 50 g per day)
- Olives (limit to 50 g per day)
- Nuts and seeds (limit to 60 g per day)
- Celery
- Cucumber
- Capsicum
- Low carbohydrate dip / sauce (e.g. guacamole or salsa)
- Berries (limit to 50 g per day)
- Heavy whipping cream
- 90% dark chocolate (limit to 20 g per day)
- Pork rhind
- Beef jerky
- Kale chips

Some foods are slightly higher in carbohydrate, this means you cannot snack limitlessly. It's also possible that you find yourself snacking less. If this is the case, we will need ensure you overall energy intake is adequate to prevent weight loss.

How to read food labels

It's probable that the majority of food you eat will not come with packaging and labels. However, if you're looking to include alternative options into your diet, which come with a food label, here's what to look out for.

NUTRITION INFORMATION		
Servings per can: 2		
Serving size: 210g		
	Average Quantity Per serving	Average Quantity Per 100g
ENERGY	895kJ	425kJ
PROTEIN	10.8g	5.1g
FAT: TOTAL	1.2g	0.6g
-SATURATED	0.2g	0.1g
CARBOHYDRATE	33.7g	16.1g
-SUGARS	15.5g	7.4g
DIETARY FIBRE	11.9g	5.7g
SODIUM	1300mg	620mg
POTASSIUM	650mg	310mg
IRON	2.7mg	1.3mg

Always ensure total carbohydrate is very low or less than 2 g per 100 ml or 100 g. For some products, food manufacturers manipulate their portion size to make it appear low in carbohydrate or sugar. In this case, consider how much you're likely to eat. If you're unsure whether you should buy or eat it, it's best to ask Dave first.

How to prepare and recover from exercise

Nutrition during and around training sessions will be very different compared to your usual strategies. Fuelling up with carbohydrate is no longer required as your body becomes adapted to increase its ability to burn fat. This means consuming carbohydrate during and after training sessions should be avoided. However, there are some key strategies to use, these include

- Stay well hydrated by consuming sufficient water and consider a (no carbohydrate) electrolyte solution.
- Consume 20g of protein after exercise bouts to help with muscle repair.
- If your training load is increasing, add more fat to your diet to maintain energy balance. Consider low carbohydrate, low fat shakes for recovery.

Recipes

If you want to increase your dietary variety, countless ketogenic recipes can be sources from different websites. **Please refer to the recipe only rather than reading the content of the site.** If you would like to search for ideas online, simply Google "keto" or "ketogenic" followed by a meal idea. If you're unsure if the recipe is suitable, please send the link to Dave to double check first – it's better to be on the safe side rather than undoing all your good work!

A useful recipe book is called “The Easy 5 Ingredient Ketogenic Diet Cookbook” and can be sourced here <https://www.amazon.com/Easy-5-Ingredient-Ketogenic-Diet-Cookbook/dp/1939754445> (NZD \$~12.00 on Kindle / Kindle app).

Other recipes can be found here <https://www.dietdoctor.com/low-carb/keto/recipes> and here <https://ketosummit.com/ketogenic-diet-recipes>.

Chocolate Fat Bomb recipe (What The Fat book) (makes 20 x ~30 g serves)

- 1 cup (250 ml) Cream
- ½ cup (50 g) Butter
- ⅔ cup (100 g) Coconut oil
- ¾ cup (200 g) 85% dark Chocolate, broken in pieces
- 1/3 cup (20 g) desiccated coconut
- Cocoa or coconut threads for rolling

In a pot add the cream, butter and coconut oil and slowly heat until it begins to bubble. Pour the hot mixture over the chocolate pieces and whisk vigorously to incorporate the ingredients are nicely mixed together. Add to the mixture the desiccated coconut, mix well and set in a container or in individual moulds like an ice cube tray. Chill the mix completely in the fridge before cutting into bite size pieces or roll into bite-sized balls. At this point you can roll them in some coconut threads or cocoa – or simply transfer them as they are into a container with a tight fitting lid and storing them in the fridge for up to 2 weeks.

7-day example meal plan

Below is a weekly plan to provide ideas for meals and snacks. All options are very low carbohydrate and ketogenic diet alternatives for high carbohydrate options. Please note that **this is not a personalised plan** specific to your calorie requirements. For this, refer to next section.

	Mon	Tue	Wed	Thurs	Fri	Sat	Sun
B/fast	Vegetable omelette including mushrooms, spinach and courgette	LCHF muesli, unsweetened Greek yoghurt, unsweetened almond milk	Bacon, avocado, tomato, spinach	Cauliflower rosti with egg / bacon and hollandaise sauce	Berry and coconut parfait	Low CHO bread toasted with salmon and avocado	Cream cheese pancakes
Snack	Veggie sticks and guacamole	Peanut butter cookies	Nuts and brie cheese	Kale chips	Low carbohydrate muffin	Boiled eggs	Pork rind
Lunch	Turkey and brie sandwich with low CHO bread	Caesar salad with chicken and olive oil	Bunless burger	Eggplant pizza	Veggie and tofu stir-fry with peanut sauce	Frittata with veggies, avocado and olive oil	Creamy mushroom and bacon soup
Snack	Fat bomb	Creamy berry protein shake	Nuts and cherry tomatoes	Jerky	Tin of tuna	Pepperoni slices	Salty soup
Dinner	Pizza with cauliflower base	Courgette noodles with chicken and pesto	Chicken curry with cauliflower rice	Salmon with salad	Beef and cauliflower fried rice	Meatballs with tomato sauce and salad	Lettuce wrap enchiladas
Snack	Berries and whipped cream	Avocado pudding	Keto lava cake	Keto chocolate mouse	Pina colada fat bomb	Keto cookie	90% dark chocolate
Fluid	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink	Water Salty soup Electrolyte drink

Portion size guide specific to your requirements

Below is a tool to guide your portion sizes to ensure you consume sufficient calories (~3500 kcal or ~14,000 kJ), protein, fat and micronutrients, whilst restricting your carbohydrate intake to less than 50 g per day. **All weights and amounts are based on raw food** (i.e. prior to cooking). Keep in mind this is a starting point and will likely change day-to-day based on your hunger, weight and feedback. Please feel free to ask questions.

	Day 1	Day 2	Day 3
B/fast	Omelette / scrambled eggs <ul style="list-style-type: none"> 4 size 6 eggs 30ml olive oil 40g mushroom (raw) 40g spinach (raw) 	Cereal <ul style="list-style-type: none"> 50g Original Crunch Cereal (Clean Paleo) 150ml unsweetened almond milk 100g unsweetened Greek yoghurt 	Eggs on toast <ul style="list-style-type: none"> 1 slice Freya's Lower Carbohydrate Soy and Linseed bread 3 size 6 eggs (fried or scrambled) 30g spinach 30ml olive oil or 30g butter
Snack	<ul style="list-style-type: none"> 50g almond roasted with salt 50g Brie cheese 	<ul style="list-style-type: none"> 2 eggs (boiled / fried / scrambled) 3Tbs coconut oil or butter (to cook or eat) 	<ul style="list-style-type: none"> 100g carrot 100g Guacamole
Lunch	Chicken salad <ul style="list-style-type: none"> 150g chicken (breast / thigh, raw) 30g butter 50g lettuce 15 olives (optional) 1 medium tomato 30ml olive oil 	Bacon butty <ul style="list-style-type: none"> 2 slices Freya's Lower Carbohydrate Soy and Linseed bread 80 bacon, with fat 50g avocado 20g Best Foods Real Mayo 	Bunless burger (homemade) <ul style="list-style-type: none"> 100g mince 1 egg (to make into burger) 20g lettuce 30g Best Foods Real Mayo 30g mushroom 30g butter
Snack	<ul style="list-style-type: none"> 90g tuna (tin) 30g Fat bomb 75g carrot 	Shake <ul style="list-style-type: none"> 100g cream 30g frozen blueberry 1 scoop Whey Protein isolate / concentrate (not mass gainer) 150ml unsweetened almond milk 3Tbs coconut oil 	<ul style="list-style-type: none"> 70g Edam cheese 50g Almonds, roasted and salted 30g Fat Bomb
Dinner	Salmon and sautéed veg <ul style="list-style-type: none"> 150g salmon fillet (raw) 30g butter 40g spinach 40g capsicum 30ml olive oil 	Baked chicken and sautéed veg <ul style="list-style-type: none"> 150g chicken breast 100g courgette 100g capsicum 30ml olive oil 30g butter 	Sausages and veg <ul style="list-style-type: none"> 3x sausages (high quality) 100g broccoli 100g cauliflower 30g butter
Snack	<ul style="list-style-type: none"> 70g creamed (whipped) 40g strawberries 	<ul style="list-style-type: none"> 100ml cream (whipped) 20g 85% (or higher) dark chocolate (grated) 	<ul style="list-style-type: none"> 2x 30g fat Bomb
Fluid	<ul style="list-style-type: none"> Water 3 cups of salty fluid e.g. Miso, soup or broth 	<ul style="list-style-type: none"> Water 3 cups of salty fluid e.g. Miso, soup or broth 	<ul style="list-style-type: none"> Water 3 cups of salty fluid e.g. Miso, soup or broth

Example shopping list

In addition to your vegetable, meat, chicken and fish purchases, below is a list of products you may consider adding to your shopping list. These are not essential for a ketogenic diet, but are here to provide ideas. **Please note that some items contain 3-7 g of carbohydrate per 100 g or 100 ml, which is relatively high if consumed in large amounts** (e.g. Carbonara sauce). This will mean you will need to manage your portion sizes for these foods.

Please note, alternative brands to those shown may possess different nutritional value. Please check nutrition label prior to consumption.

- **Chilled and deli section**



- **Canned and prepared food**



- **Frozen food**



Appendix H: Guidelines for conducting an image-assisted weighed dietary record

How to do an image-assisted weighed dietary record

Accurate and complete recording of your dietary intake is important. This will allow for reliable information to be collected about how your nutrient intake changes when you follow a ketogenic diet compared to your habitual diet. The days you will need to record your diet will be as follows;

- Five day initial experimental block. This includes
 - 3 days prior to your VO_{2max} test (i.e. Days -5, -4 and -3)
 - Day of VO_{2max} test (i.e. Day -2)
 - Day of stool collection (i.e. Day -1)
- Two self-selected, nonconsecutive days during the following periods
 - Days 1-7
 - Days 8-14
 - Days 15-21
- Five day follow up experimental block. This includes
 - 3 days prior to your VO_{2max} test (Days 26, 27 and 28)
 - Day of VO_{2max} test (i.e. Day 29)
 - Day of stool collection (i.e. Day 30)

Below is a guide on how to provide an image-assisted weight dietary record.

1. Ensure you record **everything** you eat and drink, with the exception of plain water, during the entire 24 h.
2. Be as honest, accurate and descriptive as **possible**. If possible, include preparation techniques, cooking modes and additions / condiments, peeled / unpeeled, skin on / off etc.
3. If you are unable to weigh ingredients, provide standardised house-hold measures (e.g. cup, Tbsp, tsp).
4. **Record / report your diet across the day to show real-time dietary intake**, rather than pre weighing and reporting several hours early. This will allow you to change your dietary intake and report leftovers.
5. For the self-selected days, ensure these are not consecutive days and are **reflective of your typical intake** (i.e. do not record if you are calorie restricting, travelling, out of routine etc).

Step 1. Weigh all your food and/or ingredients.

Use a reliable kitchen scales. If you are consuming food with a known weight / serving size (e.g. packaged food), you can report this rather than weighing the contents.

Step 2. Take of picture of your food and drink including the fiducial marker.

Take a picture on a 45 degree angle as this will provide an informative view of portion sizes. Also, please take photographs in sufficient light and capture all your food and fluid in the shot.



Step 3. Using WhatsApp, send Dave the picture and a written or verbal description of your meal, snack or drink (as per the previous guidelines).

Step 4. Using a similar method, send Dave a picture and description of any leftovers.

Here's an example of a diet entry →

Step 5. Dave will review your dietary intake and code it to quantify your nutrient intake. If it appears unusual or you are underreporting, Dave will contact you.

