Dietary manipulation of brown adipose tissue (BAT) thermogenesis in healthy adult volunteers

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Abstract

Brown adipose tissue (BAT) is one of the three different types of adipose tissues found in mammals and is only present in small amounts in the adult human (~100 g), of which the main site is the supraclavicular neck region. As BAT possess a unique mitochondrial uncoupling protein (UCP1), it can have a pronounced influence on energy balance due to the capacity of UCP1 to uncouple aerobic respiration that results in the free flow of protons across the inner mitochondrial membrane thereby bypassing the need to convert ADP to ATP. One of BAT's main functions is to transfer energy from food into thermal energy, and this is primarily regulated through the sympathetic nervous system (SNS). The activation of non-shivering thermogenesis in BAT can be measured indirectly by thermal imaging (TI). Two important factors that influence the activity of BAT are diet and cold. My thesis aimed to investigate the effect of cold and individual dietary compounds, i.e. galacto-oligosaccharide (GOS) prebiotic and extra virgin olive oil (EVOO) on BAT thermogenesis in healthy adult volunteers by using TI. In mice, the end product of microbial fermentation of the GOS-prebiotic, short chain fatty acids (SCFA), and the phenol content in EVOO (oleuropein aglycone) have both been shown to increase UCP1 expression in BAT, and therefore, promote thermogenesis.

In my first pilot study, one individual was exposed to seven days of intermittent cold exposure that significantly increased BAT supraclavicular temperature. Then to study the influence of the GOS-prebiotic on BAT function, two studies were performed. The first was a feasibility study that recruited 14 participants and aimed to establish whether daily consumption of a GOS-prebiotic was practical and feasible as part of a daily routine. Each participant was studied before and during GOS-prebiotic consumption, i.e. at 0, 7 and 14 days. All participants were readily able to consume the GOS-prebiotic each day. However, there was no detectable difference in BAT temperature after 7 or 14 days of consumption. The second study was a randomised controlled trial to further assess the effect of GOS-prebiotic. It involved 63 participants, divided into three groups; one received the prebiotic, another a placebo (maltodextrin) and another did not receive any supplement. There were two study

sessions, before and after consumption, i.e. at 0 and 14 days. Again, there was no effect of the GOS-prebiotic on BAT temperature after 14 days. Finally, I examined the effect of EVOO by examining 58 participants, who were divided into two groups one consumed 25 ml of EVOO for 21 days and the other was a control group. There was no effect of EVOO on BAT temperature after 21 days.

In conclusion, adding potential thermogenic compounds to the diet appear to not affect BAT thermogenesis as determined by TI. Future studies should examine the effect of longer-term use of other dietary compounds, e.g. tea catechin or caffeine, and its impact on BAT thermogenesis. Cold challenges seem to be more effective in increasing BAT thermogenesis assessed with TI than diet. However, the sample size was too small to consider it significant. More studies are needed with more participants per group to demonstrate causality.

Declaration

The work in this thesis was performed within the Child Health, Obstetrics and Gynaecology Division, School of Medicine, University of Nottingham between October 2014 and October 2017

This thesis illustrates my work, completed under the supervision of Professor Helen Budge and Professor Michael Symonds This report is an accurate representation of the work performed and no other study reproducing this work, to my knowledge, has been carried out within the University of Nottingham.

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Abbreviations

AC	adenylate cyclase
AgRP	agouti-related protein
ARC	arcuate nucleus of the hypothalamus
AT	adipose tissue
AUC	area under the curve
BAT	brown adipose tissue
BIA	bioelectrical impedance
BMI	body mass index
BMP's	bone morphogenetic proteins
cAMP-PKA-HSL	camp-protein kinase A-hormone-sensitive-lipase
CoV	coefficient of variation
DEXA	dual energy- energy x-ray
DMH	Dorsomedial nucleus of the hypothalamus
DIT	diet-induced thermogenesis
ECBS	endocannabinoid system
EE	energy expenditure
EMR	electromagnetic radiation
EVOO	extra virgin olive oil
EFSA	European food safety authority
FDA	food and drug administration
F-FDG	f-18 fluorodeoxyglucose
FGF21	fibroblast growth factor
FLIR	forward Looking Infra-Red camera
FFA	free fatty acids
FFAR	Free fatty acid receptors
FFM	free fat mass
FPA	focal plane array

FWIR	far wave infrared
GI	gastrointestinal
GOS	galacto-oligosaccharides
IL-6	interleukin
ILSI	international life sciences institute
IR	infrared radiations
LDL	lipoprotein lipase
LHA	lateral hypothalamic area
LWIR	long wave infrared
MD	Mediterranean diet
NAE	N acylethanolamine
NIR	near infrared
MWIR	mid wave infrared
NE	noradrenaline
NIR	near infrared
Nrg4	nauregulin
NST	non-shivering thermogenesis
PEP	phosphoenolpyruvate
PGC1α	peroxisome proliferator activated receptor gamma coactivator 1 alpha
PET-CT	positron emission tomography
POA	preoptic area
PPG	optoelectrical plethysmograph device
PRDM16	PR domain containing 16
PVH	paraventricular nucleus of the hypothalamus
ROI	region of interest
SFCA	short chain fatty acids
SNS	sympathetic nervous system
SWIR	short wave infrared

TF	transcriptional factor
TI	Thermal imaging
TITCH	thermal imaging conversion hub
T_{REF}	reference temperature
T_{REL}	relative temperature
TRP	transient receptor channels
TRPA	transient receptor potential ankyrin 1
TRPV1	vanilloid 1
T_{SCR}	supraclavicular temperature
UCP1	uncoupling protein 1
VEGF	vascular endothelial growth factor
VMH	Ventromedial nucleus of the hypothalamus
VLDL	very low-density lipoproteins
VLWIR	very long wave infrared
WAT	white adipose tissue

1 Introduction

1.1 Brown Adipose tissue (BAT) concept

Adipose tissue (AT) is an organ located in different places within the body. The subcutaneous AT can be detected under the skin and comprises 80% of total body fat (Tsiloulis and Watt, 2015). Intra-abdominal AT includes the adipose tissue located around digestive organs and kidneys; together making up 20% of total body fat (Townsend and Scriven, 2014).

Mammals possess three different types of fat cells in the body; one is white adipose tissue (WAT) which is usually referred for its energy storage functions and its role in the inflammatory process like obesity (Trayhurn and Beattie, 2001). The other two types of fat cells regulate energy homeostasis; one being brown fat (BAT) and the other is beige or bright fat (Ikeda et al., 2018).

BAT specialises in energy expenditure (EE) through non-shivering thermogenesis and is an important regulatory tissue for core body temperature (Cannon and Nedergaard, 2004). BAT depots are located mainly in the supraclavicular region and around the kidney. BAT's main characteristics include the fact that its mitochondria possess a unique uncoupling protein one (UCP1) (Cannon and Nedergaard, 2004). On the other hand, beige adipocytes are located in discrete locations within WAT, and their mitochondria possess only 10% of the total amount of UCP1 in BAT. However, BAT and beige appearance are stimulated by environmental factors, like chronic cold acclimation or dietary influences (Harms and Seale, 2013). Table 1.1 shows the functional and developmental characteristics of BAT and beige fat.

Table 1. 1 Functional characteristic of thermogenic fat.

	Brown	Beige
Development	During embryogenesis	By environmental and endogenous stimuli
Location	- Dedicated depots (infants) - Relatively homogenous	-Adults (mice and humans) -Highly heterogeneous
Maintenance	Constitutive	Transient
Thermogenic mechanisms	UCP1 dependent	UCP1 independent

Distinct characteristics in the development and regulation of the two cell types: brown and beige (Ikeda et al., 2018).

1.1.2 BAT's thermogenic route

BAT's thermogenesis is activated with cold, sympathetic stimulation and dietary compounds (Bartness et al., 2010). To fully understand how BAT's thermogenesis is activated it is essential to start at the control centre of the process, the hypothalamus (Contreras et al., 2017).

The hypothalamus is comprised of neural populations which are scattered in different hypothalamic nuclei and interconnected to each other (Lechan RM, 2016). The neural populations which are involved in BAT thermogenesis are:

-The preoptic area (**POA**), is the principal nucleus which feels temperature and regulates the body temperature by the outside environment (Boulant, 2000). Once cold and prostaglandin E2 signals are detected by POA, this activates hypothalamic mechanisms to induce thermogenesis in BAT (Nakamura and Morrison, 2008).

-The arcuate nucleus of the hypothalamus (**ARC**), it contains a diverse population of neurons like agouti-related protein (AgRP) and neuropeptide Y (NPY). These neurons control feeding behaviour and energy expenditure (Waterson and Horvath, 2015). To give an example, the work of leptin in the ARC is necessary for the start of potential action of sympathetic nerves sub-serving BAT (Harlan et al., 2011).

-The dorsomedial nucleus of the hypothalamus (**DMH**), is implicated in the central control of thermogenesis in brown and beige adipose tissue throughout sympathetic transmission

(Morrison et al., 2014). It is obstructed under norm-thermic conditions by a GABAergic tone which is disinhibited by POA stimulation inducing thermogenesis (Osaka, 2008).

-The ventromedial nucleus of the hypothalamus (**VMH**), in this site, many peripheral signals connect in order to regulate BAT thermogenesis: thyroid hormones (THs) (López et al., 2010), leptin (Tanida et al., 2013), estradiol (E2) (Martínez de Morentin et al., 2014) and glucagon-like-peptide-1 (GLP1) (Beiroa et al., 2014). All these hormones are required to inhibit AMP-activated protein kinase (AMPK). The suppression of AMPK activates sympathetic fibres to BAT and WAT (van Dam et al., 2015, Martínez-Sánchez et al., 2017).

-Lateral hypothalamic area (**LHA**), it is involved in the thermogenesis of BAT and browning of WAT when neurons manifest orexins (OX) (Sellayah and Sikder, 2012).

-The paraventricular nucleus of the hypothalamus (**PVH**), its specific control of the thermogenic process is arguable. Different researchers have found that PVH indeed stimulates BAT thermogenesis (Amir, 1990). However, different researchers have found that inhibit (Madden and Morrison, 2009) or there is no effect on BAT thermogenesis (Holt et al., 1988).

Once the hormones and nutritional signals mentioned above integrate all the information that is needed to be transmitted, it reaches the brainstem nuclei (posterior part of the brain) (Morrison et al., 2014). In the brainstem nuclei, rRPa sympathetic premotor neurons integrate a wide number of signals from peripheral thermos-receptors, the hypothalamus and other sides of the brain which are involved in the regulation of body temperature to control sympathetic output which activates BAT (Nakamura, 2011).

BAT and WAT are both innervated by the sympathetic nervous system (SNS) (M et al., 2018). Once BAT is stimulated through the activation of transient receptor channels (TRP), which in return secretes catecholamine, specifically noradrenaline (NE). NE activates β3-adrenergic receptors and couples to a G-protein that activates adenylate cyclase (AC), in turn, AC catalyses the conversion of ATP into cAMP. This process leads the activation of protein kinase A (PKA) (Contreras et al., 2016).

PKA is the main inducer of lipolysis because it activates lipases like adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Duncan et al., 2007). These lipases hydrolyse triacylglycerol, diacylglycerol and monoacylglycerol into free fatty acids (FFA) (Puigserver et al., 1998). Subsequently, carnitine palmitoyltransferase 1a (CPT1a) inserts the FFAs into the mitochondria where they are oxidised through β-oxidation and reduced from NAD^+ and FAD to NADH and $FADH_2$ (Schönfeld and Wojtczak, 2012). These two components serve as combustible of the electron transport chain.

Oxidation takes the protons out of the matrix of the mitochondria, and over there, they are reintroduced by UCP1 and dissipate energy as heat (Saito, 2013) (Figure 1.1).

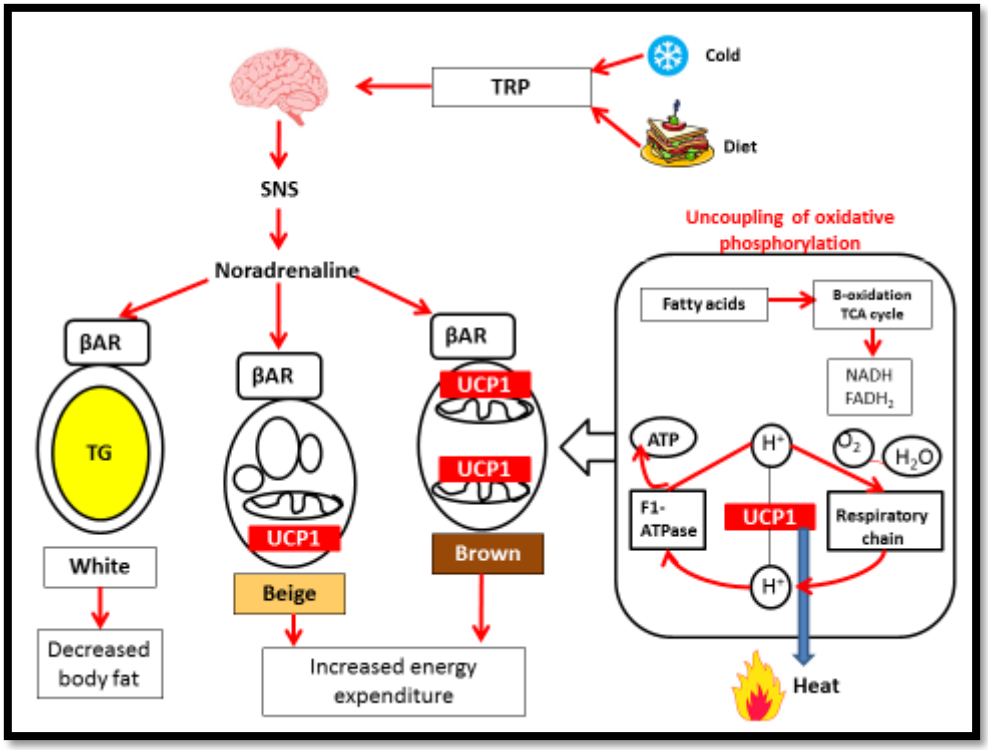


Figure 1. 1Physiological control of BAT thermogenesis.

Sympathetically activated thermogenesis in brown adipose tissue, lipid mobilisation from white adipose tissue and induction of beige cells (Saito, 2013)

1.1.3 Metabolic function of BAT

When BAT is maximally activated, it can contribute to up to 20% of the basal metabolic rate (Rothwell and Stock, 1983). It is estimated that cold-induced thermogenesis in BAT can burn, approximately, 25-400 kilocalories per day (Cypess et al., 2014) and has the potential to regulate long-term energy balance and body weight by converting excess food energy into thermal energy (Cypess et al., 2009, Cypess and Kahn, 2010a).

Brown adipocytes not only can produce heat but they also secrete hormones that influence metabolic functions; therefore this can have implications in the future for use as a therapeutic tool (Wang et al., 2015a).

Some of these factors include:

- **Neuregulin (Nrg4)** which is part of the epidermal growth factor family (EGF) of proteins. It is induced during the adipogenesis of brown adipocytes, and acute cold exposure stimulates its expression in BAT (Rosell et al., 2014).
- **Fibroblast growth factor (FGF21)** is produced by a range of tissues like liver, BAT and skeletal muscles (Hondares et al., 2011). In rodents exposed to prolonged cold, there is an increase of FGF21 mRNA expression in BAT (Stanford et al., 2013). The mechanism in which FGF21 regulates BAT thermogenesis is through the central nervous system (Owen et al., 2014).
- **Vascular endothelial growth factor (VEGF)** is expressed in BAT following cold exposure and mediated by the β-adrenergic pathway (Asano et al., 2001).
- **Bone morphogenetic proteins (BMP's)** are a family of extracellular signalling proteins that were initially involved in the regulation of bone and cartilage rehabilitation (Carreira et al., 2015). The BMP'S implicated in BAT functioning are BMP7 and BMP4; both promote the formation of brown adipocytes from skeletal muscle and WAT during development (Schulz et al., 2011).
- **Interleukin (IL-6)** is produced by the adipose tissue, and one of its main functions is to control the metabolism of glucose (Kristiansen and Mandrup-Poulsen, 2005).

When activated, IL-6 increases the browning of WAT and energy expenditure (Sanchez-Delgado et al., 2015).

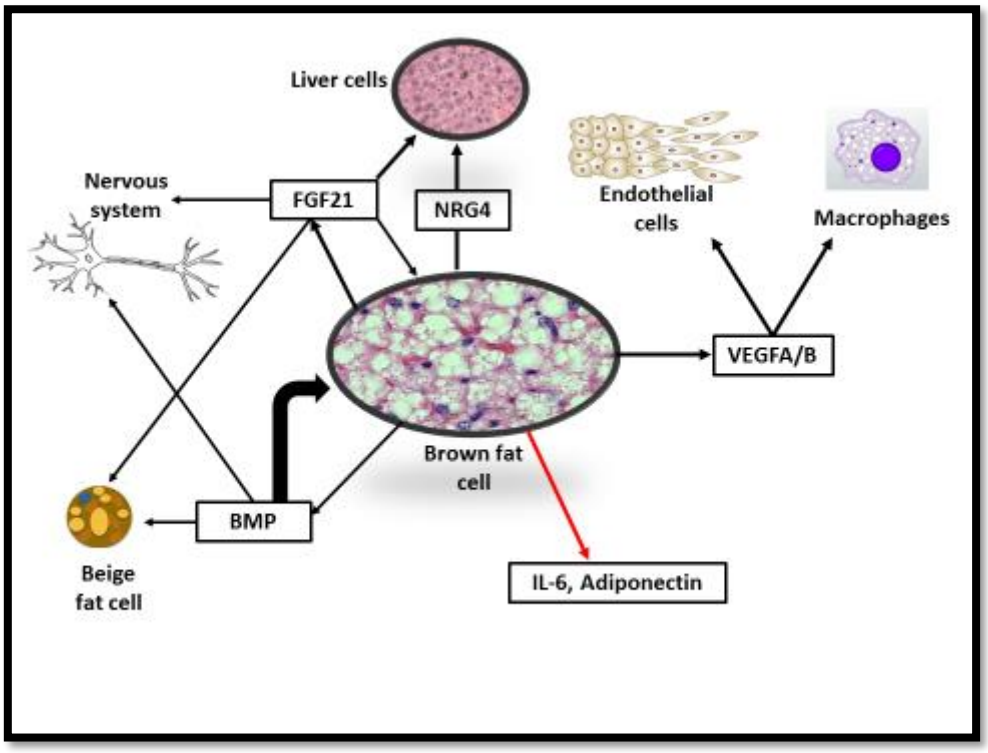


Figure 1. 2 Secreted factors delivered by BAT.

These factors can influence energy metabolism beyond BAT's thermogenesis (Wang et al., 2015a).

The effect of the thermogenesis of BAT in the control of obesity and other metabolic diseases such as insulin resistance, hyperlipidaemia, hyperglycaemia or hypertriglyceridemia relies on the capability of the thermogenic adipocytes to uptake lipids and glucose from the circulation (Bartelt et al., 2011a, Guerra et al., 2001, Gunawardana and Piston, 2012, Chen et al., 2013)

1.1.4 BAT's anatomical location and prevalence

Heaton (1972) first described the anatomical location of BAT in adults by a series of human autopsies, and it was found to vary between species and with age. In human neonates, BAT depots are located along the back, along the upper half of the spine, the shoulders and the supraclavicular fossa, where its main function is to ensure that the neonate remains warm (Dawkins, 1965). In adults, the region where BAT is most active, according to F-

fluorodeoxyglucose (FDG) tracer studies, is in the supraclavicular and neck regions and can extend towards the thorax and on visceral areas (Nedergaard et al., 2007).

BAT can also be found in the perivascular area, specifically around the aorta and carotid arteries. It also surrounds the epicardium around the heart and in the esophagus-tracheal groove, as well as in solid organs like kidneys, pancreas, liver and splenic hilum (Sacks and Symonds, 2013).

The prevalence of BAT varies considerably between individuals and decreases with age, as well as being more prevalent in adults with a normal body mass index (BMI) (18.5-24.9 Kg/m^2) (WHO, 2014) and lesser total body fat (Leitner et al., 2017). This finding is valid for both Caucasians (Bakker et al., 2014) and younger adults (van Marken Lichtenbelt et al., 2009b). Some different authors including Wang et al. (2015b), who studied 4011 participants (both female and male) aged 18-89 for BAT activity, showed that a decline in BAT quantities for elderly subjects as well as BAT positive subjects having lower BMI and less visceral and subcutaneous fat areas was noted.

Matsushita et al. (2014) studied 268 volunteers (males and females, between 20–72 years old) and assessed BAT activity with FDG tomography after two hours of cold exposure. They found that BAT activated by cold was detected in 48% of the subjects. When compared with subjects without detectable BAT, those with detectable BAT were younger and showed lower adiposity parameters such as BMI, body fat mass percentage and fat around the abdominal area. When BAT is stimulated by cold, the amount is lower in obese (Orava et al., 2013) and morbidly obese (Vijgen et al., 2011) individuals respectively, as compared to lean individuals.

Several studies have also suggested that the amount and activity of BAT are higher in women than in men (van den Beukel et al., 2015, Cypess et al., 2009, Pfannenberg et al., 2010) because females are more sensitive to cold than males.

BAT regulation is affected by different factors such as environmental temperature. It has been found that cold temperatures stimulate BAT thermogenesis more than warm

environments (Lee et al., 2014). It is also influenced by feeding status with BAT activity increasing after a meal (Scotney et al., 2017, Vosselman et al., 2013).

1.2 Imaging techniques for the analysis of BAT

1.2.1 Positron emission tomography (PET-CT)

Previous attempts to study BAT have relied on invasive or expensive techniques. The main methods that have been used include post-mortem studies (Kortelainen et al., 1993) and positron emission tomography-computed tomography (PET-CT) (Cypess et al., 2009, Santhanam et al., 2018).

Currently, PET-CT is considered the gold standard method for the detection of BAT. PET scan uses a small amount of an injected radioactive drug to show where cells are actively metabolising glucose in the body (Enerbäck, 2009). However, PET-CT also has some disadvantages: it is expensive, involves the use of ionising radiation and can only be used to detect metabolically activated BAT in the fasted state and tends to ignore smaller depots (Izzi-Engbeaya et al., 2014).

Therefore, imaging studies of this type have been limited by the number of participants studied since is not ethically acceptable to expose volunteers repeatedly or in large numbers to an amount of radiation when such investigations have not been clinically validated.

It has been proposed to use a Thermal Imaging camera (TI) as an alternative method to assess BAT thermogenesis due to its non-invasive, non-radioactive properties and infrared properties (Law et al., 2018b).

1.2.2 Electromagnetic radiation

To comprehend how a TI camera works, it is important to start by understanding what electromagnetic radiation is.

Electromagnetic radiation (EMR) is a type of energy spread through a material medium in the form of electromagnetic waves and possess two types of properties: wave and photon (Cloude, 1995). In figure 1.3 is possible to see an example of an electromagnetic wave.

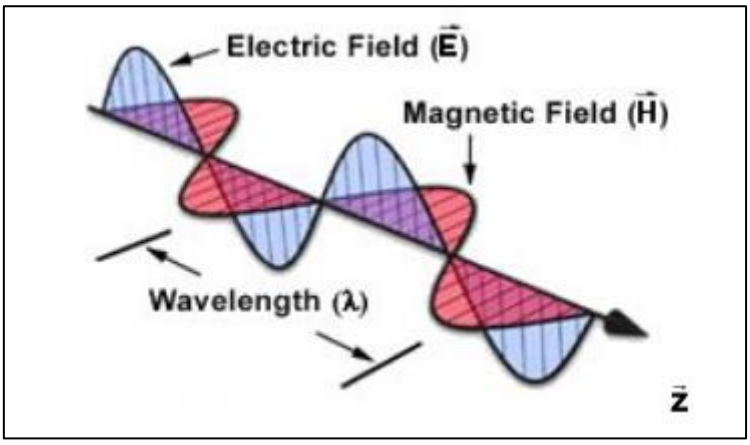


Figure 1. 3 Schematic representation of an electromagnetic wave.

The wave is propagating along the z-axis while the electric and magnetic field are propagating in the x-y plane (Prado-Gonjal, 2014).

The main characteristics of the waves are frequency, wavelength, speed and phase (Lloyd, 2013).

Frequency is defined as the number of waves per second that pass a given point in space (Avison, 2014).

Wavelength is the distance between two consecutive peaks in a wave (Reading, 1987).

Figure 1.4 shows a representation of a wavelength

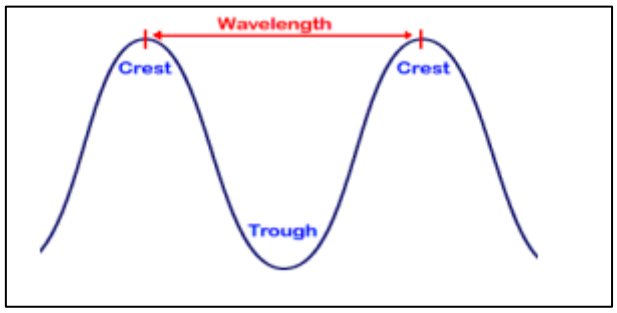


Figure 1. 4 Schematic representation of a wavelength.

A wavelength can be assessed between two points, either between crests or between troughs (Cassidy et al., 2002).

A **wavenumber** is the number of wave crests or wave troughs in a determined unit of length (Weisstein, 2007). Wavelength and wavenumber units are as follow:

Wavelength (and its type of radiation):

- Angstrom (A) : 1 A = 10^{-10} (X ray)
- Nanometre (nm): 1 nm=10^{-9} (ultraviolet, invisible)
- Micrometre (µm): 1 µm = 10^{-6}(infrared)
- Millimetre (mm): 1 mm = 10^{-3} (infrared)
- Centimetre (cm): 1cm = 10^{-2} (microwave)
- Meter (m): 1m = 1 (tv, radio).

Wavenumber: inverse length (often in cm^{-1}) (Cassidy et al., 2002).

The waves per second are assessed as **frequency units** (unit cycles per second 1/s (or s-1) is called hertz (abbreviated Hz) (Britannica, 2018). On Table 1.2 is possible to see the frequency units and their frequency (as cycles or seconds).

Table 1. 2 Frequency units (Dicke, 1982)

Unity	Frequency (cycles/sec)
Hertz, Hz	1
Kilohertz, KHz	10^3
Megahertz, MHz	10^6
Gigahertz, GHz	10^9

1.2.3 Spectrum of electromagnetic radiation

The spectrum of electromagnetic radiation is the distribution of electromagnetic radiation according to energy, wavelength or frequency (National Aeronautics and Space Administration, 2010). Infrared radiation (IR) lies between the visible and microwave portions of the electromagnetic spectrum as it can be seen in Figure 1.5

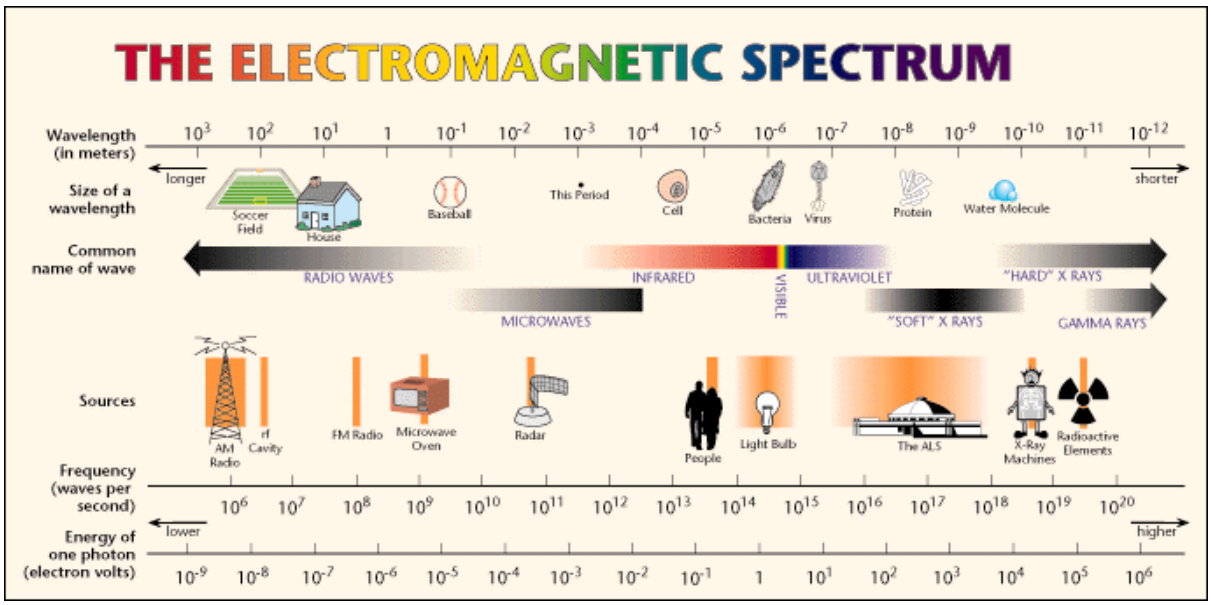


Figure 1. 5 The electromagnetic spectrum

The electromagnetic spectrum is composed of all forms of EMR, and each corresponds to a different section of the spectrum (National Aeronautics and Space Administration, 2010)

IR spectrum is divided into the following categories (Henneges et al., 2010):

•Near Infrared (NIR), 0.75 µm to 1.4 µ m

•Short Wave Infrared (SWIR), 1.4 µ m to 3 µ m

•Mid Wave Infrared (MWIR), three µm to 5 µm

•Long Wave Infrared (LWIR), eight µm to 12 µm

•Very Long Wave Infrared (VLWIR), 12 µm t 25 o µm

•Far Wave Infrared (FWIR), 25 µm to 1 mm

The primary source of IR is heat or thermal radiation, and this is what a TI camera measures.

1.2.4 Thermal Imaging (TI) Camera

A thermal imaging camera is a device that documents the intensity of radiation in the infrared part of the electromagnetic spectrum and transforms it into a visible image (Vollmer and Mallmann, 2017).

In 1800, Sir William Herschel discovered IR, but it was not until 1956 that the first Forward Looking Infra-Red camera (FLIR) was built at the University of Chicago (Meola, 2012).

When FLIR systems started, the resolution and thermal responsiveness were poor, so the quality of the images was not good (McLean, 2012). As the technology advanced and low-noise, high-resolution components were developed, the quality of the images subsequently improved.

The mechanisms in which FLIR works is illustrated in Figure 1.6

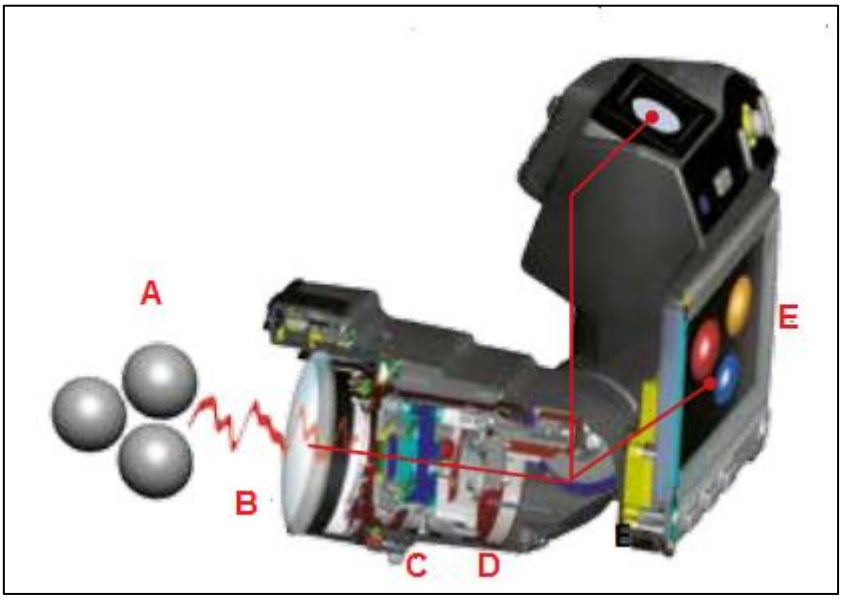


Figure 1. 6 FLIR's mechanism

Firstly (A) the infrared energy comes from an object and is focused by the optics (B) onto an IR detector (C). Immediately the detector sends the information to sensor electronics (D) for the processing of the image. Lastly, the electronics translate the information that comes from the detector into an image (E) (FLIR Systems, 2018).

Stefan–Boltzmann derives FLIR physical principles from the Total Radiation Law. This law implies that "an object reacts to incident radiation from its surroundings by absorbing and reflecting a portion of it, or allowing some of it to pass through (as through a lens)" (Siegel R, 1992).

The formula of the law can be simplified to give: $\mathbf{1 = \alpha + \rho + \tau}$

In which **α** stands for absorption, **ρ** stands for reflection and **τ** stands for transmission (Lloyd, 1975). Each coefficient can have a value from zero to one, depending upon how well an object reflects or transmits radiation. Human bodies, with normal temperature (36°-37°C), will radiate about 0.5 kW/m^2 (Ring and Ammer, 2012b). To obtain the temperature of an object

with FLIR, the equation must permit for its emissivity and include the influence of atmospheric temperature on it.

Emissivity is the proportion of energy that an object radiates to the exterior and is a number between zero and one (Collins, 2018). The more radiative properties an object has, the higher is its emissivity; the human skin emissivity is of 0.98 (Watmough and Oliver, 1968).

Atmospheric influence: IR enters FLIR from the object that is the subject of analysis as well as other objects nearby. FLIR is influenced by the environmental conditions when the measurements are taken; these conditions imply the background noise and the atmospheric temperature (Vollmer and Mallmann, 2017)**.** They are also influenced by humidity, higher humidity results in less IR reaching the sensors of the camera, and object distance, when the distance between the subject and the camera increases, there are fewer chances that the attenuation of IR is influenced by water vapour present; the ideal distance should be of less of two metres (Law et al., 2018a).

To reduce background noise, there are some methods that the researcher or the person in charge of the camera needs to follow:

1. There must be no sun shining on the back of the measured object, and there should be no direct sunlight that strikes FLIR.
2. Dust and vapour attenuate the infrared signal, therefore is recommended to do not allow these obstacles to interfere between the measured object and the FLIR.
3. All participants should be imaged at the same distance from the camera so that in every case, the image of the subject covers the same number of detector elements (FLIR Systems, 2018).

Temperature

FLIR lens receives radiation from three different origins:

- The target object
- Radiation from its surroundings
- Atmospheric temperature (FLIR Systems, 2018)

FLIR cameras require inputs of the emissivity of the object, atmospheric attenuation and temperature of the ambient surroundings. Once all the inputs are adjusted and then inserted in the camera, it is possible to use TI to obtain thermal measurements of different parts of the human body.

A typical FLIR assesses temperature with an accuracy of 2% when it is calibrated against a levelled source (Jones and Plassmann, 2002).

1.2.5 Types of IR cameras

A TI camera can be built either as a scanning device, which means that it can capture one image at a time or as a two-dimensional infrared focal plane array (IRFPA). On this last one, all images are reproduced at the same time with each detector element in the array (Gade and Moeslund, 2014).

Nowadays, IRFPA is the ruling technology as it is faster and has better resolution than scanning devices (Kaplan, 2007).

There are two types of detectors used in TI cameras: photon detectors or thermal detectors. Both detectors absorb EMR. However, photon detectors convert EMR into a change of the electronic energy distribution while thermal detectors convert EMR in thermal energy (Vollmer and Mallmann, 2017)

A thermal detector assesses radiation in the LWIR band and use different detector types like uncooled thermal detectors. These detectors have been composed mainly of two different types: ferroelectric detectors and microbolometers.

One of the main differences between both detectors is the material used in each one. Ferroelectric detectors use barium strontium titanate (BST) while microbolometers use vanadium oxide (VOx) and amorphous silicon (a-Si) (Gade and Moeslund, 2014). IR radiation modifies the electrical resistance of the material, which can be converted to electrical signals and processed into an image.

Microbolometers are more used in today's market due to its higher sensitivity and low noise, they also have a smaller pixel size on the detector, allowing a higher spatial resolution (Rogalski, 2011).

The applications of TI cameras are wide and include electrical, security, building diagnostics, science, maritime, transportation, law enforcement, firefighting and medical uses (FLIR Systems, 2018).

1.2.6 Medical applications of thermal imaging

Infrared cameras have been commonly used since 1990 to solve thermal patterns generated by skin vessels located in the surface of the body (Ring, 1990). However, most recently, there is an advancement to standardise protocols to assess thermogenesis with a TI camera for medical use. The International Standards Organization has published two documents, one in 2008 and which recounts the design and execution of the TI camera for screening (IEC, 2008) and one in 2009 which defines how the system must be tested and how to train its users (ISO/TR, 2009).

The guidelines for TI in medicine have incorporated the position of the patients to obtain the necessary images for medical thermography as well as data obtained from left and right sides of the body which have shown no significant difference at all (Ring and Ammer, 2012a).

The University of Glamorgan released the "reference atlas for clinical tomography" which began in 2001 and was published in 2008. This atlas contains a total of 24 positions and 90 regions of interest, and it has already made a difference on the correctness of the temperature measurements acquired from TI (Ammer, 2008).

The applications of TI cameras in medicine are wide. They can be used to assess pain, as pain pathologies are associated with an alteration of the thermal distribution of the body (Herry and Frize, 2004). They can also be used to determine the functionality of anti-allergy medicine (Hara et al., 2014).

Other medical uses of TI cameras include inflammatory arthritis (Bird et al., 1979), osteoarthritis (Varju et al., 2004), soft tissue rheumatism (Vecchio et al., 1992), peripheral

circulation (Ammer, 2009), fever screening (Chiu et al., 2005). TI cameras offer a bigger advantage of real-time two-dimensional temperature quantification as the measurements can be recorded in seconds (Ring and Ammer, 2012a) and can be used either as a diagnostic tool or as a result for clinical trials.

1.2.7 Thermal Imaging (TI) for the analysis of BAT thermogenesis

For the reasons mentioned above, it has been proposed to use FLIR to measure BAT activation, as it does not require the use of ionizing radiation, the process can be undertaken within minutes and, in general, is a more flexible tool for studying BAT function and activity (Jang et al., 2014b). Another advantage of using of FLIR, on assessing BAT thermogenesis, is that they can monitor changes in BAT activity over minutes or hours (Lee et al., 2011) and it can assess heat directly from the supraclavicular area of the subjects (Ang et al., 2017).

FLIR detect changes in temperature over the infrared range of the electromagnetic spectrum. Infrared radiation is emitted by objects at temperatures above zero, with an increased noted in the amount emitted as the object's temperature rises (Khanna and Branca, 2012). Several authors have shown positive results in the use of FLIR for the detection of BAT by using different types of participants i.e. children, healthy adults, adolescents with hypothyroidism (Symonds et al., 2012, Jang et al., 2014a, Kim et al., 2014, Nirengi et al., 2015), and it is currently in use in a range of studies at the University of Nottingham (Symonds et al., 2012, Robinson et al., 2014, Scotney et al., 2017).

To determinate if FLIR can detect BAT in real time in comparison to the gold standard PET-CT, a study was performed by Law et al. (2018b) that compared FLIR and PET-CT data from 8 healthy male volunteers. The study aimed to determine the regions of interest (ROI's) in the supraclavicular area where BAT is mainly located, using FLIR and finding out if it similarly corresponds to the ROI's of maximal glucose uptake within PET-CT. When the images of both techniques were overlapped, the hotspots noticed were located in the same ROI, i.e. supraclavicular area, specifically when the participants were stimulated with a cool challenge.

The researchers analysed the hottest ten per cent of points within each ROI and then calculated the medians of these points.

Although the benefits of FLIR overshadow the disadvantages, one of its major problems is that they only detect BAT located superficially such as in the supraclavicular fossa, although this is the major BAT depot in adult humans. Another disadvantage is that FLIR mostly detect BAT thermogenesis only in normal body weighted subjects as it can be more easily detected than in subjects with thicker supraclavicular skin (Gatidis et al., 2016).

A disadvantage of both imaging techniques to detect BAT is that they cannot classify the two BAT cells (brown and beige) in healthy human volunteers, the only possible way to do this is by using biopsy material (van der Lans et al., 2014).

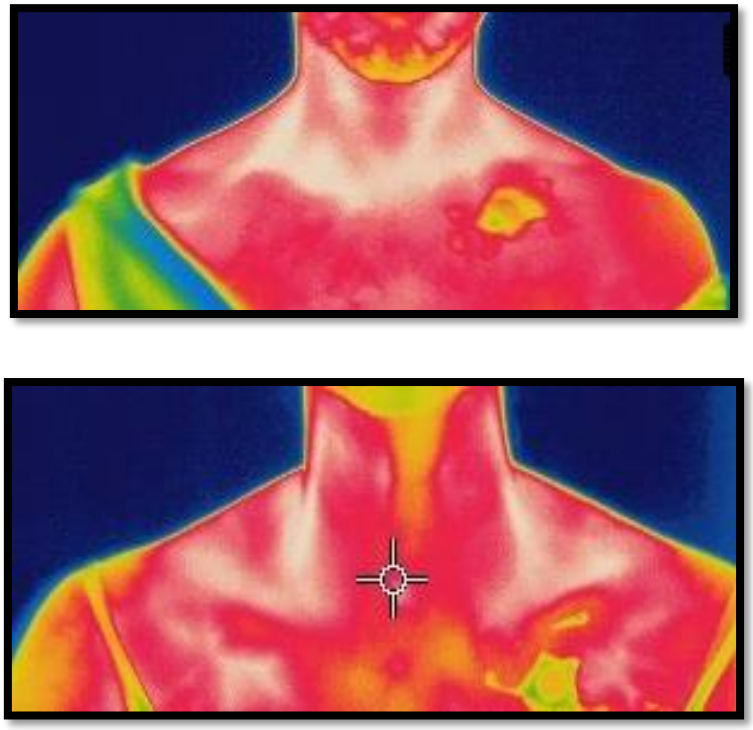


Figure 1. 7 Thermal images of the supraclavicular area of the body of two healthy adult volunteers.

Images obtained with FLIR camera E-60.

1. 3. Environmental and dietary activators of BAT thermogenesis

1.3.1 Cold as a stimulating factor of BAT thermogenesis

Cold is the most widely used activator of BAT in humans (Harms and Seale, 2013) and has been adopted in a wide range of studies both animal and human (Wang, 2009, van Marken Lichtenbelt et al., 2009a, Barbatelli et al., 2010, Lee and Gallagher, 2008, Lim et al., 2012, Robinson et al., 2014).

Cold exposure for several hours (acute cold) activates existing UCP1, whereas long-term cold exposure for several weeks (cold acclimation) induces the recruitment of new brown adipocytes leading to hyperplasia in the tissue as well as hypertrophy (Cameron and Smith, 1964). Furthermore, chronic cold exposure induces beige cells in white fat so that there is an increase in body energy expenditure and potentially, reduction in body fat (Nedergaard et al., 2007).

BAT thermogenesis is activated by cold temperature through the triggering of transient receptor channels (TRP), these receptors are V1, M8 and A1 (Saito et al., 2009). V1 is a calcium channel located on sensory neurons all around the body (Holzer, 2011). M8, mostly know as cold and menthol receptor 1, is the number one molecular converter of cold sensations in humans (Olsen et al., 2014). A1 is a sensor for environmental irritations like cold (Hojland et al., 2015). These receptors navigate through the sympathetic nervous system (SNS), which in return, secretes catecholamines such as NE in this case. NE activates β3-adrenergic receptors and initiates a cascade signalling for triglycerides hydrolysis (Puigserver et al., 1998). Once the fatty acids are released, they activate UCP1 and work as an energy origin for thermogenesis. This lipolysis is fundamental for cold-induced non-shivering thermogenesis (NST) (Cannon and Nedergaard, 2004). The activation of UCP1 uncouples oxidative phosphorylation from ATP synthesis and dissipates energy as heat (Saito, 2013)

The effect of cold exposure on increasing the BAT activity can have a direct benefit on the glucose and lipid metabolism, and therefore, have a therapeutic effect on metabolic disorders related to obesity like diabetes, dyslipidaemia and stroke (Cypess and Kahn, 2010b). The

evidence of this effect can be seen in animal models when they are exposed to cold; they increase the rate of plasma clearance of triglycerides because of the increased uptake into BAT (Bartelt et al., 2011a). This process depends on the action of lipoprotein lipase (LPL), which is located at the endothelial cell surface and is the major source of free fatty acids (FFA) in BAT (Bartelt et al., 2012). After a regular meal, lipids in the diet are carried out by chylomicrons and very low-density lipoproteins (VLDL) through the lymphatic vessels into the bloodstream. Once triglycerides get into the bloodstream, LPL hydrolyses them into FFA for BAT uptake. Subsequently, hyperlipidaemia is mitigated, and an improvement in the effects of insulin resistance is noted (Festuccia et al., 2009). When exposed to cold, FFA uptake is higher in BAT than in skeletal muscle (Bartelt et al., 2011a).

1.3.1.1 Cold exposure variability

Several authors have found variability in non-shivering thermogenesis and BAT activity in different individuals, Vijgen et al. (2011) found that obese subjects have a delayed onset of shivering. In a later study, the same author found that when obese subjects lose weight, there is an increase in BAT activity (Vijgen et al., 2012). The ambient temperature also plays a significant role in the thermogenesis of BAT in adult humans. Huttunen et al. (1981) described a greater presence of BAT adipocytes on the neck region of Finnish outdoor workers compared with indoor workers. Additionally, the cold exposure of human subjects increased their BAT depots and EE when assessed in winter compared with summer (Yoneshiro et al., 2016). This variation indicates the change in the role of BAT with seasonal alterations leading to a thermogenic response to cold exposure.

The cold temperature protocols used to detect the thermogenesis of BAT in humans vary considerably according to the literature. Some authors used acclimatised rooms that range in temperature from 18 to 22°C (Romu et al., 2016). Others used water perfused body suits (Iwen et al., 2017, Law et al., 2018) while the immersion of limbs in cool water is also another prevalent technique (Romu et al., 2016, Robinson et al., 2014).

When BAT thermogenesis is assessed, using cold temperatures as stimulation of thermogenesis, it is important to distinguish between shivering thermogenesis (ST) and non-shivering thermogenesis (NST). Involuntary muscle contractions cause ST while on the other hand, NST is the extra heat produced by BAT (Cannon and Nedergaard, 2004). NST is defined by Cannon and Nedergaard (2004) as “the thermogenesis that replaces shivering and is UCP1 dependant”. Ideally, human BAT metabolism should be studied in the absence of shivering, although it is difficult to measure and even some acclimation protocols are insufficient to abolish shivering response (Blondin et al., 2015). van der Lans et al. (2014) recommend doing a combination of asking subjects, visual inspection and electromyography to check the absence of shivering while applying a cooling protocol when NST is studied.

1.3.2 Dietary regulation of BAT

Besides cold, BAT thermogenesis can be induced by different factors like diet. Diet-induced thermogenesis (DIT) is defined as the increase in energy expenditure (EE) that follows food intake (Rothwell and Stock, 1983).

DIT constitutes 10% of the energy content of the food consumed during the day (Westerterp, 2004) and consists of the digestion and absorption of nutrients (Bonet et al., 2017).

Rothwell and Stock (Rothwell and Stock, 1997) were the first researchers to discovering that BAT might be associated with DIT. During the author’s study, they put rats in cages and fed them with a “cafeteria diet”. This diet was high in fats and carbohydrates (palatable food). They observed that the rodents that consumed the diet had an increase in plasma noradrenaline and oxygen consumption and more BAT in the interscapular region and the abdomen directly over the liver. The effect of the diet lasted after the cafeteria diet was stopped and the DIT effect was more prominent in the male animals.

The same effect has been seen in studies with humans (Hibi et al., 2016b), where DIT and fat utilisation was higher in BAT positive subjects after the consumption of a high caloric diet.

However, not all the studies in humans have shown similar results. Schlogl et al. (2013) performed a study in 16 healthy adult volunteers and overfed them with a diet that consisted of 50% of carbohydrates, 30% of fat and 20% of protein. They identified BAT deposits after three different times: after 24 hours of overfeeding and at four and 12 hours after the last meal. The researchers discovered that, after the assessment with PET-CT, there was no difference in BAT activity after the overfeeding in the three occasions when measured during a state of fasting.

On the contrary, apart from the high caloric diets that can increase DIT, macro and micronutrients also can do it thanks to their individual properties; although the latter's notion is not generally accepted and remains controversial (Symonds et al., 2015).

Some compounds have thermogenic potential, although the type of evidence collected thus far has been proven predominantly for animals (Table 1.3).

Table 1. 3 Functional compounds of foods and their proposed action on BAT thermogenesis.

Functional compound	The proposed mechanism of action	Reference
Skin of grapes (Resveratrol)	AMP-activated protein kinase (AMPK)	Wang et al. (2015b)
Olive (oleuropein)	Sympathetic Nervous System	Oi-Kano et al. (2008a), Oi-Kano et al. (2007a)
Fish oil (EPA, DHA)	Sympathetic Nervous System, UCP1 proton conductance, PPARs ligands	Oi-Kano et al. (2007a), Sadurskis et al. (1995)
Hot pepper: capsiate	Sympathetic Nervous System	Masuda et al. (2003)
Coffee (caffeine)	Sympathetic Nervous System	Kogure et al. (2002)
Fatty acids	UCP1 proton conductance	Ježek and Freisleben (1994)
Sweeteners: sucrose and saccharin	Sympathetic Nervous System	Kawada et al. (1991)
Dietary fibre	cAMP-PKA-HSL pathway	Han et al. (2017)

The study with dietary fibre aimed to evaluate the effects of high fibre cereal on the levels of lipolysis and thermogenesis in WAT and BAT. The researchers used mice for this experiment (12 animals per group) and divided them into four different groups: chow group, high-fat diet

group, high-fat diet group plus supplementation with oat and a high-fat diet supplemented with wheat bran fibre. The supplementation with oat and wheat was 0.8% of the total diet. As a result, both dietary fibres increased lipolysis and browning process. However, oat was more effective at increasing protein expressions of UCP-1 and PGC-1α than the wheat group in WAT. On the contrary, the wheat group was more effective in the protein expressions of the cAMP-PKA-HSL pathway in BAT. Both fibres contributed to the development of BAT.

1.3.2.1 Gut Microbiota

The gut microbiota has been described as the “forgotten organ” and contains an estimated amount of 10^{14} cells; 10 times more than the total number of human cells (Qin et al., 2010). Each person possesses a unique microbiota which is affected by many factors like diet, exercise, weight, health status, drugs usage, geographical location, sex, stress, and age (Harvie et al., 2017). However, the diet is believed the factor with the most significant impact on the microbiota (Xu and Knight, 2015) (Figure 1.8).

Gut microbiota contains approximately one and a half kg of bacteria residing in the human gut and 50% of human faecal matter being composed of bacterial cells (Sekirov et al., 2010) and it is favourable for bacterial growth due to factors like the availability of nutrients and the pH (Cummings and Macfarlane, 1991). Due to its bacterial population, the microbiota can have an impact on metabolic homeostasis and contribute to human health (Al-Assal et al., 2018)

Microbiota is affected by selected substrates such as antibiotics, minerals, vitamins and bacteriophages (Gibson et al., 2017). To maintain such a high population density of bacteria, these bacteria need nutrients which can only come from food sources such as prebiotics (Annalisa et al., 2014).

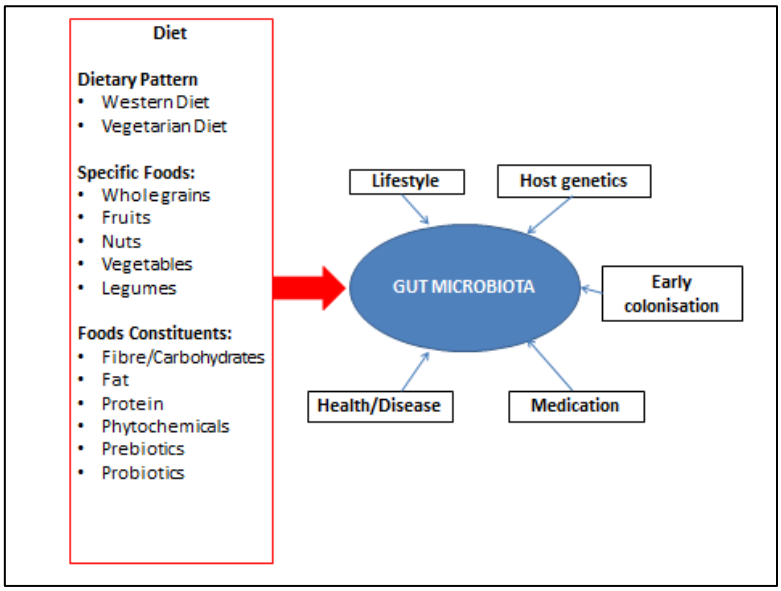


Figure 1. 8 Factors which influence the composition of the human gut microbiota, with a special focus on the diet (Geurts, Neyrinck et al. 2014).

Other factors which influence gut microbiota, and are not related to the diet, are genetics, the use of medications, the lifestyle of each person and even the method of delivery (C-section vs natural birth).

1.3.2.2 Prebiotics

Prebiotics are non-digestible compounds that, through its metabolization by microorganisms in the gut, modulate the composition and activity of the gut microbiota, thus conferring a beneficial physiologic effect on the host (Bindels et al., 2015)

The International Life Sciences Institute (ILSI) described them as “the product which has beneficial effects on one or more specific functions on the body, beyond the nutritional effects, this being relevant to improving the health and wellbeing and reducing the risk of disease”(ILSI, 2013). According to a recent consensus, prebiotics can help on the control of blood lipids, insulin resistance and even mental health (Gibson et al., 2017b).

Any dietary component that reaches the colon intact is a potential prebiotic, but to be classified as such, it must also (Gibson and Roberfroid, 1995, Wang, 2009):

1. be indigestible; this means being able to resist host digestion (i.e. gastric acidity),
2. be fermented by the microbiota, colonising the gastrointestinal system,
3. selectively stimulate the growth and activity of specific bacteria within the gastrointestinal system,
4. provide a beneficial effect on the host’s health and

5. be stable in various food processing conditions.

There are different types of prebiotics, for many years only fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin were considered prebiotics. However, new products have been shown enough evidence to be considered prebiotics (Carlson et al., 2018). These products are:

Beta-glucans, these compounds are located in the endosperm of grains like oats and barley (Cloetens et al., 2012).

FOS, oligofructose and inulin are oligosaccharides composed of linear chains of fructose units and reach the colon intact. In the colon, they are metabolised by the gut microbiota to produce short-chain carboxylic acids, L -lactate, CO_2, hydrogen and other metabolites (Sabater-Molina et al., 2009).

Isomaltooligosaccharides, are glucose oligomers with α-D-(1,6)-linkages (Crittenden and Playne, 1996). They are present in foods like rice miso, soy and sake (Tokuoka et al., 2017).

Guar gum, it is a polysaccharide removed from guar gum and which is commonly used in dairy products, bakery or cereals (Mudgil et al., 2014).

Resistant starch, there are many types of these and can be either synthetic or naturally present in foods. However, one thing in common is that all of them escape digestion in the upper gastrointestinal tract (Sharma et al., 2008).

Xylooligosaccharides and arabinooligosaccharides, their composition is made of an estimated amount of two and ten xylose monomers linked with β 1,4 bonds with DP≤ 20 (Makelainen et al., 2010). They are present in dairy products, sports drinks and isotonic beverages (Vázquez et al., 2000).

Galacto-oligosaccharides (GOS), are non-digestible carbohydrates that are derived from milk and consist of chains of galactose monomers (Roberfroid, 2005). As a food ingredient, GOS has been commercially available since the 1990s (Akiyama K, 2001). In Europe, the European Food Safety Authority (EFSA) has authorised three health claims on GOS for the

knowledge of the consumers: “maintains a healthy digestive system”, “prebiotic/bifidogenic” and “calcium absorption” (EFSA, 2010).

One of the main positive attributes of GOS is the ability to change gut microbiota after a short period (seven days) and at low levels of consumption (two to five g/day) (Bhatia et al., 2015, Li et al., 2017a). They are not toxic, and the only known adverse effect is diarrhoea when GOS are consumed in excess. At an estimated dose of 0.3 g/kg to 0.4 g/kg of body weight (T. Sako, 1999), for a 68 kg adult, this equates to ~20.4 g. GOS reach the small intestine intact because of their chemical structure. This action occurs as well because the small intestine villi do not provide enzymes that are required to split and hydrolyse them (Macfarlane GT, 2011).

A recent systematic review and meta-analysis demonstrated that the consumption of fructans and GOS led to a bigger abundance of *Bifidobacterium* and *Lactobacillus* compared with a placebo. In the same article, the researchers found no difference in SCFA concentrations in either prebiotics or dietary fibre (So et al., 2018).

1.3.2.3 Mechanism of action of prebiotics

The mechanism of action of the prebiotics start when they reach the colon, unaltered, and in there they are fermented by bacteria (*Bifidobacteria* and *Lactobacillus*) to disintegrate substrates (Markowiak and Śliżewska, 2017).

Lactobacillus contains over 180 species and has been shown to be protective against pathogen infections (Hirano et al., 2003). They are not only present in the microbiota but also the rectal, vaginal and oral mucosa (Petricevic et al., 2012).

Bifidobacteria is believed to play a key role in human health, they are gram-positive and non-spore forming microorganisms (Pokusaeva et al., 2011). The bacteria deteriorates polymeric carbohydrates into low molecular weight oligosaccharides, which can, later on, be degraded to monosaccharides (de Vrese and Schrezenmeir, 2008).

These monosaccharides are converted to intermediates of the hexose fermentation pathway (or “bifid shunt”) and lastly converted into short chain fatty acids (SCFA) through the enzyme fructose-6-phosphoketolase (de Vries and Stouthamer, 1967).

SCFAs are available by about 36% (acetate), 9% (propionate) and 2% (butyrate) in the host respectively (Simpson and Campbell, 2015). They affect the composition of the gut microbiota and change its metabolic function (Van Loo et al., 2005) by regulating lipid metabolism, increasing the absorption of calcium, modifying the bowel function and even having an effect on the immunological system (Carlson et al., 2018).

As each prebiotic has a different structure, this determines their effects in the physiology of the host and the type of microorganisms that can use them as an origin of energy in the bowel (Macfarlane et al., 2008). Figure 1.9 shows the different types of prebiotics and its effects on health through SCFAs production.

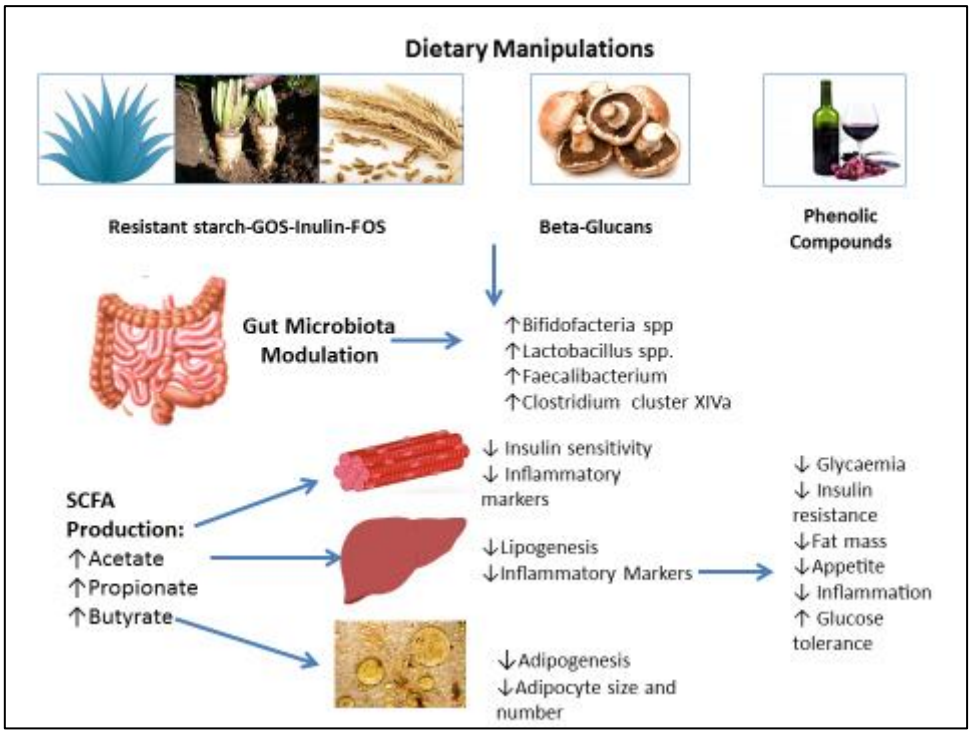


Figure 1. 9 Effect of non-digestible nutrients with prebiotic properties on host pathophysiology (Geurts et al., 2014).

The consumption of prebiotics shifts the composition of the gut microbiota by conferring health benefits to the host.

1.3.2.4 Short Chain Fatty Acids

SCFA are saturated aliphatic organic acids, and the most abundant types are acetate, propionate and butyrate (Cook 1998). Its measurement *in vivo* is not possible; the only way to measure SCFA concentrations is in faeces and *in vitro* studies. Faecal SCFAs do not reflect their total concentration as most of the SCFAs are taken by the host (den Besten et al., 2013). The faecal content of SCFA varies depending on the consumption of fibre by the host. When high amounts of fibre are consumed (>30 grams of fibre per day), the SCFA range is of 10-30 mmol/day when compared to control diets (5-15 mmol/day) (Jenkins et al., 1998).

There are some vital factors which influence the production of SCFA:

1. The type of fibre: pectin produces acetate, while guar gum propionate and resistant starch butyrate (Henningsson et al., 2002, Metzler-Zebeli et al., 2018).
2. The structure and function of the gut microbiota: as mentioned before, there are a diverse series of factors that can affect the gut microbiota. However, one of the most important factors is the use of drugs that can slow the colonic transit like loperamide and cisapride; this drugs can alter the production of SCFA (El Oufir et al., 1996)
3. Colonic pH: butyrate and acetate are produced at different colonic pH, 5.5 and 6.5 respectively (Walker et al., 2005).

Butyrate is considered the most important SCFA because it is the main nutritional provider of the colonocyte and its production may have an impact role in satiety and adipose tissue remodelling (Denise Robertson, 2007).

Acetate is the principal SCFA found in the circulation, and consequently, is the primary inducer to a significant metabolic modulation in peripheral tissues, resulting in decreased fatty acid accumulation (Kondo et al., 2009). Propionate has the same anti-inflammatory effect as acetate in humans and is an immune cell regulator (Cox et al., 2009).

The microorganisms in the gut benefit the host by utilising energy from the fermentation of undigested carbohydrates and the subsequent absorption of SCFAs. Once SCFAs are

fermented, they produced the equivalent of ~10% of the human caloric requirements (Bergman, 1990) (Figure 1.10).

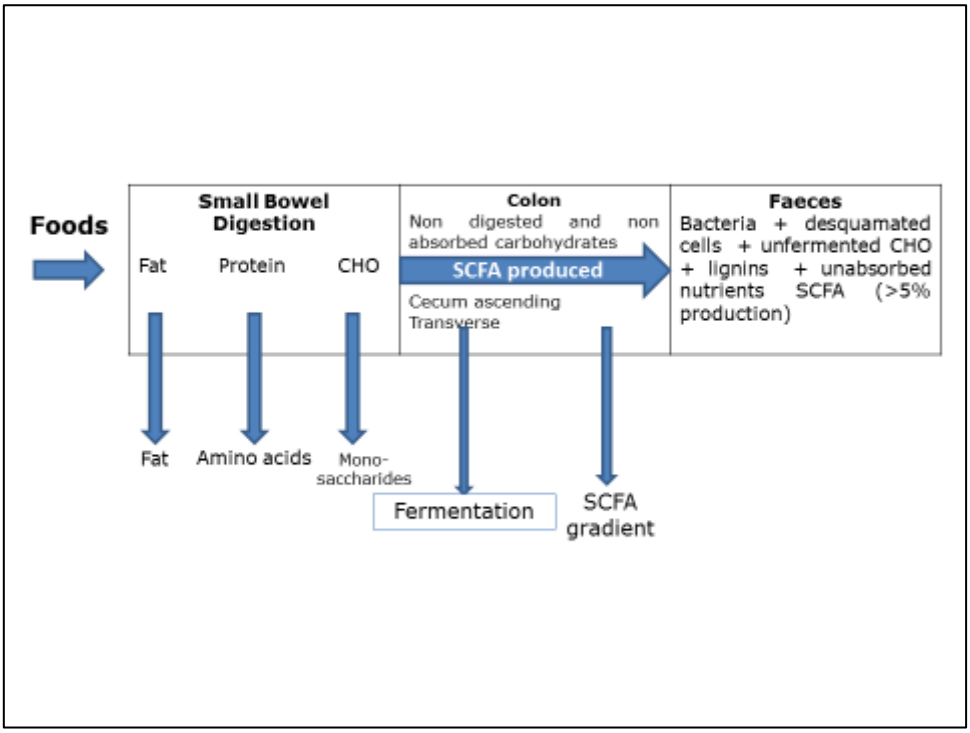


Figure 1. 10 The overall scheme of nutrient processing and transit through the gastrointestinal tract and excretion.

SCFAs are obtained predominantly from fermentable fibres. However, they can also be obtained from fasting conditions and after consumption of alcohol (Topping and Clifton 2001).

1.3.2.5 Metabolism and excretion of short chain fatty acids.

Once SCFAs are produced in the colon, they evacuate through the gut lumen into the portal vein. Then, portal venous SCFAs pass to the liver where SCFAs enter into a different set of pathways; this process depends entirely on the energy levels of the host (Morrison and Preston, 2016). In the liver, most of the SCFAs are metabolised, and only the acetate has a significant concentration in the peripheral circulation (Frost et al., 2014).

Acetate can be formed by hydrolysis of acetyl-CoA or from CO_2 by the Wood-Ljungdahl pathway (Ragsdale and Pierce, 2008). In the Wood-Ljungdahl pathway, CO_2 is diminished to CO and transformed with a methyl group and CoASH to acetyl-CoA (Figure 1.11a) (Pryde et al., 2002).

Propionate can be formed through the primitive electron transfer chain using PEP (Phosphoenolpyruvate) or when there is a depletion of lactate to propionate (the acrylate pathway) (Figure 1.11b) (Miller and Wolin, 1996).

The formation of butyrate initiates from the condensation of two molecules of acetyl-CoA and subsequent reduction to butyryl-CoA (Figure 1.12) (Duncan et al., 2004).

As acetate and butyrate can be converted to acetyl-CoA, thus, these SCFAs can be used to develop lipids and ketone bodies (Henning and Hird, 1972). SCFAs can also enter the citric acid cycle and be used to produce glucose through gluconeogenesis (den Besten et al., 2013a).

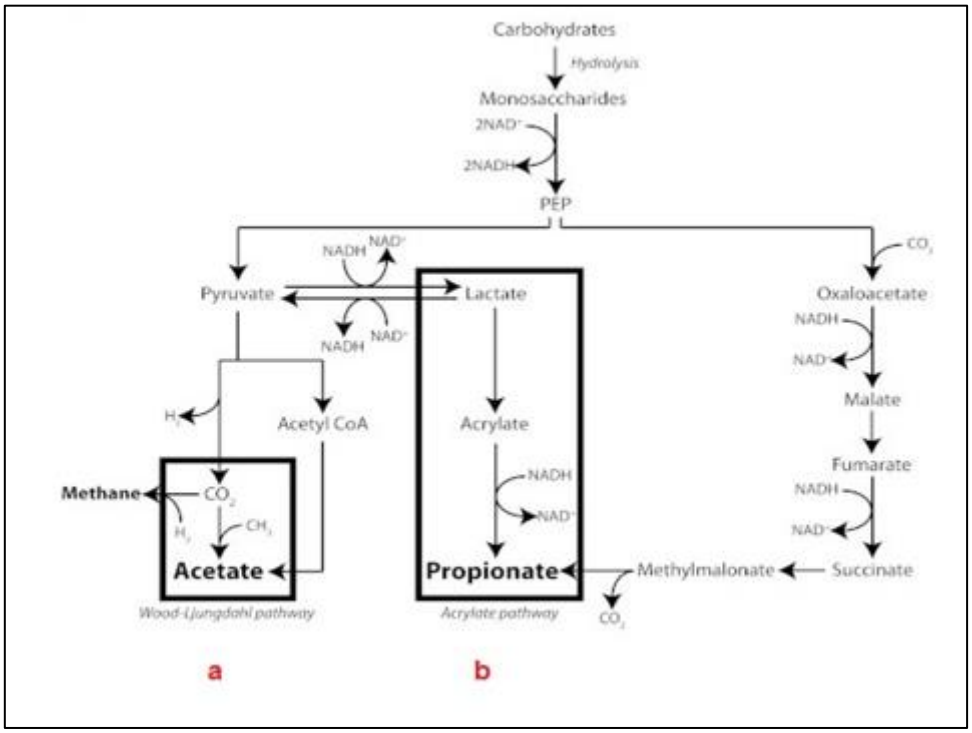


Figure 1. 11 Overview of the production of acetate and propionate from carbohydrates (den Besten et al., 2013b)

The microbial transformation of dietary fibres/prebiotics in the gut affects the production of SCFAs. a) acetate and b) propionate.

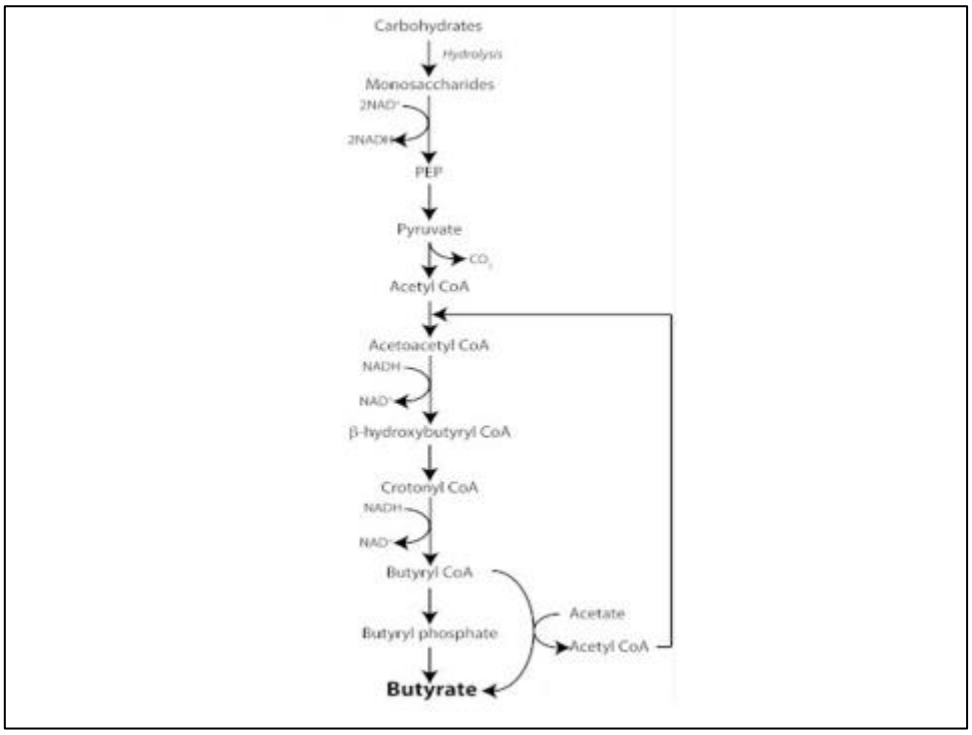


Figure 1. 12 Overview of the production of butyrate from carbohydrates (den Besten et al., 2013b)

Another characteristic of SCFAs is that they can modulate extracellular activity through FFA receptors (FFARs)

1.3.2.6 FFAR2 and FFAR3 receptors

FFARs represent the largest family of cell surface receptors in eukaryotes (animals, plants, fungi and protozoa), and can be estimated to comprise approximately 1000 distinct gene products or three per cent of the human genome (Kuaushansky, 2010). FFARs consist of a single polypeptide that is folded into a globular shape and embedded into a cell's plasma membrane (Trzaskowski et al., 2012). They are also called 7TM receptors because of seven segments of this molecule span the entire width of the membrane and the intervening portions loop both inside and outside the cell. Consequently, the extracellular loops form part of the pockets where signalling molecules bind to the FFARs (Iismaa and Shine, 1992).

FFARs interact with G proteins in the plasma membrane. When an external signalling molecule binds to an FFAR, it causes a conformational change in the GPCR (Dorsam and Gutkind, 2007); and the presence of FFARs in the adipose tissue suggests that this may be a

target for metabolites produced by the gut microbiota (Lin et al., 2012). $FFAR_3$ is activated by propionate and butyrate, which in turn activates the SNS, increasing energy expenditure and energy utilisation (Figure 1.13).

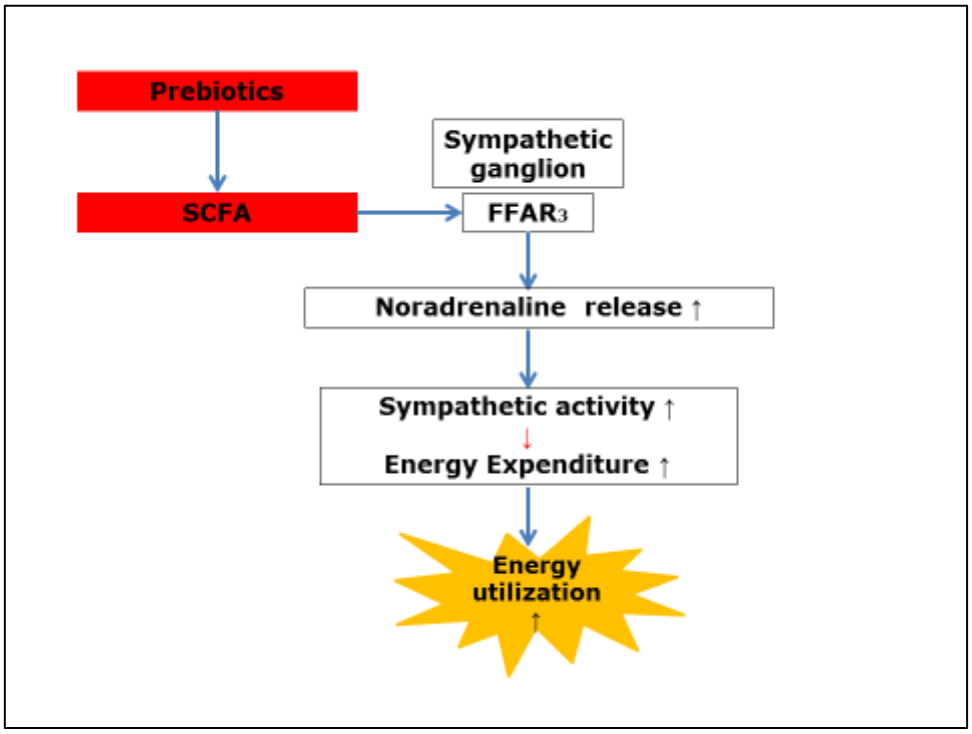


Figure 1. 13 Effects of SCFA in energy utilisation mediated by FFAR3 (Al-Lahham, Roelofsen et al. 2010).

SCFAs increase energy utilisation by activating the sympathetic nervous system by stimulating $FFAR_3$ in the sympathetic ganglion, leading to an increase in energy expenditure and utilisation.

However, when there is inhibition of $FFAR_3$ function, this leads to lower energy expenditure and ultimately obesity (Bellahcene et al., 2013). The $FFAR_2$ receptor is also required for the control of energy expenditure, Hu et al. (2016b) fed mice with acetate (150 mM) for six weeks and discovered that $FFAR_2$ mRNA expression was raised in BAT as was UCP1, PPARγ and PGC-1α gene expressions, respectively. They concluded that SCFA could act as regulators of adipogenesis in brown adipocytes development. Nevertheless, apart acetate which has the capability of increasing the amount of BAT, butyrate has also the capability to increase energy expenditure and therefore BAT thermogenesis in animal models by increasing PGC-1α and UCP1 (Gao et al., 2009). In one study, mice were fed with a diet composed of 58% of fat and mixed with 5% of sodium acetate. There was an increase in

rectal temperature after 60 minutes when animals were fed with the high-fat diet and the acetate. There was also an increase in UCP1 mRNA when animals were exposed to the cold environment (room temperature of 4°C for 90 minutes).

As supplementation with prebiotics increases the expression of both $FFAR_2$ and $FFAR_3$, some authors have hypothesised that this effect occurs through the modulation of gut microbiota and SCFA production (Bindels et al., 2013, Dewulf et al., 2011). Another hypothesis that has been proposed to explain the effect of short-chain fatty acids on BAT thermogenesis are transcription factors.

1.3.2.7 BAT's transcription factors

Adipogenesis is the process in which preadipocytes become adipocytes (Sarjeant and Stephens, 2012). BAT is regulated by several transcription factors like PPAR-γ and CCAT/enhancer-binding proteins (Hu et al., 2016a). PPAR-γ is the main regulator of adipocyte differentiation, and one of its main functions is the activation of UCP1 (Sears et al., 1996).

On the other hand, PPAR-γ coactivator-1α (PGC-1α) is high in BAT but not in WAT, and the shifts in nutritional status can regulate its expression (Fisher et al., 2012). Different authors have found that the consumption of acetate (Hu et al., 2016a) and butyrate (Gao et al., 2009) activates PGC-1α mRNA expression and these SCFA's act as regulators of brown adipocyte's expansion and differentiation.

1.3.3 Extra Virgin Olive Oil (EVOO)

1.3.3.1 The Mediterranean Diet

The Mediterranean diet (MD) is the type of diet consumed in countries that border the Mediterranean Sea; which include France, Greece, Italy and Spain. These countries have an average consumption of 15 kg per year of olive oil; while areas outside the Mediterranean area, the consumption is much lower (Visioli et al., 2000). The MD includes a high consumption of vegetables, fruits, legumes, grains, fish and unsaturated fats like extra virgin olive oil (EVOO) (Estruch, 2010).

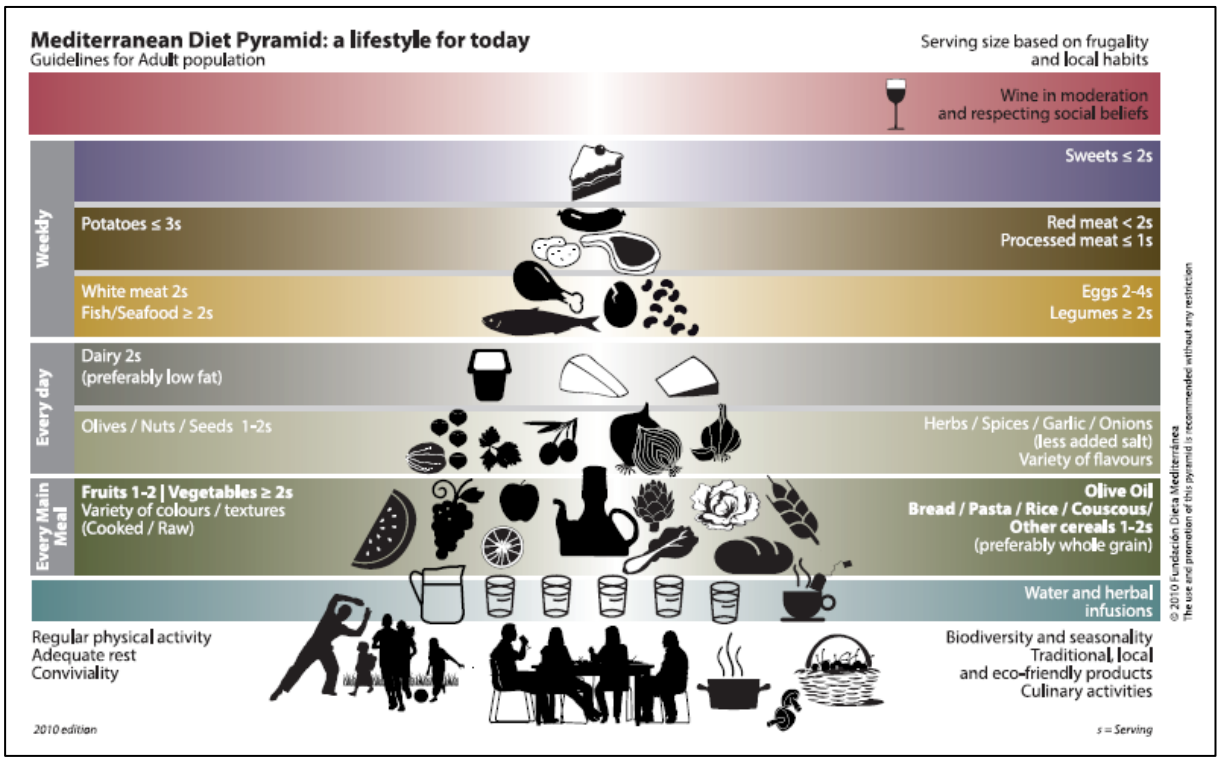


Figure 1. 14 The Mediterranean Diet pyramid (Cosimo Lacirignola & Roberto Capone 2015).

Many epidemiological studies have shown that there is an association between the consumption of the MD and a lower occurrence of cardiovascular disease (de Lorgeril and Salen, 2006, Estruch et al., 2013) and some cancers such as colon cancer (Galeone et al., 2007). The MD also has benefits for the prevention and treatment of diabetes (Rossi et al., 2013), hypertension (Estruch, 2010) and metabolic syndrome (Kastorini et al., 2011).

1.3.3.2 Differences between olive oil and EVOO

To understand the difference between EVOO and olive oil is important to know the process by which the oils are obtained. The process starts with the olive milling, and the product is then divided into three categories: extra virgin oil, virgin oil and lampante, Lampante can be consumed after a refining process called “refined olive oil” (Peri, 2014) (Figure 1.15)

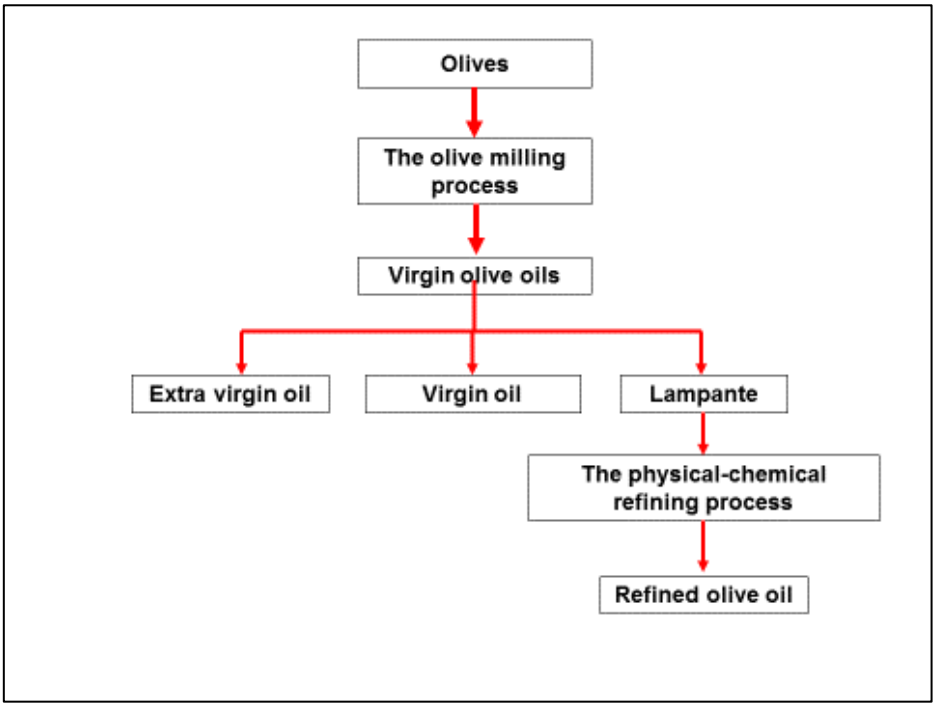


Figure 1. 15 Flow-chart of olive oil production.

The categories mentioned above are defined based on quality criteria. The Commission Regulation (EC), No. 702/2007 of June 2007, defined the chemical standards of all the categories of olive oils as it can be seen in Table 1.4.

Table 1. 4 Chemical standards of virgin oil.

Chemical standard	Extra-virgin	Virgin	Lampante
Free acidity (%), per litre	≤0.8%	≤2.0	>2.0
Peroxide index, per litre	≤20	≤20	----

The commission also defined extra virgin oils as “the oil obtained of the olive tree solely by mechanical or other physiological means under conditions that do not lead to alterations in the oil. The oil has not undergone any treatment other than washing, decantation,

centrifugation or filtration, to the exclusion of oils obtained using solvents or adjuvants having a chemical or biochemical action, or by the re-esterification process and any mixture with oils of other kinds" (Union, 2007).

Olive oil and extra virgin olive oil (EVOO) are the principal sources of fat in the MD, and it has shown to have several health benefits for humans (Waterman and Lockwood, 2007). Olive oil is associated with a lower mortality rate from cardiovascular diseases (Estruch et al., 2013). Results of EPIC study cohorts have shown an inverse relationship between olive oil consumption and coronary heart disease mortality and incidence (Bendinelli et al., 2011). Results of the PREDIMED study show that consumption of EVOO in line with MD recommendations reduces the risk of atrial fibrillation in older and high cardiovascular risk individuals (Martinez-Gonzalez et al., 2014).

Olive oil consumption has proven benefits regarding lipid profile, insulin sensitivity, lipid and DNA oxidation, inflammation, endothelial function, thrombotic factors and blood pressure (Lopez-Miranda et al., 2010). Studies have suggested that these effects are linked to various qualities of EVOO, including the high proportion of monounsaturated fatty acids, the high levels of vitamin E and phenolic compounds (Table 1.5)

Table 1. 5 Human studies on the effect of olive oil on health parameters.

Authors	Location	Participants	Age (years)	Study type	Results
Estruch et al. (2013)	Spain	7,477 men and women	55-80	Multicentre trial	Reduced the incidence of major cardiovascular events.
Buckland et al. (2012)	Spain	40,622 men and women	29-69	Prospective multicentre cohort	Decreased risk of overall mortality and reduction in CVD mortality.
Bendinelli et al. (2011)	Italy	32,578 women	35-74	Prospective	The inverse association between increasing consumption of olive oil and CHD risk
Martinez-Gonzalez et al. (2014)	Spain	7,477 men and women	55-80	Multicentre trial	EVOO in the context of an MD pattern may reduce the risk of atrial fibrillation
Storniolo et al. (2017)	Spain	90 women at high cardiovascular risk	60-80	Prospective	The supplementation with EVOO reduced the blood pressure among hypertensive women.

The main and most important difference between EVOO and olive oil is the amount of phenolic content (PC). At least thirty phenolic compounds are contained in olive oil (Oi-Kano et al., 2008a). These compounds are high in anti-oxidants both *in vivo* and *in vitro* (Alarcón de la Lastra et al., 2001). EVOO's phenolic content is estimated in 50 to 800 mg/kg (Sanders, 2001) while olive oil PC content is estimated in 196 mg/kg (Owen et al., 2000). Oleuropein is the main PC found in olive oil (Omar, 2010). However, in EVOO the main PC is found in the form of oleuropein aglycone (OA) (Leri et al., 2018) (Figure 1.15). The countries that comprise the Mediterranean region consume, from their diet, an approximate amount of 100 mg of oleuropein per day (Andreadou et al., 2006).

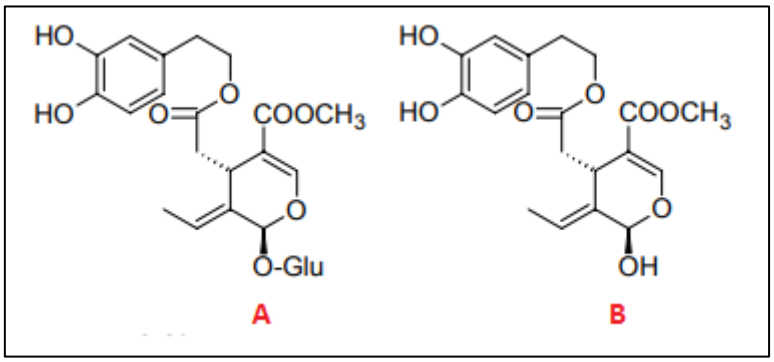


Figure 1. 16 Chemical structures of A) oleuropein and B) oleuropein aglycone (Omar 2010).

More recently, Neveu V (2010) determined the difference in phenolic content between EVOO and refined olive oil as seen in Table 1.6

Table 1. 6 Concentration phenolic compounds in olive oils.

Phenolic compound	EVOO (mg/100 g)	Olive oil refined (mg/100 g)
Flavonols:		
Apigenin	1.17	0.03
Luteolin	0.36	0.12
Polyphenols:		
3,4 DHPEA-EA	7.22	1.09
Oleuropein	0.17	---
Tyrosol	1.13	0.41

EVOO is composed mainly of monounsaturated fats and oleic acid, making it less vulnerable to oxidation and can be used for all types of cooking, even frying, without losing its properties (Owen et al., 2000).

1.3.3.3 Oleuropein aglycone as an activator of BAT thermogenesis

The phenols obtained from olive oil and EVOO are absorbed in the small intestine, whereas oleuropein and OA are hydrolysed in the intestinal tract (Oi-Kano et al., 2008a). In humans, although the majority of the phenols are absorbed in the small intestine, it is estimated that oleuropein's absorption in the body is around of 66 to 73% and only a small portion is secreted through urination (Vissers et al., 2002). When compared to other types of fats like sunflower oil, palm oil or beef tallow, EVOO has the unique capability to increase the UCP1 expression in interscapular BAT (Rodriguez et al., 2002).

The process by which OA increases BAT thermogenesis starts when OA is absorbed. Once OA is absorbed, the plasma concentrations of noradrenaline and adrenaline increase and then act through the beta-adrenergic receptors (β2 and β3) after an increase in the transient receptor potential ankyrin 1 (TRPA) and vanilloid 1 (TRPV1). These are allocated to the plasma membrane of animals and humans cells (Clapham et al., 2005). The rise in noradrenaline, in response to the stimulation of the sympathetic nervous system, activates UCP1 expression in BAT and increases its thermogenesis (Cannon and Nedergaard, 2004). The subsequent rise in thermogenesis reduces the amount of visceral fat in obese rats (Oi-Kano et al., 2017) after the consumption of OA for seven weeks (Figure 1.17).

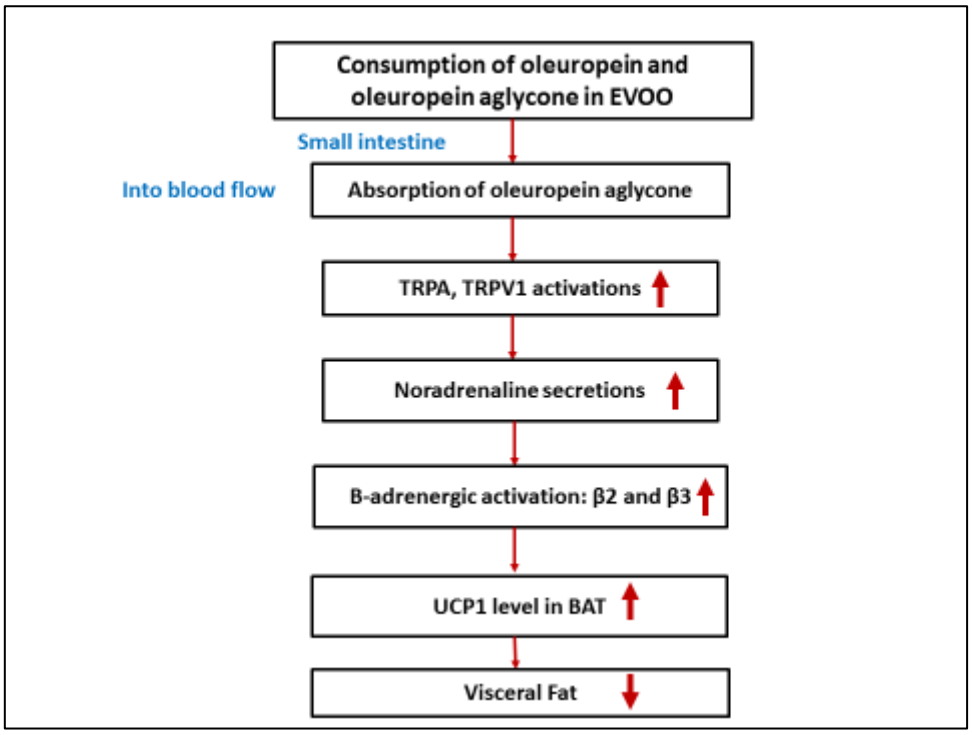


Figure 1. 17 Phenolic compounds of EVOO and their effect on BAT.

The figure shows the mechanism through which oleuropein and OA increase BAT thermogenesis and its effects on the body composition (Oi-Kano et al., 2017).

The amount of EVOO necessary to increase the plasma concentrations of noradrenaline and adrenaline to rise UCP1 levels, and therefore BAT thermogenesis, is approximately 20 or 30 g per day (Oi-Kano et al., 2007a).

1.3.3.4 Dietary polyphenols and their effect in the gut microbiota

Most recently, studies have been shown the interaction between dietary polyphenols and gut microbiota (Thilakarathna et al., 2018). These effects have been seen in both animal (*in vitro)* and human studies, that when phenolic compounds enter the gut microbiota, there is an alteration in the Bacteroides/Firmicute equilibrium (Lee et al., 2006, Hervert-Hernández et al., 2009). Therefore, polyphenols are now considered as prebiotics (Gibson et al., 2017b).

The process in which polyphenols can switch the microbiome to aid the colonisation of beneficial gut microbes (Mueller et al., 2017) start when polyphenols enter the gut microbiota, there, microbes degrade the polyphenols and produce phenolic metabolites (Bhat and Kapila, 2017). These metabolites, are capable of remodelling the epigenome by affecting enzymes (i.e. methyltransferases, acyltransferases, and phosphotransferases) which are

involved in the DNA. Consequently, there is a shift in gene expression (Bhat and Kapila, 2017). Figure 1.18 shows the road in which polyphenols bio-transform in the gut.

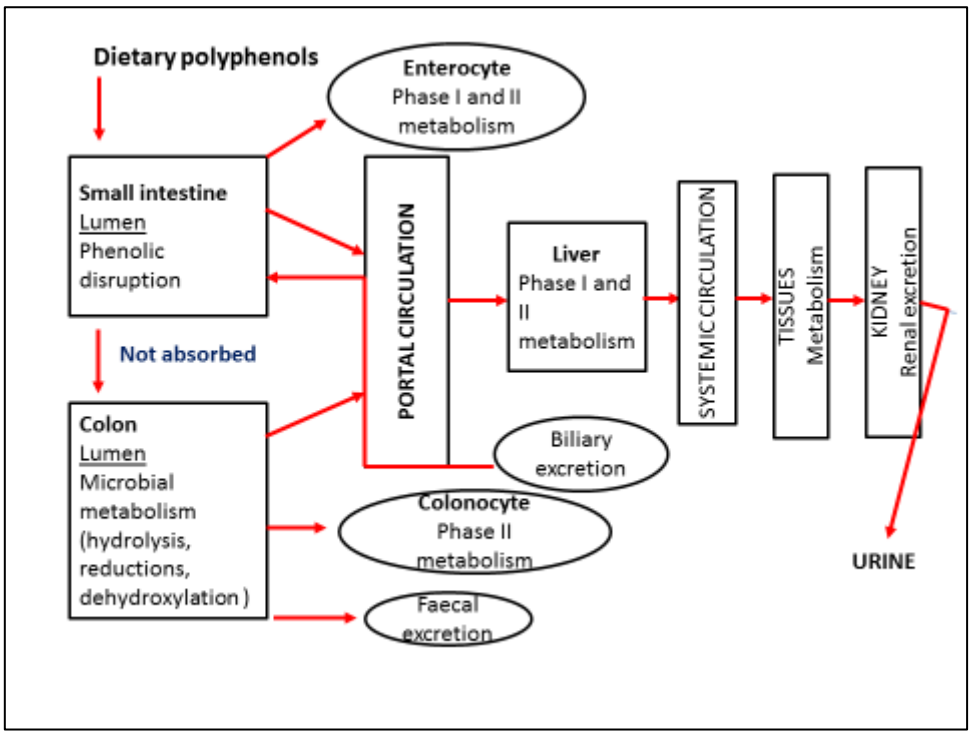


Figure 1. 18 Dietary polyphenols and their metabolites in humans.

Only five to ten per cent of dietary polyphenols are absorbed in the small intestine. After they are absorbed in the small intestine, a small portion of them are put through to an extensive Phase I and Phase II biotransformation in the enterocytes and then followed by hepatocytes. This process results in a sequence of metabolites (methyl, glucuronide, sulfate) and which are quickly released to the systemic circulation and distributed to the organs and excreted in urine. Phase I, involves oxidation, reduction and hydrolysis; while Phase II involves conjunction. In the colon, the colonic bacteria act enzymatically on the remaining polyphenols (90-95%) producing another type of metabolites (Cardona et al., 2013).

The type of polyphenols which have shown a prebiotic effect in the gut microbiota are diverse, and range from the kernel of several nuts (almonds, hazelnuts), composed mainly of tannin structures (Mandalari et al., 2010, Oliveira et al., 2008), cocoa (Massot-Cladera et al., 2012), cabernet sauvignon grapes (Forester and Waterhouse, 2008), red wine polyphenols (Queipo-Ortuño et al., 2012) and tea phenolic compounds (Lee et al., 2006).

The main effect observed by the researchers in most of the studies mentioned above was the shift in the gut microbiota from the prevalence of *Bacteroides, Clostridium* and *Propionibacterium* to a predominance of *Lactobacillus* and *Bifidobacterium.*

1.4 Main hypothesis and aims

As described, certain dietary compounds and cold have been shown to affect the increase of BAT thermogenesis in animal models. However, limited studies are describing the influence of these factors in humans and assessed with thermal imaging. To further elucidate this relationship, four studies were used in the course of my PhD.

- BAT thermogenesis assessed with TI can be activated by cold challenges in a healthy adult volunteer.
- BAT thermogenesis assessed with TI can be activated by the supplementation of a GOS prebiotic in healthy adult volunteers. A pilot study and a randomised controlled trial study. The GOS prebiotic was chosen because it induces similar modifications in the gut microbiota composition as fermentable fibres (Delcour et al., 2016). GOS can also increase the rate of SCFA in faeces (Wang et al., 2017).
- BAT thermogenesis assessed with TI can be activated by the consumption of EVOO in healthy adult volunteers.

2 Materials and methods

This chapter describes the materials and methods for the studies included in this thesis, i.e. for chapters 3-6 which examine the effect of cold, prebiotics (PRE-BAT studies) and extra virgin olive oil effect on brown adipose tissue (EVOO-BAT study).

2.1 Thermal Imaging (TI) study regime

2.1.1 Location of the Thermal Imaging Studies

The cold (Chapter 3) and pilot PRE-BAT studies were performed in the Human Physiology laboratory of the Division of Child Health, Obstetrics & Gynaecology, University of Nottingham. This room was used as it belonged to the division, however, after performing diverse series of studies there, it was necessary to use another room which can be thermally controlled as the room temperature can influence BAT. The University of Nottingham provided the department with a thermally controlled room, where me and my colleagues, could develop more TI studies.

The PRE-BAT randomised controlled trial and EVOO studies were performed in the Academic Imaging Suite (B floor, Queens Medical Centre), University of Nottingham.

The main difference between these rooms was that the Academic Imaging Suite was thermally controlled (at 22°C) while the Human Physiology was not temperature controlled resulting there is a small variation in room temperature between thermal imaging

2.2 Selection and withdrawal of participants

2.2.1 Recruitment

The studies were conducted using healthy adult volunteers after the ethics approval. Participants were recruited by word of mouth and posters/flyers.

- Cold stimulated by BAT approved on the 14th of July 2012. Ethics no. D19062014 SoM Child.
- PRE-BAT pilot study approved on the 24th of August 2015. Ethics no. E11082015 SoM CHOG PhD.

- PRE-BAT randomised control trial approved on the 3rd of March 2016. Ethics no. E11082015 SoM CHOG PhD.
- EVOO-BAT study approved on the 21st of December 2016. Ethics no. B08122015 CHOG.

In the case of the EVOO-BAT study, participants were also recruited through the "call of participants" website from the University of Nottingham (Molina, 2017).

Once participants showed interest in any of the different studies, they contacted the research team by email and a screening questionnaire was sent to them through their email address to assess their eligibility for participation (Section 2.2.2). If the potential participants were eligible to take part in any of the studies, they were provided with an electronic copy of the participant information leaflet in a pdf format (Appendix 1, 2 and 3)

Eligible participants had the leaflet for at least 24 hours before they were approached to give informed consent. Details of the study were explained in person, and a printed participation leaflet was provided. The investigator answered any questions that participants had concerning study participation. Potential participants were asked if they were willing to participate in the study in question and to attend the designated number of sessions for that study.

Then, once participants had sufficient time to consider whether to participate in the study or not, they each provided written informed consent. The consent form was signed and dated by the participant, and the researcher before they entered the study. Informed written consent was received from each participant before they underwent any part of the study (including physical examination and history taking). The participant kept one copy of the signed consent form, and the investigator kept another.

2.2.2 Eligibility criteria

Inclusion criteria

Participants were eligible for the studies if they:

- Had a BMI between 18.5-24.9 kg/m^2 (Normal weight).
- Were aged between 18 to 29 years old.
- Were healthy.

Exclusion criteria

Each study had its exclusion criteria. However, participants were not eligible for all studies if they:

- Had a diagnosis of hypothyroidism, hyperthyroidism or diabetes; as these conditions may affect BAT activation.

PRE-BAT studies:

- Potential participants were taking any antibiotics, beta-blockers or any food supplement that contained pre or probiotics by a month prior to starting the trial. Antibiotics, pre and probiotics can affect the host's gut microbiota, and beta-blockers affect BAT activation.
- Had an allergy, intolerance or dietary restriction to prebiotics, maltodextrin or lactose.
- Had a pacemaker

EVOO-BAT study

- Had an allergy, intolerance or dietary restriction to olive or olive-derived products.
- Had a pacemaker
- Normally used extra virgin olive oil as part of their diet.

After participants gave informed consent, they changed into standardised clothing: a light cotton vest-style top to expose the area of the shoulders and the collarbone. Any jewellery around the neck and ears was removed, and long hair was placed behind the shoulders so as not to obscure the region of interest.

Non-invasive anthropometric and physiological measurements were taken: height, weight, tympanic temperature, adiposity (PRE-BAT and EVOO studies) and stress flow (EVOO-BAT study).

2.3 Anthropometric and physiological measurements

2.3.1 Height

Participants were asked to remove their shoes and any head accessories for measurement of height. Height was assessed using a stadiometer (SECA 213 Leicester height measure, Birmingham, United Kingdom).

They were asked to stand with their back, buttocks, feet, and calves against the stadiometer and look directly forward during the measurement. They were positioned underneath the drop down measuring device of the stadiometer. Then, the measuring device was lowered until it rested gently on top of the head of the participant.

The measurement was recorded in metres and centimetres (Hall and Gripp, 2006).

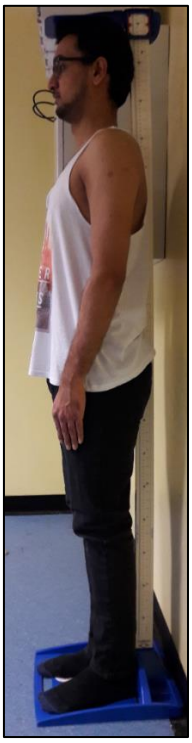


Figure 2. 1 Height measurement.

Measuring of the height of an adult using SECA 213 stadiometer. Image used with permission of participant Zaid Janjua©. Ms Lucia del Carmen Vazquez Rocha.

2.3.2. Weight

Participants were asked to remove their shoes and heavy items from their pockets like keys, wallets and mobile phones; once they did that, the researcher set to zero the scale so the participants can step onto it.

This parameter was measured to the nearest 0.1 kg with a flat scale with cabled remote display (SECA 899, Birmingham, United Kingdom). Then, they were asked to step onto the scale and look straight ahead, remaining still. The measurement was recorded in kilograms after the digital screen settled (Hall and Gripp, 2006). The scale was calibrated every six months through the SECA® calibration check service, which is ISO® accredited and member of the UK Weighing Federation®.

The steps of the calibration are as follow. Firstly, the scale has to turn on and let to warm up. Secondly, it is important to locate the calibration button of the scale (represented as “Cal”). Then, the calibration button needs to be pressed down until the digits displayed on the screen are zero. At this point, the scale is ready for the calibration test.

The calibration test involves placing the known weight objects on the central part of the scale, to get the most precise readings. The reading should last five seconds, and the calibration button should be pressed once more to end the session. Once it’s finished the following word is displayed on the monitor: “End”.

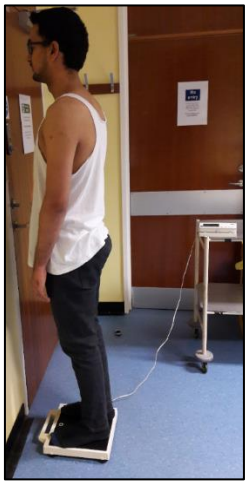


Figure 2. 2 Weight measurement.

Measuring the weight of an adult using a SECA 899 scale. Image used with permission of participant Zaid Janjua©. Ms Lucia del Carmen Vazquez Rocha.

2.3.3. Tympanic membrane temperature

Participants were seated in a chair and advised to do not move their head while the researcher was taking the measurement; temperature was measured using a digital infrared ear thermometer (Braun ThermoScan® ear thermometer IRTS 6520, Braun GmbH, Kronberg, Germany).

The researcher took the thermometer out of its holder and put a new throwaway cover over the tip of the thermometer; then, put the covered tip into the ear opening.

Once the thermometer was inside the ear, the measurement was taken and recorded in °C and taken for two additional times (Hall and Gripp, 2006).

As BAT participates in the regulation of body temperature (Ravussin and Galgani, 2011), the tympanic temperature was assessed in all the studies to see if there was a change of it before and after the interventions.

2.3.4. Adiposity (percentage of body fat)

This evaluation was used in the PRE-BAT placebo and EVOO-BAT studies. In both studies, adiposity was measured using a tetrapolar bioelectrical impedance machine (BIA).

BIA Is a safe, rapid, validated and non-invasive technique which provides reliable and accurate estimates of human body composition (Gomes et al., 2015, Goncalves et al., 2013, Kusztal et al., 2015, Neves et al., 2015). Resistance and reactance compose BIA. Resistance is determinate by the total amount of water in the body (around 73% in subjects which are normally hydrated) (Hoffer et al., 1969). Reactance is the resistance in a variation of voltage across an object and occurs due to the ability of cell membrane to store an electric charge (capacitance) (Kyle et al., 2004). BIA assess the resistance of the body tissue through an electrical signal that is small and harmless (1-10 μA) which passes through water present in free fat mass (FFM). However, meets resistance when it hits fat mass (FM) (De Lorenzo et al., 1997). This resistance is what is known as ***impedance*** is measured and the data into scientifically validated equations to calculate body composition measurements (Lukaski et al., 1986).

BIA uses, as an electrical conductor, four disposable electrodes positioned in hand and foot respectively. The electrodes used were attached to the skins participants due to its ability to stick.

As BIA is inexpensive, non-invasive and suitable for use in human volunteer studies, it is frequently used to assess body composition in preference to other techniques such as tomography, isotope dilution of total body water, DEXA and air displacement plethysmography (von Hurst et al., 2015). Pacemaker placement and pregnancy are the only documented restrictions to the use of BIA.

For the PRE-BAT placebo study, Maltron BF-906 Body Fat Analyser (Maltron International Ltd, 20 Sirdar Road, Rayleigh, Essex SS6 7XF UK) was used. This device has been utilised in some published articles (Doornweerd et al., 2016, Muskiet et al., 2016, ter Horst et al., 2016) (Figure 2.3).

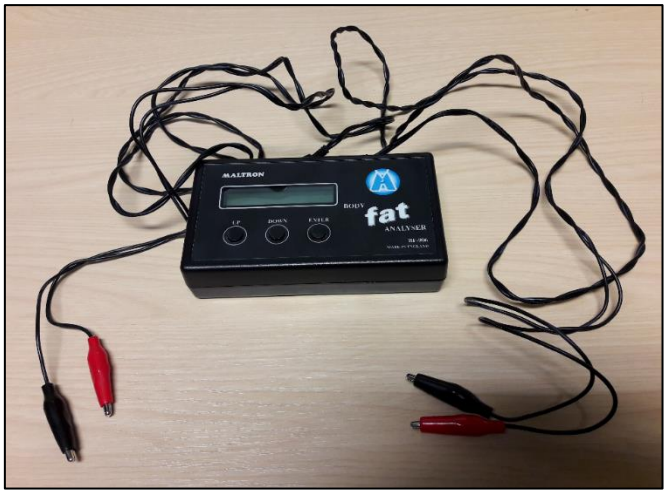


Figure 2. 3 Maltron Tetrapolar Bioelectrical Impedance.

A device used for the body composition assessment in the PRE-BAT randomised control trial.

For the EVOO-BAT study, Biotekna BIA-ACC (Biomedical technologies, 510 Cannon Wharf, Pell Street SE8 5EN – London UK) was used. The device has been used in different published data (Kyrou et al., 2017, Stefanaki et al., 2016, Peppa et al., 2017).

Both appliances were used because they conformed to ISO standards (ISO 9001-2000 and EN ISO 13485) (Figure 2.4).

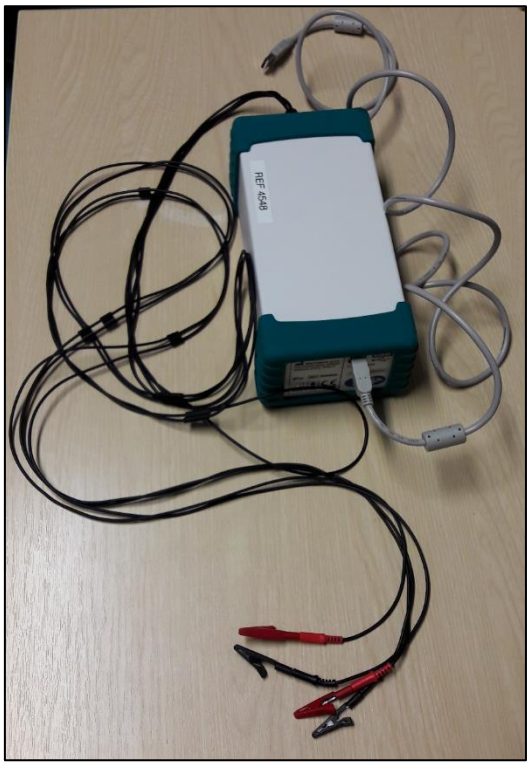


Figure 2. 4 Biotekna Tetrapolar Bioelectrical Impedance.

A device used to obtain body composition analysis in the EVOO-BAT study.

The guidelines for body composition analysis with BIA used in both machines are the same. The only difference is that the Biotekna was connected to a computer to show the results obtained from the analysis; while Maltron showed the results directly on the machine screen. In both procedures, participants were positioned away from the screen of the equipment so they could not see the results obtained and therefore avoid altered behaviour between visits.

BIA procedure:

1. Participants were asked to remove their right sock and shoe as well as any jewellery or metallic items on their bodies like belts.
2. Participants lied on their backs on the exam table with arms by their side and their legs separated from each other.
3. Participants were exhorted to clean the skin areas where electrodes were placed (dorsal of the hand and foot) with an alcohol-based swab for adhesive removal and skin cleansing (steret).

4. Two disposable electrodes were placed on the dorsal surface of the right hand: the first electrode on the distal end of the third metacarpal bone between the second and third finger (metacarpophalangeal joint), and the second electrode between the distal prominences of the radius and ulna of the wrist (radio-ulnar joint). The distance between the electrodes was fixed at 5 cm.

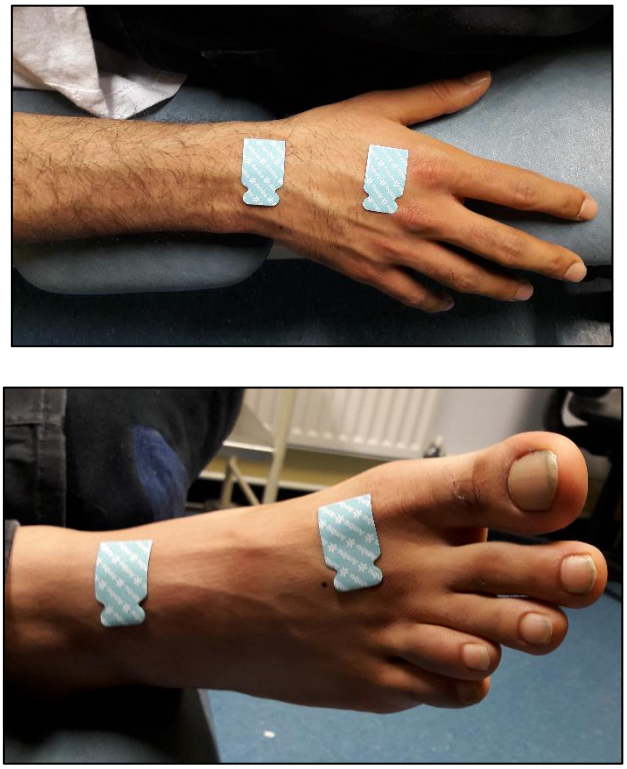


Figure 2. 5 Application of electrodes for the assessment of body composition.

The correct form of application of electrodes for BIA in both hand and foot. Image used with permission of participant Zaid Janjua©. Ms Lucia del Carmen Vazquez Rocha.

5. Leads were attached to the electrodes.
6. After the participant was suitably dressed, the test started. The researcher entered height (cm.), weight (kg.), age and sex in the machine (Maltron) and computer program (Biotekna), respectively.
7. After the data was entered and the participant connected, the program started running.
8. After the test was completed, the participants were asked to remove the electrodes and to sit in an upright position. The test lasted around 30 seconds.



Figure 2. 6 Position of the electrodes on the right side of the participant's body.

Participants have to lay down for the researcher to apply the electrodes on the right side of the body. Image used with permission of participant Zaid Janjua©. Ms Lucia del Carmen Vazquez Rocha.

2.3.5. PPG stress flow

As the sympathetic nervous system (SNS) affects BAT activity, sympathetic and parasympathetic activity indicators were evaluated using an optoelectrical plethysmograph device (PPG).

The PPG stress flow device monitored the participant's autonomic nervous system. This device was used only in the EVOO-BAT study after carefully evaluating the importance that the assessing of the SNS is in BAT studies. The results were expressed in low (LF) and high (HF) frequency power.

PPG measures changes of the volume within an organ, in this case, the heart. From this blood flow (F), volume change (dV) over time (dt) can be calculated: dV= F x dt (Hertzman, 1937).

For this study, a commercially available PPG such as the PPG Stress Flow© (Biotekna S.r.l.®, Italy) which conforms to ISO standards and is CE marked (e.g. PPG Stress Flow© CE0476) was used.

The following steps were followed to assess the SNS:

1. The PPG device was placed on a flat surface.
2. Participants were asked to remove any metallic jewellery and to be seated in a comfortable position with their forearms on the table. They were advised not to move during the test; in particular, their fingers.
3. The sensors were connected to the right and left index fingers of the participant.

4. A USB cable was connected to the back panel of the PPG device, and then the test was commenced.
5. After the test was completed, the sensors were removed from the participant.

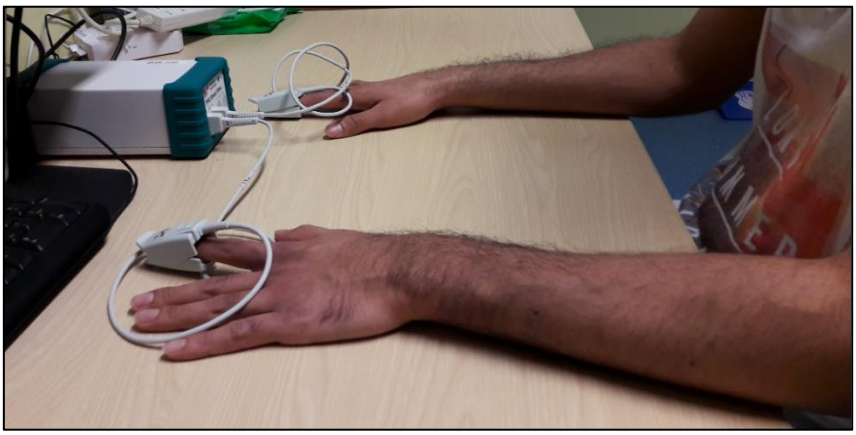


Figure 2. 7 PPG sensors.

The correct form of application of PPG sensors to a volunteer.

This technique has been used in healthy individuals and patients (Aliverti et al., 2000, Hull et al., 1985).

Once all the physiological and anthropometric measurements were taken in the study session, the Thermal Imaging (TI) assessment was introduced.

2.4 Thermal Imaging assessment

TI was the technique used to assess BAT thermogenesis in the supraclavicular area of the participants. The camera used for all of the studies was a forward-looking infra-red camera (FLIR) (FLIR E-60; FLIR Systems, Danderyd, Sweden) (Figure 2.8). The camera was calibrated every six months by Inspec Calibration® UK and Ireland, a service centre for the repair and calibration of TI cameras.

The TI camera was tested against the manufacturer's specification to identify its existing operating parameters.



Figure 2. 8 E-60 FLIR camera.

This model was used in all the TI studies described in this thesis.

The camera was chosen because it can detect BAT in real time as well as the gold standard PET-CT (Positron emission tomography); a study performed by Law et al. (2018b) compared the TI and PET data from 8 healthy male volunteers. The study aimed to recognise the regions of interest (ROI's) in the supraclavicular area, where BAT is located, using TI and if it similarly corresponds to the ROI's of maximal glucose uptake in PET. When the images of both techniques were overlapped, the hotspots were located in the same ROI, supraclavicular area, specifically when the participants were stimulated with a cool challenge ($p<0.05$). The researchers analysed the hottest ten per cent of points within each ROI and then calculated the medians of these points.

The operation system of the TI camera is similar to a digital optical photographic camera: i.e. lens, electronics and software that process the signals and images. However, the main difference is that the FLIR camera operates with a focal plane array (FPA) and is sensitive to infrared radiations (IR) wavelengths. The characteristics of the E-60 FLIR camera are listed in Table 2.1

Table 2. 1 Characteristics of the E-60 FLIR camera.

Frame rate	60 Hz.
Temperature range	-4 to 1202°F (-20 to 650°C)
Thermal sensitivity (N.E.T.D)	<0.05°C at 30°C
Detector Type - Focal plane array; (FPA) uncooled micro bolometer	320 x 240 pixels
Video Camera w/Lamp & Laser	3.1MP/LED Lamp/Laser pointer
Digital Zoom	4X Continuos
Data Communication Interface	USB-mini, USB-A, Composite Video, Bluetooth, Wi-Fi.
Field of view/Focus	25° x 19°/Manual (minimum focus distance 0.4 m).
Spectral range	7.5 to 13µm.
Display	Built-in 3.5" color LCD.
Image modes	Thermal, visual, MSX and image gallery.
Image storage	>1000 radiometric JPEG images.
Set-up controls	Mode selector, colour palettes, configure image info, units, language, date and time formats, and image gallery.
Measurement mods	Auto hot/cold spots, Isotherm (above/ below/ interval).
Measurement correction	Reflected ambient temperature and emissivity correction.
Battery time/Operation time	Li-Ion/ >4 hours.
Charging system	In-camera AC adapter/2 bay charging system.
Dimensions/Weight	9.7x3.8x7.2" (246x97x184mm)/<1.82lbs (825g), including battery.

FLIR physical principles are derived from the Total Radiation Law; this law implies that "an object reacts to incident radiation from its surroundings by absorbing and reflecting a portion of it, or allowing some of it to pass through (as through a lens)" (Siegel R, 1992).

The formula of the law is simplified as:

$$1 = \alpha + \rho + \tau$$

In which **α** means absorption, **ρ** reflection and **τ** transmission. Each coefficient can have a value from zero to one; this depends on how well an object reflects or transmits radiation.

Human bodies, with normal temperature (36°-37°C), will radiate about 0.5 kW/m2 (Ring and Ammer, 2012). The radiation absorbed by the camera is affected by emissivity, atmospheric influence (surroundings) and temperature. Therefore, relevant settings were applied to the camera before each study session and recorded in each participant's datasheet (Appendix 4 and 5) i.e. reflective temperature, emissivity (0.98, this number remained constant during all sessions as this is the emissivity number used for human skin (Fernández-Cuevas et al., 2015)), room temperature and humidity.

Room temperature and humidity were registered using a moisture meter (Pinless Moisture Meter, Extech Instruments, A FLIR Company, Stockholm, Sweden).

The external temperature was recorded to analyse wheatear this parameter influenced the final result in the studies as some authors have stated in the past (Lee et al., 2014). The outside (ambient) temperature was taken from the MET office website (https://www.metoffice.gov.uk/). Once the settings were inserted in the camera, the TI session commenced.

2.4.1. TI procedure

After all the settings were inserted in the camera, the participants were seated at a distance of 1 metre from it. The camera itself was placed on a tripod, 1 metre vertically above ground level. The participant's right hand was placed in their lap, and the left hand was placed inside an empty receptacle (Figure 2.9).

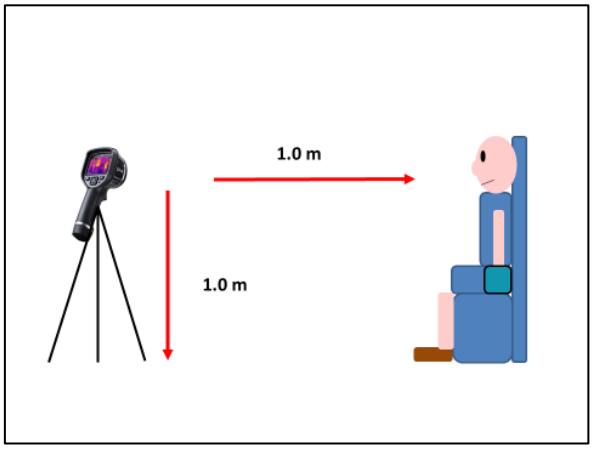


Figure 2. 9 Thermal imaging technique.

Schematic representation of the technique used to assess BAT thermogenesis with a thermal camera.

After a period of resting (30 minutes), pre-stimulation images were taken for 10 minutes, followed by the introduction of a cool stimulus. The stimulation period resulted from the immersion of the participant's hand in cold tap water (typically between 16-20°C) for 20 minutes. The temperature of the water was assessed with a thermometer (Riester, Jungingen, Germany) before and after the participants placed their hand on the water. Imaging continued for a further 20 minutes after the hand was removed from the cold water. Images were taken every 30 seconds for the cold and PRE-BAT studies; and 20 seconds for the EVOO-BAT study. This timing was chosen based on the operating procedure of previous published data using TI techniques (Robinson et al., 2014, Robinson et al., 2016).

2.5 Data analysis

2.5.1 Methods of analysis

There are two ways to analyse the images from TI measurements. The first used pre-installed ThermaCAM Researcher Pro 2.10 (FLIR system AB, Täby, Sweden) and the other one is an alternate technique that involves the use of Matlab (MATLAB and Statistics Toolbox Release 2017a, The MathWorks, Inc., Natick, Massachusetts, United States).

Dr James Law and Assistant Professor David Morris from the University of Nottingham developed Matlab software for the analysis of TI images.

Matlab has advantages over ThermaCAM. The first is that it is a pioneer and readily available program for all the TI users in the Child Health Department in their personal computers. Secondly, the software analyses image more automatically and rapidly than FLIR. In an exercise performed with a set of 30 images using both programs, ThermaCAM's analysis took an estimated time of 36 minutes to finish, while the same analysis only took 10 minutes to complete with Matlab.

Another main difference in the analysis is that with ThermaCam the Regions of Interest (ROIs) of BAT have to be calculated manually; while for Matlab the researcher needs to place a circular figure in the ROIs and the computer program will then define the area automatically (Figure 2.10).

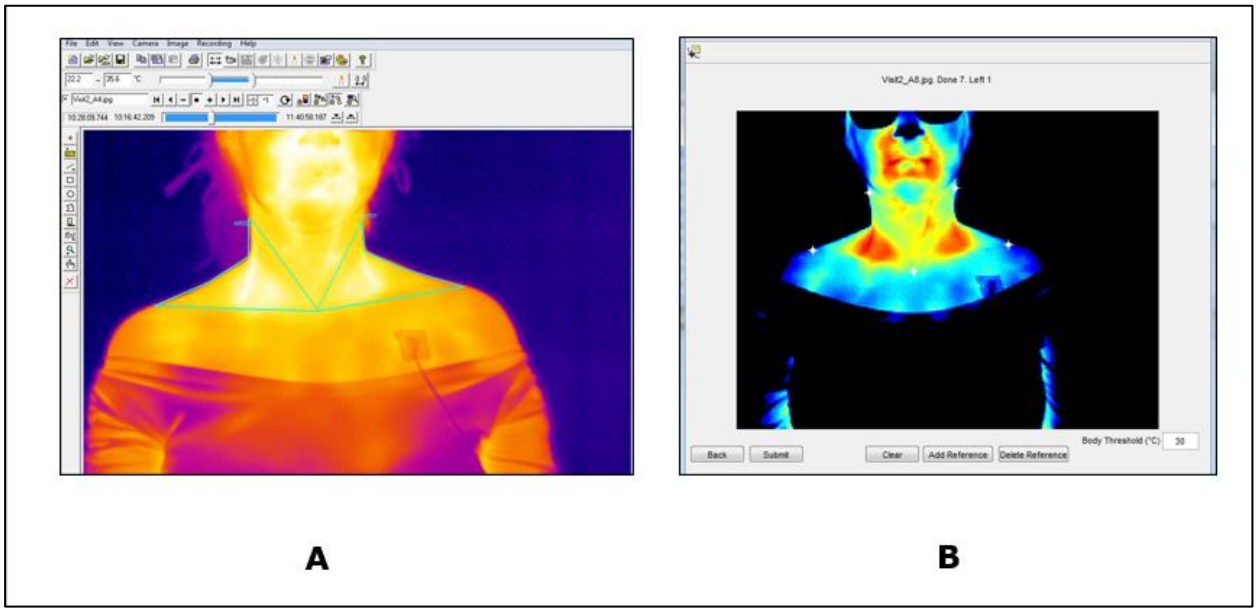


Figure 2. 10 Delimitation of ROIs for the analysis of BAT thermogenesis using two different methods.

A)ThermaCAM, the delimitation of ROIs, triangle figure, is done manually. B) Matlab, the delimitation of ROIs is done automatically by the computer program.

Matlab is the program most recently used in publications by the department (Scotney et al., 2017, Law et al., 2018b). Alternatively, though, an analysis conducted with FLIR has also been previously published (Robinson et al., 2014, Robinson et al., 2016). Therefore, after considering these facts, I decided to use Matlab for my analysis.

2.5.2 Procedure to analyse the images

Thermal images were saved in a memory stick that was attached to the thermal camera while each study was running and immediately after the images were taken, the data was collected and stored in a secure place (investigator's site). Images were then converted to a .png format with a Thermal Imaging Conversion Hub (TITCH), essentially a Raspberry Pi^{TM}-based device that extracts the data from the thermal imaging camera and saves it in a format that makes it accessible to calculate the temperature from the supraclavicular area. After the images were converted, they were individually analysed using Matlab (2017a).



Figure 2. 11 TITCH Raspberry PiTM based device.

Device used to convert .jpeg images into .png.

Left and right supraclavicular ROIs of each image were defined after its conversion to:

a) Right acromion.

b) The angle where the jaw meets the neck –right.

c) ~1 cm below the sternal notch.

d) Angle were the jaw meets the neck-left.

e) Left acromion.

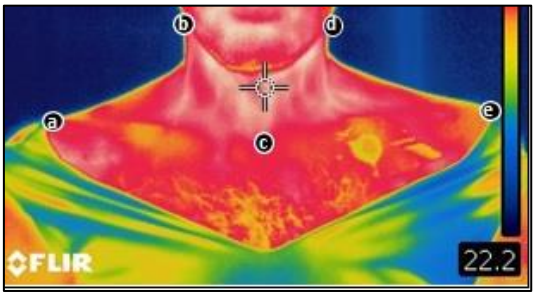


Figure 2. 12 ROIs in the supraclavicular area.

The researcher makes the localisation of the ROI's manually in each image of the participants.

After the analysis with Matlab, the images showed the ROIs, spots where the hottest BAT temperature was located as well as a reference point (lighter shade of blue). The reference (T_{REF}) point was located 1 cm below the sternal notch.

The mean BAT temperatures of the ROI, equivalent to 95th percentile, from the supraclavicular area (T_{SCR}), were obtained from an excel spreadsheet after the appropriated analysis with Matlab and separated in median right and left automatically by the program.

The data was then assessed for normality using the Shapiro-Wilk Test with SPSS 23.0 (IBM, Armonk, New York), this test was chosen because is more appropriate for small sample sizes (less than 50), but can also be used for sample sizes as big as 2000.
The temperature of the skin overlying the supraclavicular BAT was defined as T_{SCR} and was measured using TI. Mean surface temperature (T_{REF}) was evaluated with the same TI camera used to calculate $T_{SCR,}$ but its position was located 1 cm below the sternal notch. As T_{SCR} can be influenced by other factors like environmental temperature (Law et al., 2017b), T_{SCR} was adjusted by calculating the difference between T_{SCR} and T_{REF}. This result was defined as the relative temperature (T_{REL}) (Law et al., 2018b, Robinson et al., 2016). It is not possible to use for the analysis only the absolute T_{SCR} because participants exhibit a different temperature due to energy exchange with the environment, self-heating, emissivity and reflection and T_{REL} aloud the researcher to control those differences. On Figure 2.13 is possible to see where T_{SCR} and T_{REF} are located in a volunteer of one of the studies described in this thesis, as well as the average temperature calculated from that area. The result of the difference between T_{SCR} and T_{REF} is T_{REL}, or in the case of the participant, was 2.34 °C.

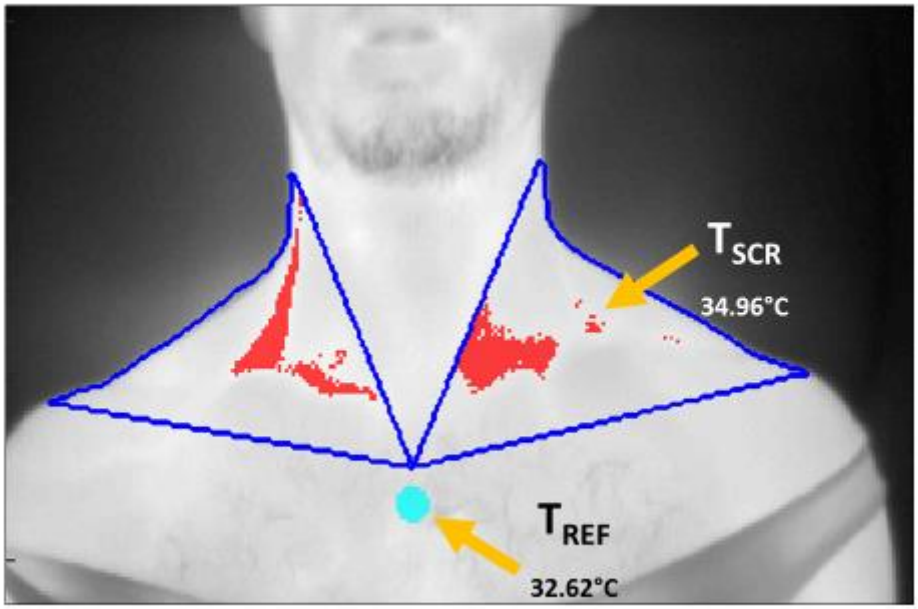


Figure 2. 13 Localization of the T_{SCR} and T_{REF} on a volunteer after the analysis with Matlab.

Once the T_{REL} was estimated, the resting temperature was defined. The resting temperature was defined as the temperature before stimulation (placing the participant's hand in cold water). The stimulation of BAT with cold water is feasible as FLIR can measure the BAT temperature in real time.

I decided to take the average T_{REL} of the last 5 minutes of pre-stimulation temperature (i.e. 10 minutes in total) as the resting temperature like previously published data (Robinson et al., 2014).

The stimulation period is defined as the average of the first five minutes of the hand in cold water. Five minutes of stimulation with cold is enough to obtain an initial response of BAT thermogenesis from the subjects when there is direct contact with the stimulus (Symonds et al., 2012).

The post-stimulation period is defined as the first five minutes after the participants removed their hand out of the water. This timing was considered to use the same time as the resting period and to compare the difference in BAT temperature between the resting, stimulation

and post-stimulation periods (Figure 2.14). Nevertheless, it is also the timing used in previous publications of the department (Robinson et al., 2014).

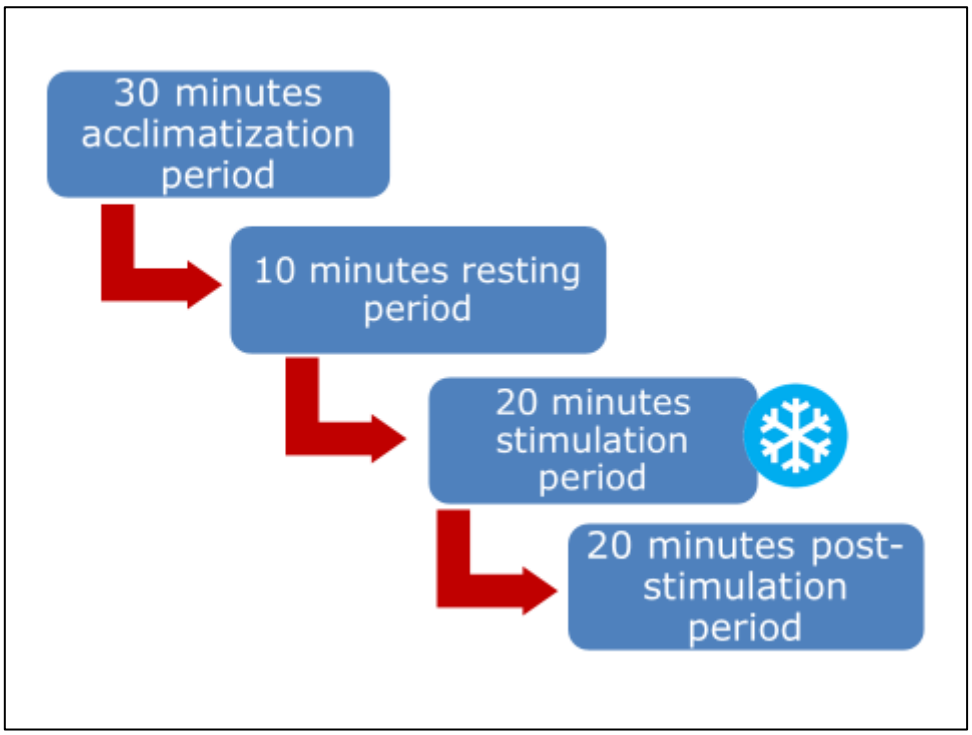


Figure 2. 14 Timings for assessment of the studies included in this thesis.
This timing included the time taken for the analysis of data.

2.5.3 Analysis of results

Once all the images were properly analysed and to see if the intervention (cold, prebiotic, extra virgin olive oil) affected BAT RT, as explained in Chapter I of this thesis; several methods were used to identify that effect.

These methods included the comparison in BAT between the baseline before and after the intervention as well as area under the curve (AUC); the indicated measurements were previously assessed in published work of the Child Health department (Law et al., 2017a, Scotney et al., 2017).

Resting temperature corresponded to the average temperature of the last five minutes of the pre-stimulation period, with a further investigation into whether the baseline measurement was capable of detecting the intervention alone. The measurement will check whether the resting temperature can increase BAT thermogenesis without cold stimulation.

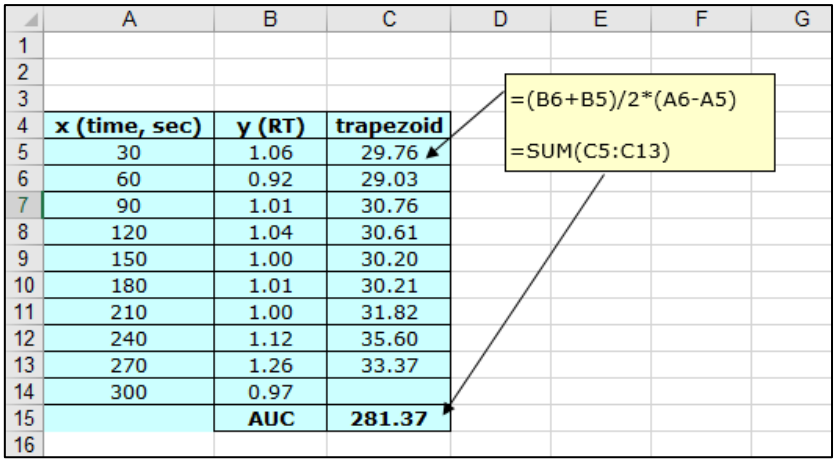
The surface temperature was assessed to detect whether it is possible to notice a change in temperature outside the ROI area.

The Area under the curve (**AUC**) technique was used because it is a reliable formula that integrates the measurement of a measurable effect, or in this case, the intervention of each study in the case of BAT temperature. The formula used to determine the AUC was the trapezoid rule which divides the curve into a series of trapezoids, each with area= (average height)(width); i.e. the sum of the areas of the strips (Turner, 2013).

The formula used in the studies was: (relative BAT temperature as **y** value)(time (seconds) as **x** value); this was then consecutively added up to give the overall area of the strip. The formula was used in the same excel file as the results obtained from the analysis of the images.

An example calculation of a participant of the PRE-BAT pilot study is shown below. The method describes how the AUC baseline period is determined.

AUC formula=sum of the first RT of y-axis + the second temperature; then divided by 2 and multiplied by the x-axis (y1+y2)/2*(60-30). Subsequently, each trapezoid was added up to obtain the AUC.



	A	B	C	D	E	F	G
1							
2							
3							
4	**x (time, sec)**	**y (RT)**	**trapezoid**				
5	30	1.06	29.76				
6	60	0.92	29.03				
7	90	1.01	30.76				
8	120	1.04	30.61				
9	150	1.00	30.20				
10	180	1.01	30.21				
11	210	1.00	31.82				
12	240	1.12	35.60				
13	270	1.26	33.37				
14	300	0.97					
15		**AUC**	**281.37**				
16							

Figure 2. 15 Determining of the AUC using the trapezoid formula in excel

The figure shows how the mathematical calculation of AUC works.

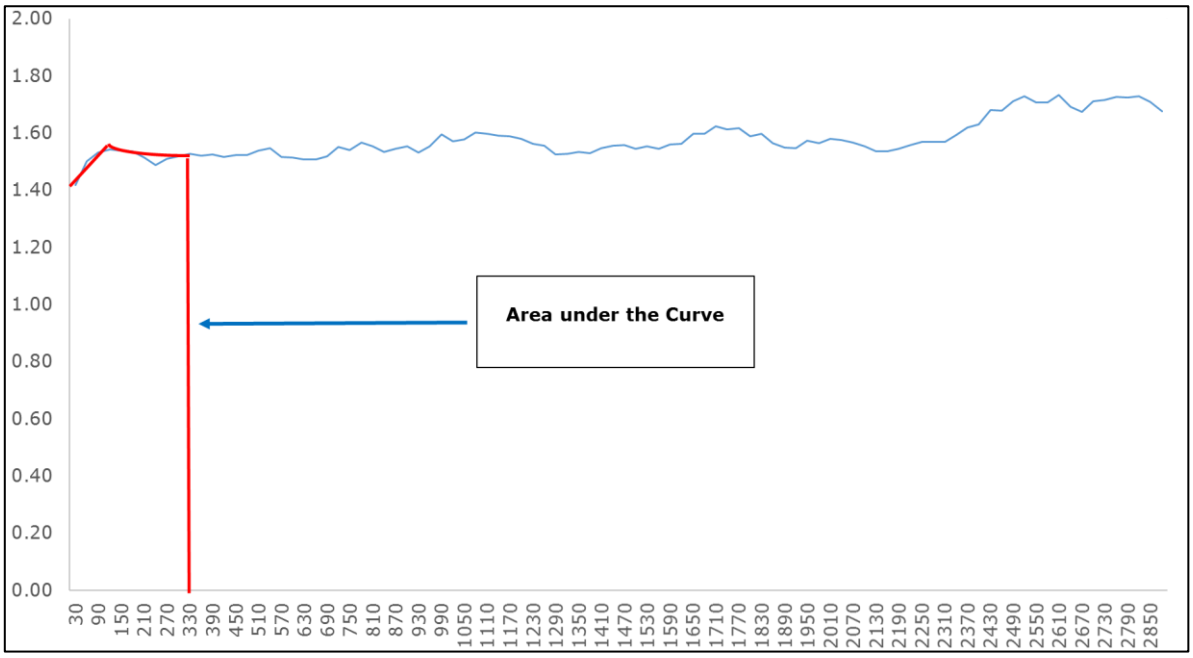


Figure 2. 16 Determining of the AUC in a graph.

In this figure, it is possible to see the AUC obtained from the formula in figure 3.8.

The change in temperature (ΔT) was considered as the difference in temperature between the first and second assessments of BAT (either RT or AUC).

Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA). A $p<0.05$ was considered statistically significant.

To determine if the external temperature influenced BAT RT; regression analysis was performed in each study. The difference in weight, core temperature, adiposity and sympathetic nervous system indicators (in the studies where these measurements were made) was calculated before, and after the intervention (cold, prebiotic, EVOO); this to see if the intervention modified those values.

3 Does a daily cold challenge, for seven consecutive days, can modulate brown adipose tissue (BAT) thermogenesis?

3.1 Introduction

As stated in chapter 1 of this thesis, introduction, chronic-cold exposure can stimulate BAT thermogenesis in both human and animal models (van Marken Lichtenbelt et al., 2009b, Hiroshima et al., 2018). This cold exposure, and consequently an increase in BAT thermogenesis, may have benefits in the energy expenditure and glucose metabolism (Cypess and Kahn, 2010b) and, therefore, coadjutant in tackling the obesity epidemic.

Obesity is related with the decline in energy expenditure, in the United States alone, the daily energy expenditure in men has decreased from 1960 (1567 kcal) to 2010 (1424 kcal) (Greenway, 2015). This country also has the highest projected rates of obesity, by 2030, 45% of their population will be obese (EASO, 2018)

In the UK the statistics are no different, it is estimated that by 2025 there will be five million people with diabetes (UK, 2017). Currently there are 3.2 million. By 2030, 35% of the UK population will have obesity (EASO, 2018).

Therefore, BAT, along with cold exposure, is an appealing therapeutic tool for generating weight loss and blood glucose control through energy expenditure.

Based on this premise, a journalist from New Scientist magazine offered to volunteer in this study. She committed to take a series of cold challenges for seven days to determine if there was a change in her BAT thermogenesis as assessed with TI.

Therefore, in this study, we hypothesised that BAT thermogenesis assessed with TI would be activated by cold challenges for seven days in a healthy adult volunteer.

This study was also treated as an initial feasibility study to assess the reproducibility of measurements using a TI camera.

3.2 Methods

The ethics committee reviewed and approved the study on the 30th of June of 2014. The ethics reference number of the study was D19062014 SoM Child. The University of Nottingham funded the study.

3.2.1 Study configuration

This study was an initial on a single volunteer (follow-up study). The study consisted of two sessions: before and after the cold challenges. Each session lasted no more than two hours and followed the overview as seen in Figure 3.3.

The first session of the study took place on the 30th of January and the second on the 6th of February 2015. During both visits, thermal imaging (TI) was assessed as well as height, weight and tympanic membrane temperature. Both studies were performed in the morning, and the participant did not fast during the studies. However, she was encouraged to have the same type of breakfast for each session.

After the first session, the participant volunteered to take a series of cold challenges for seven days. These challenges involved a cold shower every morning, switching off or lowering the central heating of her house, drinking cold (iced) water throughout the day and swimming in her local outdoor pool where the water temperature was at 3°C.

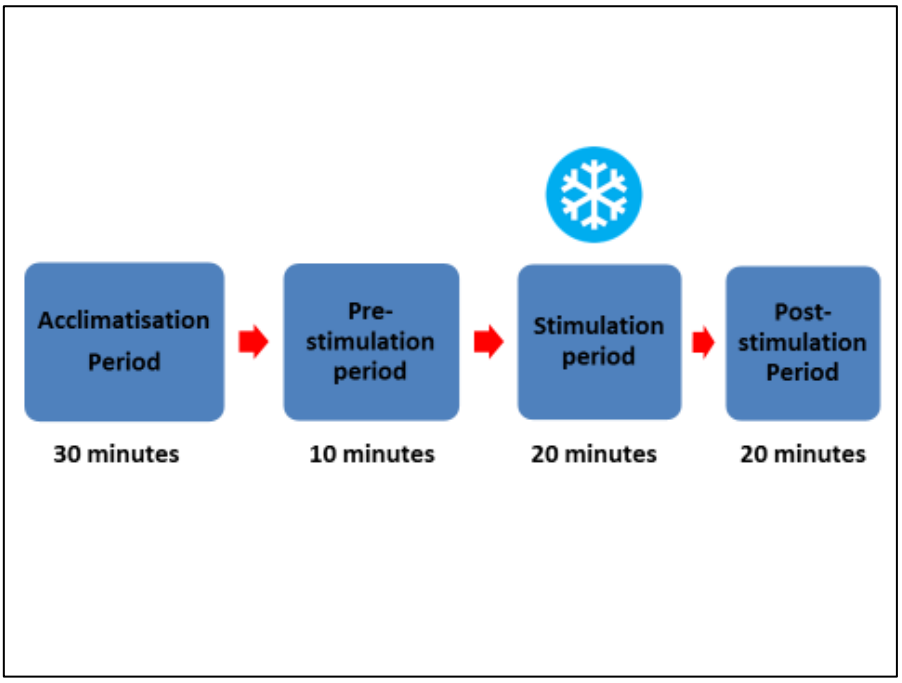


Figure 3. 1 Study overview of the cold challenges study.

The thermal Imaging method used as explained in Chapter 2.

3.2.2 Primary outcome

The primary outcome was the change in temperature (ΔT) of the tissue covering the main depot of BAT in the supraclavicular area. This change was assessed following the completion of seven days of cold challenges.

3.2.3 Secondary outcome

The second outcomes included associated changes in physical measurements: tympanic temperature and weight.

3.2.4 Statistical analysis

The results are presented in temperature of the skin overlying the supraclavicular BAT (T_{SCR}), relative temperature (T_{REL}) and area under the curve (AUC) respectively. The relative temperature can be defined as the temperature obtained from the difference between the supraclavicular area of BAT (T_{SCR}) and the surface temperature (T_{REF}) of the sternal notch. AUC is the integrated measurement of a measurable effect. The result of AUC represented a cumulative measurement of the effect of cold in BAT thermogenesis and was obtained by performing a definite integral between two points; in this case, T_{REL} and the time of assessment.

Assessments of heat production from the SCR was considered at three different times: ***resting*** or five minutes before she placed her hand in cold water, ***stimulation*** or five minutes when the participant placed her hand in the cold water; and ***post-stimulation*** or five minutes after she removed her hand from the water.

Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA). Data were assessed for normality using the Shapiro-Wilk Test. Comparisons between times of assessment were analysed using paired-samples t-test and one-way repeated measures ANOVA. To determine the reproducibility of the measurements, the coefficient of variation was calculated.

All data has been presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Baseline characteristics

The female participant was 31 years of age. Her anthropometric data, as well as the ambient conditions of both assessments, are summarised in Table 3.1.

Table 3. 1 Characteristics of the subject and the ambient conditions of the two sessions of the study.

	First session	**Second session**	**Difference**
Weight (kg)	60.8	62.3	+1.5
BMI (kg/m^2)	22	22.6	+0.6
Tympanic Temperature (°C)	37	36.9	-0.1
Room Temperature (°C)	21	20.5	-0.5
Ambient Temperature (°C)	7.1	6.9	-0.2

"*Difference"* represents the difference in the value of each parameter from the first to the second session. The + sign represents a gain and the– sign represents a loss.

3.3.2 Reproducibility of measurements using the temperature of the supraclavicular region (T_{SCR})

As mentioned in the introduction of this chapter, this study was used to assess the reproducibility of measurements using the TI camera. For this purpose, the temperatures obtained from the supraclavicular region (T_{SCR} °C), during the whole length of the study, of the first session and after seven days of cold challenges were compared in variability using the coefficient of variation (CoV) (Hyslop and White, 2009).

On Table 3.2 is possible to see the descriptive statistics of both groups as well as their CoV

Table 3. 2 Descriptive statistics of the temperatures (T_{SCR}) obtained from the two sessions of the study.

	Number of measurements	Mean	SD	CoV
0 days	30	35.44	0.20	0.6%
7 days	30	34.95	0.11	0.3%

0 days: before cold challenges; 7 days: after seven days of cold challenges. SD: standard deviation. CoV: coefficient of variation.

To determine if there was a statistical significance between the two CoV, a Levine's test for homogeneity of adjusted coefficient of variation was applied. The result was $p<0.0001$; this means that the temperatures from the first session (0 days) are statistically different than after seven days of cold challenges and therefore, it is possible to reproduce the results using this technique.

3.3.3 Effect of cold-challenges on the temperature of the surface temperature (T_{REF})

T_{REF} (°C) significantly dropped after seven days of cold challenges as determined by paired sample t-test at the three times of assessment (resting, stimulation and post-stimulation) (Table 3.3).

Table 3. 3 T_{REF} of the participant before and after seven days of cold challenges.

	0 days	Seven days	p-value
Resting	33.71 ± 0.08	31.97 ± 0.05	<0.001
Stimulation	33.62 ± 0.04	31.56 ± 0.03	<0.001
Post-stimulation	32.44 ± 0.04	31.36 ± 0.06	<0.001

0 days: before cold challenges; 7 days: after seven days of cold challenges. Data are shown as means SEM. (paired sample t-test).

3.3.4 Effect of cold challenges on T_{REL}

There was a statistically significant increase in T_{REL} temperature as determined by paired sample t-test at the three times of assessment (resting, stimulation and post-stimulation) after seven days of cold challenges (Table 3.4).

Table 3. 4 T_{REL} of the participant before and after seven days of cold challenges.

	0 days	Seven days	p-value
Resting	1.78 ± 0.07	3.20 ± 0.03	<0.001
Stimulation	1.84 ± 0.05	3.46 ± 0.02	<0.001
Post-stimulation	2.37 ± 0.04	3.56 ± 0.05	<0.001

0 days: before cold challenges; 7 days: after seven days of cold challenges. Data are shown as means with SEM (paired sample t-test).

3.3.5 Effect of cold challenges on the area under the curve (AUC)

The area under the curve (AUC) significantly increased after the participant undertook the cold challenges (Figure 3.5).

Table 3. 5 AUC of the participant before and after seven days of the cold challenge.

	0 days	Seven days	p-value
Resting	52.65 ± 1.77	94.03 ± 0.74	<0.001
Stimulation	55.35 ± 1.14	102.49 ± 0.65	<0.001
Post-stimulation	67.30 ± 1.19	107.97 ± 0.50	<0.001

0 days: before cold challenges; 7 days: after seven days of cold challenges. Data are shown as means with SEM (paired sample t-test).

3.3.6 Activation of BAT thermogenesis from stimulation of hand in cool tap water.

The stimulus used to activate supraclavicular BAT, placement of one hand in cold water (average temperature of 17°C), significantly increased the T_{REL} and AUC of the participant at the post-stimulation period alone during the first session, or before she undertook the cold challenges (Table 3.6). However, after she underwent the seven days of cold challenges, the participant's T_{REL} and AUC significantly increased in stimulation and post-stimulation periods respectively when compared with the resting period (Table 3.7).

Table 3. 6 Increase in T_{REL} and AUC during the first session of assessment after the participant placed the hand in cold water.

	Resting	Stimulation	Post-stimulation	p value
T_{REL}	1.78 ± 0.07	1.84 ± 0.05	2.30 ± 0.04	*0.001
AUC	51.11 ± 1.17	54.50 ± 1.09	67.54 ± 0.97	*0.001

Data are shown as means and SEM (one-way repeated measures ANOVA). *Significant increase in temperature between resting and post-stimulation periods alone.

Table 3. 7 Increase in T_{REL} and AUC during the second session of assessment after the participant placed the hand in cold water.

	Resting	Stimulation	Post-stimulation	p value
T_{REL}	3.20 ± 0.03	3.46 ± 0.02	3.56 ± 0.05	*<0.001 **<0.001
AUC	94.83 ± 0.79	102.28 ± 0.53	103.02 ± 0.62	*<0.001 **<0.001

Data are shown as means and SEM (one-way repeated measures ANOVA). *Significant increase in temperature between resting and stimulation periods. **Significant increase in temperature between resting and post-stimulation periods.

3.4 Discussion

3.4.1 Effect of cold stimulus on BAT temperature

We have shown a significant effect of cold stimuli on BAT supraclavicular temperature activation in one participant (tables 3.4 and 3.5). We also have shown that the reproducibly of the assessment methods is possible (Table 3.2). Our data suggest that seven days of a diverse series of cold challenges is associated with a relative increase in T_{REL} and AUC of the supraclavicular area which is a characteristic of increased BAT activity (Jang et al., 2014b). However, T_{SCR} and T_{REF} significantly decreased after the participant took the cold challenges. This effect has also been spotted in published studies like the one performed by Chondronikola et al. (2016). The group of researchers studied 18 subjects, analysed their BAT T_{SCR} and skin temperature after cold acclimatisation, and found that when subjects were exposed to a cold environment (CE) for six hours, there was a negative change (decrease) in T_{SCR} of BAT and skin temperature. Nonetheless, BAT volume was significantly associated with T_{SCR} when participants were adjusted for age and adiposity in the same study. In our results, when T_{SCR} was adjusted with T_{REF} we obtained T_{REL} which is a validated method to assess BAT activity (Law et al., 2018b). When the whole body of a subject is exposed to a CE there is a reduction in peripheral blood flow and stimulation of the sympathetic nervous system, and consequently, a decrease in the body's skin temperature (Marriott BM, 1996) like in the case of the participant of the study. The adaptation to cold takes approximately two weeks, and after that, the responses to cold are diminished (Rintamaki, 2007).

Although significant, it is important to note that the study was performed in just one person and the ΔT_{REL} resting was of 1.43°C with an SD of ±0.39. This number is much higher than the previous published data from our department when a cold stimulus was implemented, showing a change in temperature of 0.3°C with an SD of ± 0.36 (Robinson et al., 2014). The previous studies have involved just the cold stimulus, but this was a week of cold challenges, hence, maybe it produces a lasting upregulation of BAT. To secure an ideal simple size to reject the null hypothesis, I used the resting ΔT_{REL} and SD obtained from this study; the sample size obtained to have a significant statistical power from this study was of three pairs

of subjects. The ΔT_{REL} of resting was chosen because one of the aims of the study was to determinate if the cold challenges could increase BAT supraclavicular temperature T_{REL} without the need of a stimulus, or in this case, placing the participant's hand in cold tap water.

The stimulus used as an activator of BAT thermogenesis in the study, which was the participant's hand immersed in cold water for 20 minutes, increased the T_{REL} and AUC temperature of the participant. These findings support other studies that have shown that the exposure to mild cold increases the thermogenesis of BAT (Blondin et al., 2015, van der Lans et al., 2013). However, the amount of exposure in those studies was longer (>60 minutes), and was assessed with PET-CT instead of TI.

3.4.2 Clinical importance of the results obtained in the study

The importance of increasing BAT activity in humans is related to the increase in EE, as explained in the introduction of this chapter (Figure 3.2), this could have a potential beneficial effect on the metabolism of glucose and lipids regardless of the effect on body weight. The same effect, raise in BAT activity, has been proved in studies conducted in animal models under cold-induced circumstances; the studies have demonstrated that the increase in interscapular BAT temperature can help to control glucose and triglycerides (Labbé et al., 2015, Bartelt et al., 2011b). As a result, studies using cold acclimatisation have shown positive results in improving insulin resistance in patients with type 2 diabetes (Hanssen et al., 2015). In the latter research, which included cold acclimatisation for 10 days in patients with type 2 diabetes, an increase in peripheral insulin sensitivity by 43% was noted. Type 2 diabetes is a chronic disease which main characteristic is that the β-cells of the pancreas do not secrete enough insulin to keep up with the demand; this process leads to an increase of glucose in the blood (Skyler et al., 2017) and can lead to complications in the kidneys, eyes and heart. One suggestion for future studies would be to include an analysis of blood glucose and triglycerides, measuring a change in these parameters after the cold challenges to evaluate key trends.

3.4.3 Limitations of this study

The increase in T_{REL} and AUC BAT temperature did not translate into weight loss. On the contrary, the participant gained 1.5 kilograms (Table 1). During the second day of assessment, she mentioned that in addition to the external cold temperature owing to winter, the cold challenges themselves made her hungrier and she subsequently consumed more food. Several human studies have demonstrated that performing outdoor physical activities during winter can stimulate energy intake compared with warmer environments (Charlot et al., 2017, Crabtree and Blannin, 2015). This phenomenon is due to the increase in ghrelin, a hormone that stimulates appetite (Sakata and Sakai, 2010). However, the length of the exposure to cold can also have an influence on the levels of food intake. Short-term mild cold exposure increases EE but without changes in food intake; on the other hand, mild cold exposure to cold results in thermal discomfort and increase in energy intake (Langeveld et al., 2016). Nevertheless, according to the Pennington Biomedical Research Centre's weight loss/gain calculator (Hall et al., 2011), which predicts weight gain or loss based on the first law of thermodynamics which states that "the energy stored is equal to the difference of rate of energy intake and the rate of energy expanded" (Heymsfield et al., 1991). According to the calculator, the participant's daily intake should be of 2,396 calories per day; however, in order to gain 1.5 kilograms in seven days, as she did, she needed to consume 3240 kilocalories per day. It is hard to believe that there was an error on the scale where she was weighed as this scale was calibrated. In addition, for the purpose of this study, there was no assessment of the participant's diet prior to the study and during the length of it. This could have made a big difference as with a proper dietary assessment, the researcher could have noticed that in fact, the diet was an influential factor for the weight gain, which could have been due to a different factor like water retention in an alternate scenario.

One particular reason why the participant could have gained that amount of weight in such a short period was that females could gain weight due to the effects of the menstrual cycle. Previous studies have registered a significant increase in body weight particularly prior to menstruation (Kirchengast and Gartner, 2002) or during the first half of the menstrual cycle (Eck et al., 1997); these gains are due to the increase in progesterone and oestrogen that

makes the body retain fluid (Miller, 2016). A suggestion for future studies will be to assess body composition, body fat percentage and body water, before and after the cold challenges with a bioelectrical impedance (BIA) to report any difference in both variables (Bera, 2014). The process in which BIA works are explained in Chapter 2 of this thesis: materials and methods.

Another important limitation was that we could not verify the amount of cold stimulation the participant undertook in the cold challenges as she did not register them in a diary. The length of cold challenges and their effects vary in the literature. Chondronikola et al. (2014) demonstrated that the exposure to cold from 5 to 8 hours a day can increase EE by 15% in participants that were BAT positive, and as a result, there was a decrease in plasma glucose. Yoneshiro et al. (2013) showed that the daily exposition to cold at 17°C for six weeks increases EE. In both studies, there was a reduction in body fat among the participants. This study was performed during winter, and recent evidence suggest that this is an influential factor in the increase of BAT thermogenesis (Gomez de la Torre Canny and Rawls, 2015). Alternately, ambient temperatures of 24°C and above decrease the accumulation of BAT (Zukotynski et al., 2009). An ideal scenario would envisage a comparison study between the cold challenges performed during different seasons of the year (namely summer and winter), to see if the variation in the outside weather influences BAT thermogenesis or not.

The article relating to this study was published in the New Scientist magazine, with the title: “Grow fat, get thin? We put brown fat to the test” (figure 3.2).



Figure 3. 2 Cover of New Scientist magazine, No.3017 (18 April 2015).

3.5 Conclusion

Seven days of cold challenges significantly increased BAT T_{REL} and AUC in a healthy adult volunteer when assessed with thermal imaging. A study with bigger sample size, 3 participants in total, is needed. A validated method for the cold challenges assessed by using thermal imaging is required, as well as a food questionnaire and a body composition analysis. These measurements would be helpful to identify differences before and after the challenges. The present study was used to set the methods for future work.

4 Does the consumption of a bifidogenic galactooligosaccharide (B-GOS) prebiotic modulate brown adipose tissue (BAT) thermogenesis in healthy adult volunteers? A pilot study.

4.1 Introduction

The three mayor short chain fatty acids are acetate, butyrate and propionate and are mainly shaped in the gastrointestinal tract through colonic bacteria fermentation of either resistant starches, dietary fibre or prebiotics (Wong et al., 2006). Once SCFA enters the circulatory system, they are metabolised by peripheral tissues and have a direct effect in the energy homeostasis and metabolism of the host (Hu et al., 2018). Therefore, SCFA has the potential to counteract diseases like type 2 diabetes and obesity (Li et al., 2017b).

BAT it is a recent recommended therapeutic tool for obesity and its related comorbidities due to its ability to generate heat and burning calories (Poekes et al., 2018).

As mentioned in the introduction of this thesis, chapter 1, the consumption of prebiotics produce SCFA and, subsequently, these fatty acids have the potential to increase the thermogenesis of BAT in animal models (Gao et al., 2009, Hu et al., 2016a, Sahuri-Arisoylu et al., 2016) through transcriptional factors (PPAR-γ and PGC-1α), as well as through G-protein coupled receptors (GPR43) (Table 4.1). However, the same effect has not been explored as yet in humans.

Therefore, in this chapter, I hypothesised that BAT thermogenesis assessed with TI would be activated by the supplementation of a GOS prebiotic (5.5 grams) for fourteen days in healthy adult volunteers. I also hypothesised that consumption of the prebiotic for fourteen days would be acceptable and feasible for healthy volunteer participants and will not modify their daily dietary habits.

Table 4. 1 List of studies which used SCFAs and its influence on BAT

Author	SCFA used/dose	Experimental animals	Result
Sahuri-Arisoylu et al. (2016)	Acetate (administrated with lipid-based nanoparticles), 4.41mM for three weeks.	Male adult mice.	Increase in thermogenic capacity of WAT. Significantly increase of UCP1 gene expression.
Hu et al. (2016c)	Sodium acetate (150 mM) in drinking water for six weeks.	6-week old male mice	Acetate stimulated mitochondrial biogenesis with an up-regulation of PPARγ, PGC-1α and UCP1 expression in BAT adipocytes.
Gao et al. (2009)	Sodium butyrate (5 grams per kilogram per day) for 16 weeks.	4-week old male mice	Increase in the gene expression of PGC-1α and UCP1 in BAT.

4.2 Methods

The ethics committee reviewed and approved the study on the 24th of August 2015. The ethics reference number of the study was E11082015 SoM CHOG PhD. The University of Nottingham funded the study and participants were not paid for their participation.

4.2.1 Bimuno Nutri-Biotic Powder

The prebiotic chosen for the study was Bimuno Nutri-Biotic Powder® (Clasado BioSciences, United Kingdom). This product is classed as a food supplement and does not have the contra-indications that are usually associated with drugs. Bimuno® is free of artificial products, preservatives, flavours and gluten. The product can be found in most UK pharmacies, and it is easy to consume as it can be dissolved in coffee, tea or cereal. As the product contains lactose, the only contradiction in using the product is that people who are intolerant to lactose should refrain from it.

Several clinical trials and in vitro studies have successfully demonstrated that adults can safely consume Bimuno®. Its non-digestible fermentable carbohydrates are thought to benefit the microbial profile of the colon (Vulevic et al., 2013, Drakoularakou et al., 2010, D. B. A. SILK, 2009, Searle et al., 2009). The particular benefits that prebiotics confers to the

host are diverse and include the maintenance of intestinal homeostasis and the control of microbial imbalance (dysbiosis). These may have beneficial effects on health (Peterson et al., 2015).

A





B

Figure 4. 1 Bimuno® packaging (box and individual sachet).

A) Outer packing. B) Sachet of 5.5 grams of product as provided to participants in the study.

4.2.2 Study configuration

This was a single-centre, observational study composed of three study sessions. During the first session, TI was undertaken, and seven sachets of the product were given in a sealed bag to the participants for them to consume (as one sachet per day) during the next seven days.

In the second session, TI was assessed, and another seven sachets were given to the participants (to be taken one per day). They were requested to come to the third and last session of TI following in a total of fourteen days of consumption of the prebiotic in total.

Each session in the study lasted no more than two hours and followed the overview in Figure 4.2.

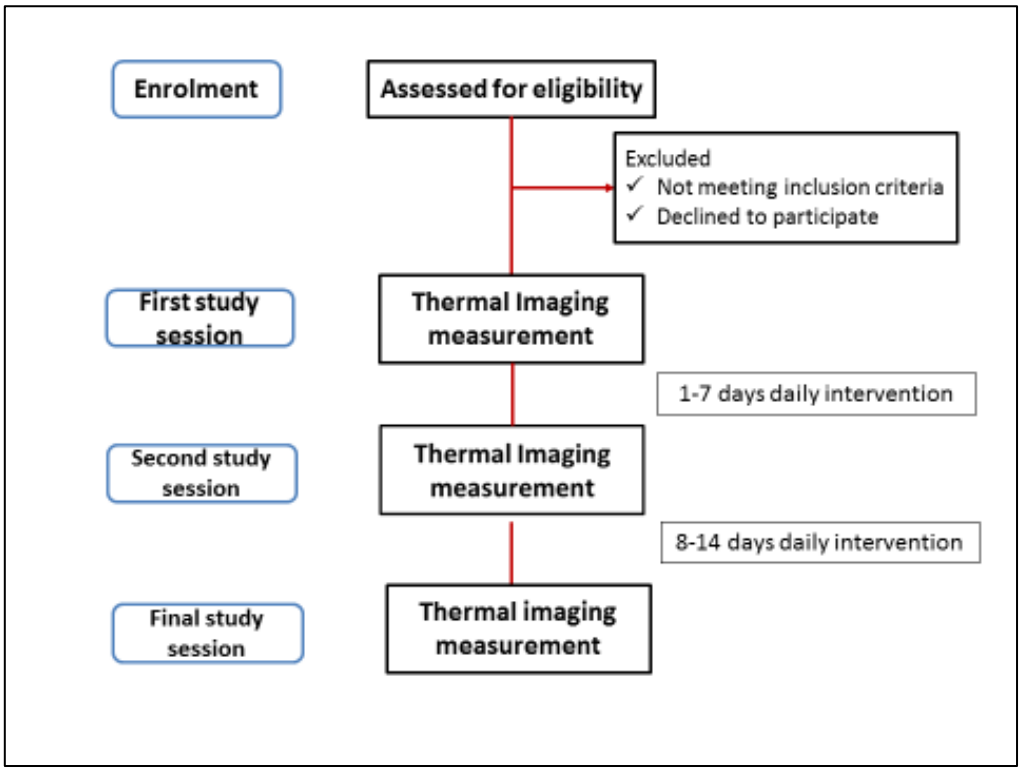


Figure 4. 2 Study overview PRE-BAT pilot study.

The figure briefly explains the study regime followed through the study.

Bimuno® claims that the effect of the prebiotic, increasing bacteria in the gut (i.e.lactobacilli and bifidobacteria), is quantifiable after just seven days of use (BIMUNO, 2017). Therefore, participants were encouraged to return for assessment after seven days of consumption and then after 14 days to reflect previous studies undertaken with this product.

The participants were evaluated between October 2015 and March 2016. One participant dropped out after the second session. However, the results obtained from this participant were included in the overall results as mentioned in the study's protocol. For this reason, I decided to do an intention to treat analysis (ITA). ITA assures that all the participants in the study are analysed within each other to represent that treatment trial (Akobeng, 2005).

4.2.3 Sample size calculation

Using data previously published by the Division of Child Health, Obstetrics and Gynaecology (Symonds et al., 2012), which demonstrated an estimated standard deviation of 0.36 for an expected change in supraclavicular temperature (T_{SCR}) of 0.3ºC fourteen participants were required to give an 80% power to detect the difference with a significance level of 5%.

4.2.4 Primary outcome

The primary outcome was the change in temperature in the tissue overlying the main depot of BAT (ΔT) in the supraclavicular area, following the consumption of GOS prebiotic. BAT activation was calculated by measuring the change in the mean temperature of the upper ten percentiles of temperature in the region of interest, the supraclavicular region, following stimulation with placing one hand in cold water.

4.2.5 Secondary outcomes

The secondary outcomes included:

- Associated changes in physical measurements: skin temperature (tympanic) and bodyweight.
- Effect of influencing factor: environmental temperature.

4.2.6 Statistical analysis

The results are presented in temperature of the skin overlying the supraclavicular BAT (T_{SCR}), relative temperature (T_{REL}) and area under the curve (AUC) respectively. The temperature of the skin overlying the supraclavicular BAT measured using TI was defined as T_{SCR} and used as an indicator of BAT activity. Mean surface temperature (T_{REF}) was evaluated at the same time using the same TI camera, but its position was located 1 cm below the sternal notch (Figure 4.3). The T_{SCR} was adjusted for the local of body surface temperature by calculating the difference between T_{SCR} and T_{REF}. This result was defined as the relative temperature (T_{REL}). AUC is the integrated measurement of a measurable effect. The result of AUC represented a cumulative measurement of the effect of cold in BAT thermogenesis and was obtained by performing a definite integral between two points; in this case, T_{REL} and the time of assessment.

In all methods, T_{SCR}, T_{REL} and AUC, three different times of assessment were considered:

- The resting period (five minutes before participants placed their hand in cold water)
- The stimulation period (five minutes when participants placed their hand in cold water).

- The post-stimulation period (five minutes after participants removed their hand of the water).

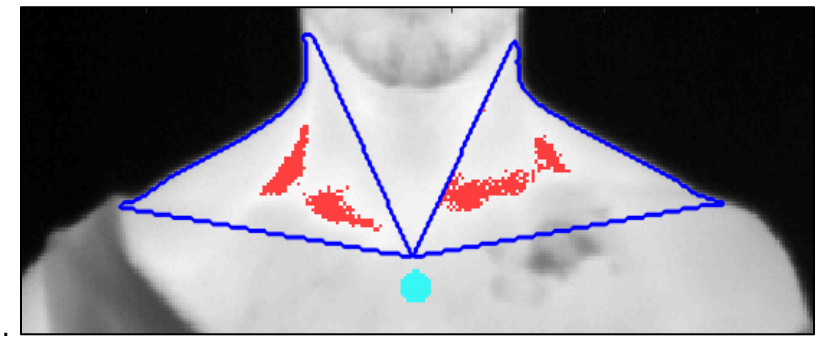


Figure 4. 3 Representative thermal image after image processing.

Blue Line: Region of Interest; Blue circle: the reference point for measurement of surface temperature (TREF); Red: the hottest point 10% of supraclavicular skin temperature (T_{SCR}). The image shows the area where brown fat is located once the analysis process is finalised. Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington,

Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA).

Comparisons between groups were analysed using one-way repeated measures ANOVA, paired sample t-test, linear regression and multiple linear regression analysis, where indicated. Data were assessed for normality using the Shapiro-Wilk Test. All data are presented as mean ± SEM. P <0.05 was considered statistically significant.

4.2.7 Compliance

All participants were asked to return unused sachets and to keep a diary of when they consumed the supplement along with which food or drink (Appendix 6 and 7). In the diary, participants noted comments regarding the attainability of consuming the prebiotic throughout the study.

4.3 Results

4.3.1 Baseline characteristics

Fifteen participants undertook the study: male (n=6) and female (n=9) with a mean age of 25.13 ± 0.70 years and a mean body mass index (BMI) of 23.21 ± 0.65 kg/m2 (by convention BMI defines the “normal weight” range as between 18.5-24.9 kg/m2) (WHO, 2011).

As the three sessions took place in a non-controlled temperature room, as expected, the ambient temperature varied between sessions. There was also a variation in external environmental temperature and the temperature of the water used to stimulate activation of BAT supraclavicular temperature. The final characteristics of the study ambient conditions are summarised in Table 4.2.

Table 4. 2 PRE-BAT study ambient conditions for the three study sessions.

Potential variable	0 (pre-consumption)	7 days	14 days
Ambient (room) temperature (°C)	21.48 ± 0.25	21.09 ± 0.34	21.27 ± 0.43
External environmental temperature (°C)	10.63 ± 0.57	10.70 ± 0.55	11.25 ± 0.79
Water (stimulation) temperature (°C)	17.77 ± 0.32	17.51 ± 0.30	17.46 ± 0.17

Data are shown as means (±SEM). There were no significant differences in any ambient characteristic between the three times of assessment ($p>0.05$: one-way repeated measures ANOVA).

The body weight (kg) (Figure 4.4), and therefore BMI, of the participants, did not significantly change with the intervention.

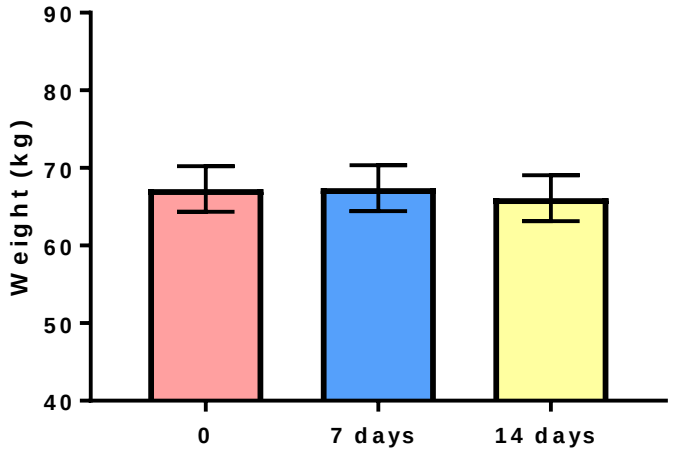


Figure 4. 4 Body weight of the participants assessed during the three sessions of the study.

0: pre-consumption; 7: seven days of consumption; 14: fourteen days of galacto-oligosaccharide (GOS) consumption. Data are shown as means, and error bars represent SEM. There was no statistical difference between the three sessions p=0.81 (one-way repeated measures ANOVA).

4.3.2 Effect of GOS-prebiotic on body temperature.

There were no effects of the consumption of the GOS-prebiotic on core (tympanic) temperature or surface (sternal) temperature (T_{REF}) (Table 4.3). Core temperature and T_{REF} are not the same. Whilst core temperature is the temperature of the inner parts of the body and was assessed with an ear thermometer, T_{REF} was the temperature of an area of the body not known to overly brown adipose tissue and representative of surface temperature, measured from the TI of the region located 1 cm below the sternal notch (surface skin temperature).

Table 4. 3 PRE-BAT study ambient conditions for the three study sessions.

Potential variable	0 days (pre-consumption)	7 days	14 days
Tympanic (core) temperature (°C)	36.73 ± 0.08	36.66 ± 0.08	36.52 ± 0.08
Surface (sternal) temperature (°C)	32.64 ± 0.23	32.50 ± 0.24	32.69 ± 0.23

Means (±SEM). There were no significant differences in either characteristic between the three times of assessment (p>0.05, one-way repeated measures ANOVA).

4.3.3 Effect of GOS-prebiotic on the temperature of the supraclavicular region (T_{SCR})

Post-hoc comparisons showed that the resting supraclavicular BAT temperature, corresponding to the average temperature of the last five minutes before each participant placed their hand in cool water, dropped after seven days of consumption of the prebiotic (0: 34.50 ± 0.10; 7 days: 34.33 ± 0.12; p=0.02). However, there was no difference in temperature after 14 days of consumption (Figure 4.5).

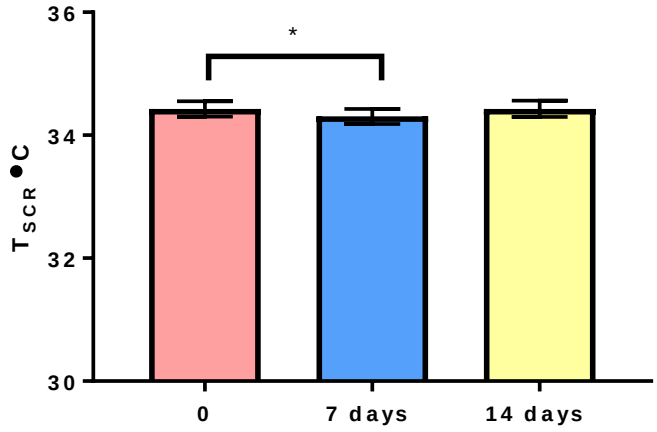


Figure 4. 5 Resting supraclavicular BAT temperature at the three times of assessment.

0: Pre-consumption; 7 days: seven days of consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption. Data are shown as means with error bars representing SEM. *p<0.05 between the first session and seven days of consumption of the prebiotic (one-way repeated measures ANOVA).

4.3.4 Effect of GOS-prebiotic on T_{REL}

There was no statistically significant difference in T_{REL} as determined by one-way repeated measures ANOVA at any of the times assessed; resting (p=0.68), stimulation (p= and post-stimulation (Figure 4.6).

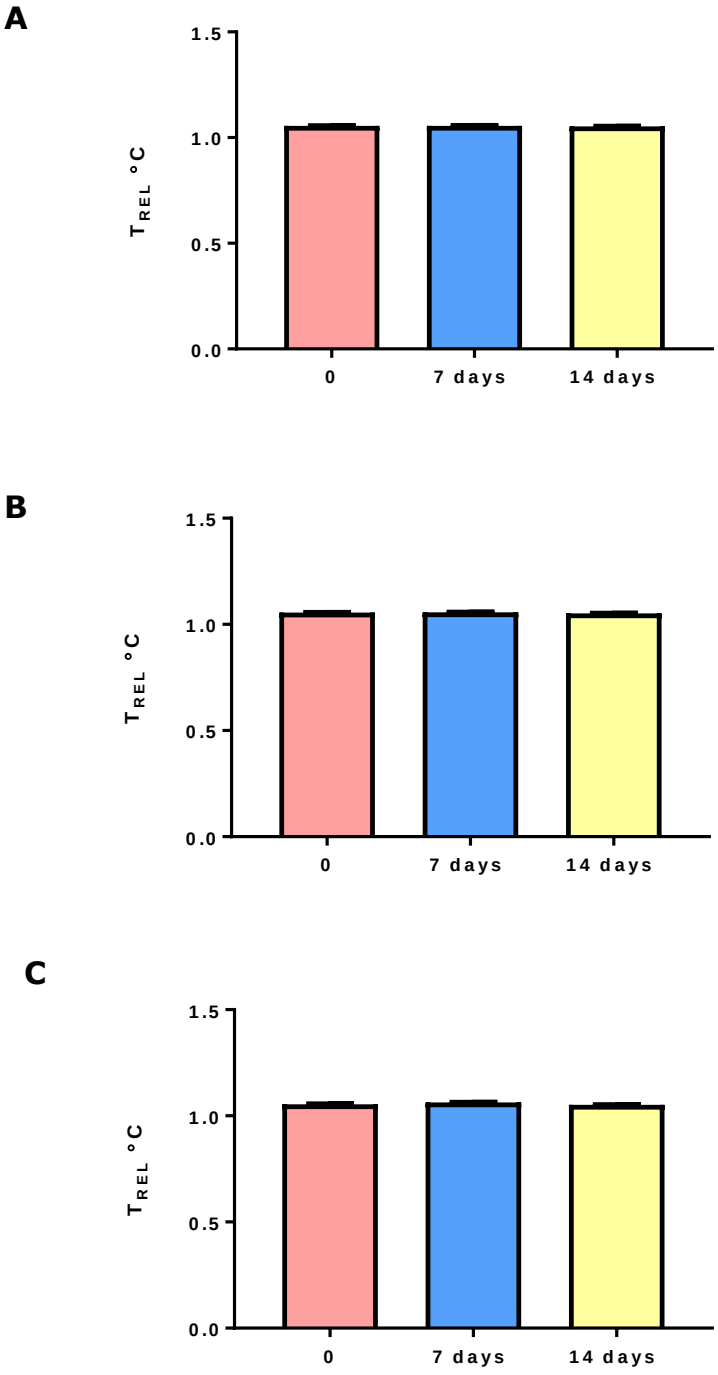


Figure 4. 6 Supraclavicular temperature relative to sternal (body surface) temperature (T_{REL}).

A) resting period (p=0.68); B) stimulation period(p=0.54); C) post-stimulation period (p=0.52). 0: Pre-consumption; 7 days: seven days of consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption. Data is shown as means, and error bars represent SEM.

Multiple regression was run to determine if T_{REL} could be predicted from external environmental seasonal and tympanic temperatures. These variables did not significantly predict T_{REL} during the first session ($p=0.32$, $R^2=0.17$), after seven days of consumption ($p=0.71$, $R^2=0.05$) or after fourteen days of consumption ($p=0.78$, $R^2=0.04$).

4.3.5 Effect of GOS-prebiotic on the area under the curve (AUC)

The consumption of the prebiotic did not increase the area under the curve (AUC) after seven and fourteen days of consumption respectively (Figure 4.7). AUC was calculated by undertaking a definite integral between two points (Chapter 2); in this case, T_{REL} and the time of assessment: resting, stimulation and post-stimulation periods.

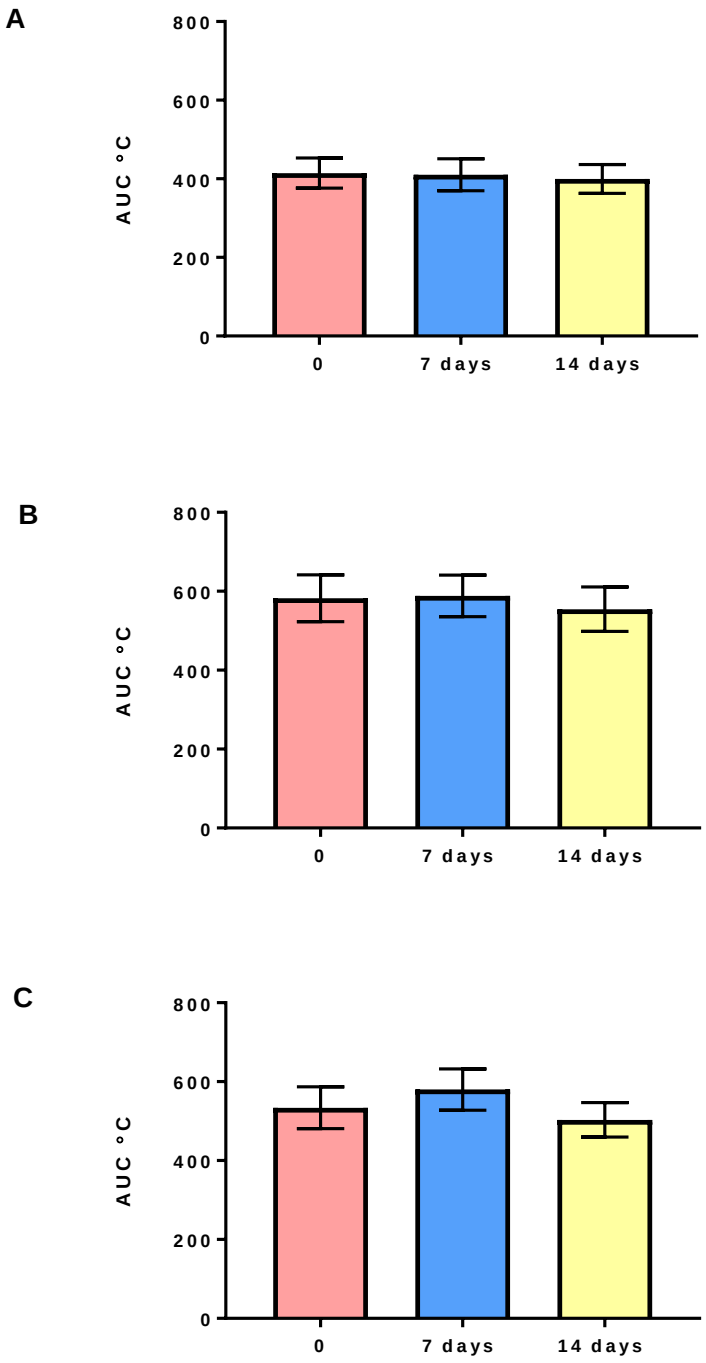


Figure 4. 7 AUC relative to sternal temperature.

A) Resting (p=0.93), B) Stimulation (p=0.78), C) Post-stimulation (p=0.28) periods. 0: Pre-consumption; 7 days: seven days of consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption. Data are shown as means with error bars representing SEM. One-way repeated measures ANOVA.

4.3.6 Differences between females and males.

As some authors have stated that women have more potential to activate BAT than men do (van den Beukel et al., 2015), a comparison between the results obtained from males and females was performed to see if there was a difference in supraclavicular thermogenic responses. Resting T_{REL} of females was statistically higher compared with males during the first session of assessment (females: 1.07 ± 0.00; males: 1.04 ± 0.00; p=0.01) and during the second session, after 7 days of consumption, (females: 1.07 ± 0.00; males: 1.04 ± 0.00; p=0.02) representing a greater difference between sternal surface temperature and the heat detected from the supraclavicular region in females than in males. However, this difference did not persist after fourteen days of consumption (p=0.06) (Figure 4.8).

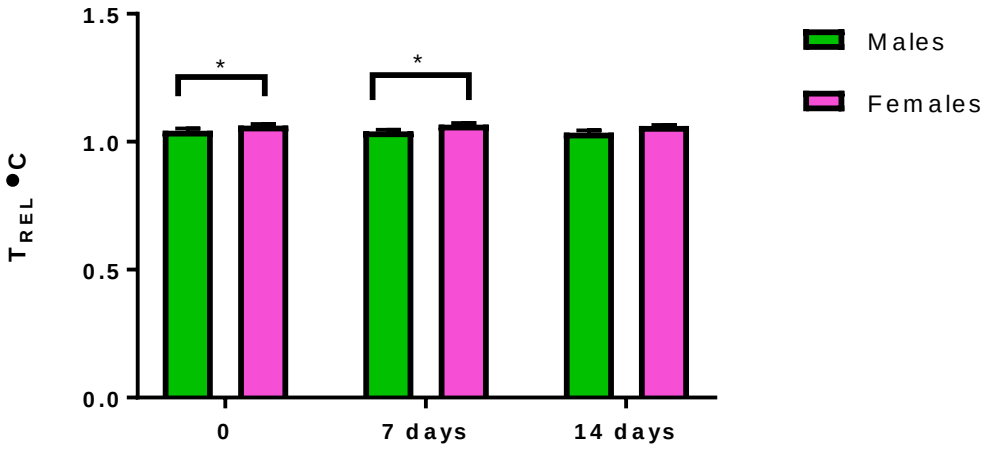


Figure 4. 8 Resting T_{REL} in males and females.

Data are shown as means with error bars representing SEM. Green: males; Pink: females. 0: Pre-consumption; 7 days: seven days of consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption. *p<0.05 between males and females during the first session and between males and females after seven days of consumption (paired sample t-test).

However, female participants had lower sternal temperatures than males and this reached statistical significance at 14 days (males: 33.37 ± 0.33; females: 31.97 ± 0.31; p=0.04) (Figure 4.9).

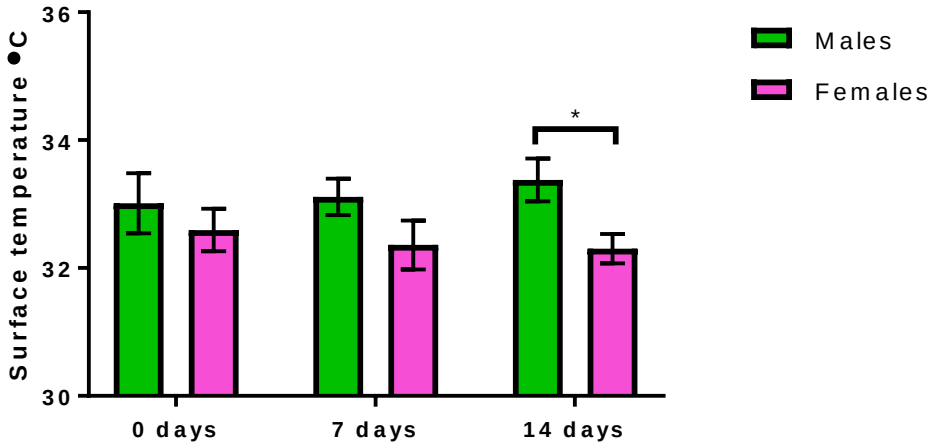


Figure 4. 9 Surface (sternal) temperature comparison in males and females.

Data are shown as means with error bars representing SEM. Green: males; Pink: females. 0: Pre-consumption; 7 days: seven days of consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption. *p<0.05 between the males and females after fourteen days of consumption (paired sample t-test).

A simple linear regression analysis was calculated to determine if whether the exterior environmental temperature influenced body surface temperature. There was no significant relationship during the first session (R^2 of 0.05, p=0.21), after seven days of consumption (R^2 of 0.04, p=0.23) or after fourteen days of consumption (R^2 of 0.07, p=0.17), respectively.

4.3.7 Activation of BAT thermogenesis from stimulation of hand in cool tap water.

4.3.7.1 Change in sternal (reference, T_{REF}), supraclavicular and relative temperature change

The stimulus used to activate supraclavicular BAT, placement of one hand in cold water, did not significantly increase participant T_{REF}, T_{SCR} and $T_{REL.}$ On the contrary, these temperatures remained the same throughout each study session (i.e. between the resting, stimulation and post-stimulation periods) as can be seen in Figures 4.10, 4.11 and 4.12 respectively.

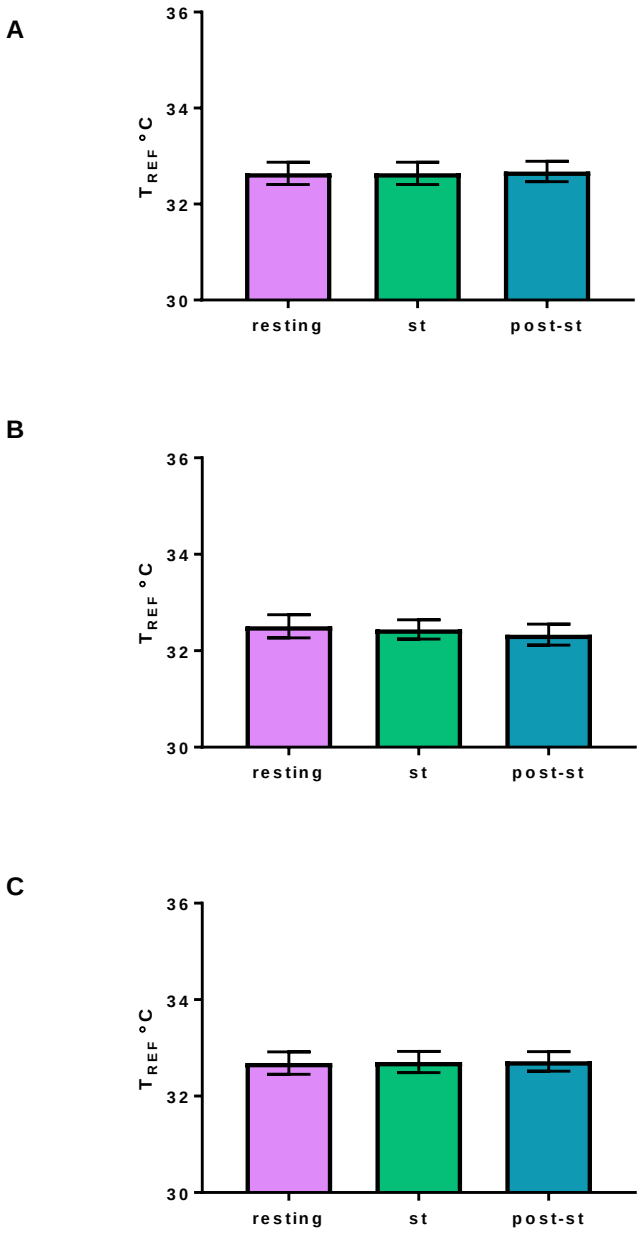


Figure 4. 10 Sternal (surface) temperature (T_{REF}) after the introduction of the stimulus (hand in cold tap water).

A) Pre-consumption (p=0.90), B) Seven days of consumption (p=0.34), C) Fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption (p=0.85). Stimulation period: st; post-stimulation period: post-st. Data are shown as means with error bars representing SEM. One-way repeated measures ANOVA.

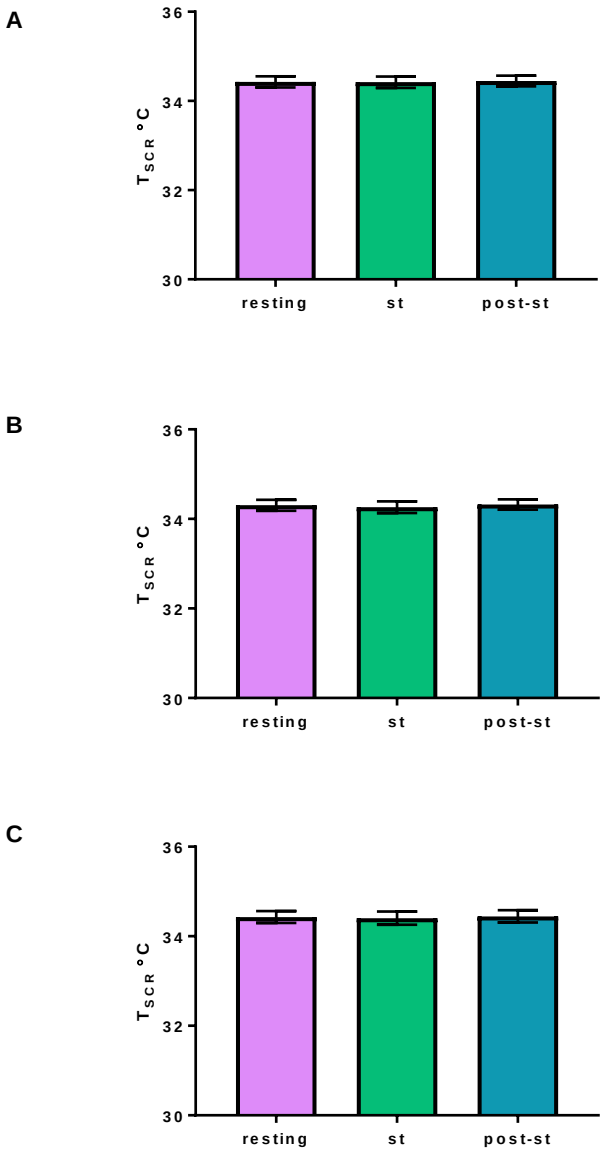


Figure 4. 11Supraclavicular BAT temperature (T_{SCR}) after the introduction of the stimulus (hand in cold tap water).

A) Pre-consumption (p=0.81), B) Seven days of consumption (p=0.17), C) Fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption (p=0.84). Stimulation period: st; post-stimulation period: post-st. Data are shown as means with error bars representing SEM. One-way repeated measures ANOVA

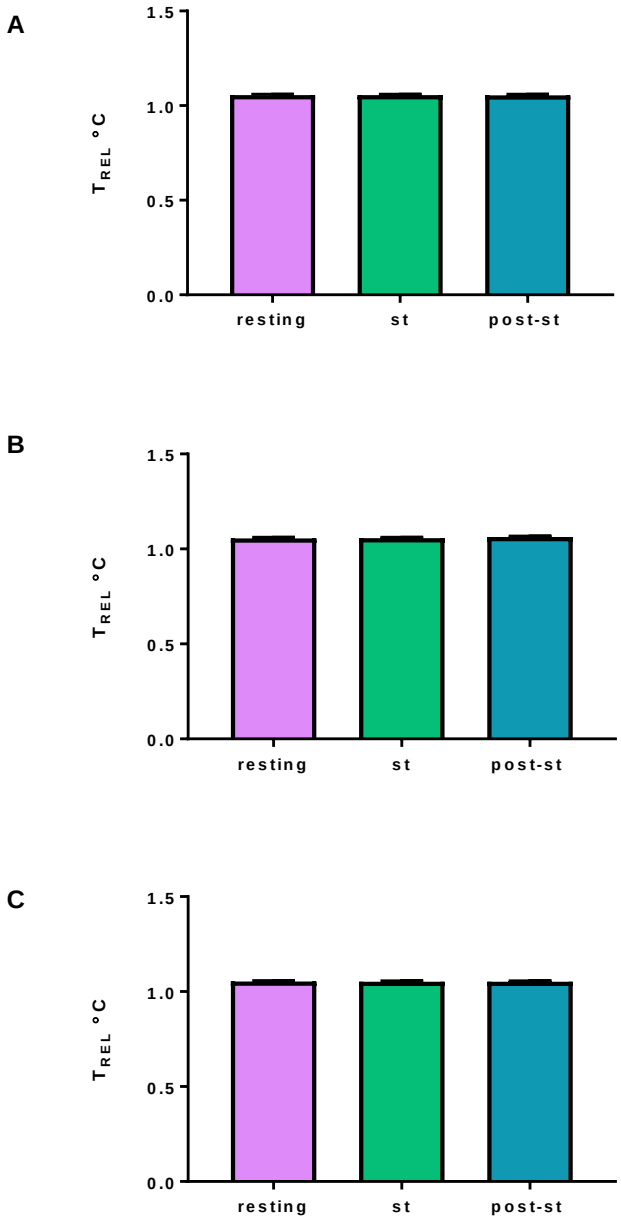


Figure 4. 12 Supraclavicular temperature relative to sternal (body surface) temperature (T_{REL}) after the introduction of the stimulus (hand in cold tap water).

A) Pre-consumption (p=0.96), B) Seven days of consumption (p=0.17), C) Fourteen days of galacto-oligosaccharide prebiotic (GOS) consumption (p=0.84). Stimulation period: st; post-stimulation period: post-st. Data are shown as means with error bars representing SEM. One-way repeated measures ANOVA.

However, the stimulus (hand in cold water) significantly increased the area under the time-

T_{REL} curve between the resting period and both the stimulation and post-stimulation periods

at all three study visits: first session, after seven days and after 14 days of consumption of the GOS prebiotic (Figure 4.13).

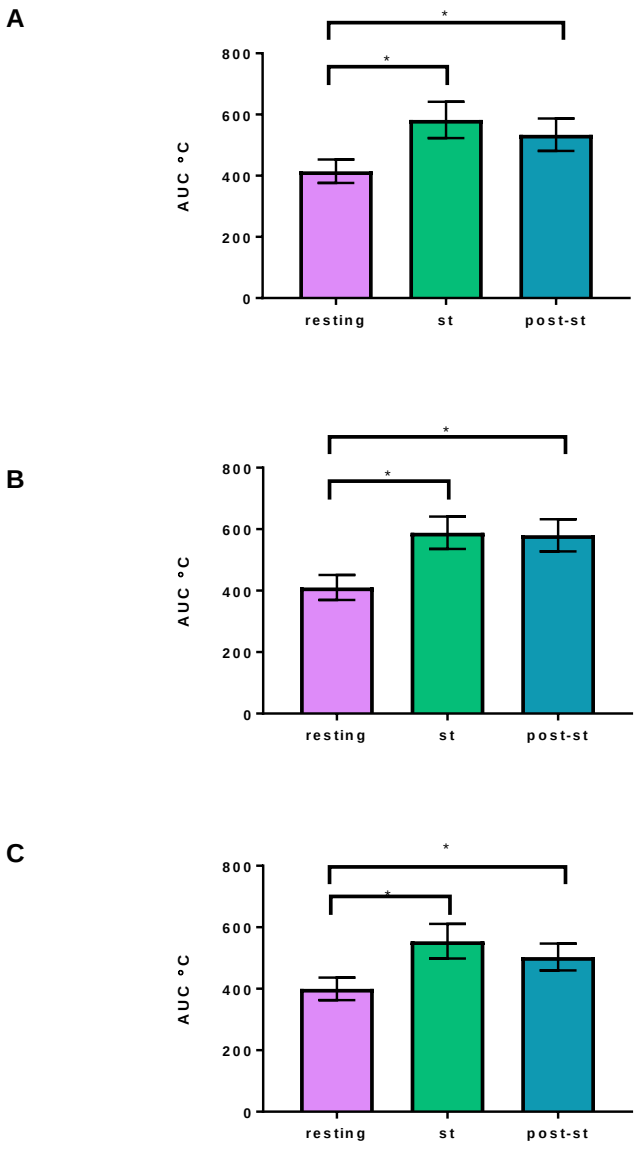


Figure 4. 13 Area under the time-$_{TREL}$ curve after the introduction of the stimulus (hand in cold tap water).

A) Pre-consumption (resting: 414.59 ± 38.26 and st: 582.49 ± 59.41; p<0.001; resting: 414.59 ± 38.26 and post-st: 533.89 ± 53.08; p<0.001); B) Seven days of consumption (resting: 410.33 ± 40.40 and st: 588.20 ± 52.67; p<0.001, resting: 410.33 ± 40.40 and post-st: 580.20 ± 52.35; p<0.001); C) Fourteen days of consumption (resting: 399.85 ± 36.55 and st: 554.47 ± 56.44; p<0.001, resting: 399.85 ± 36.55 and post-st: 503.04 ± 43.68; p<0.001). Stimulation period: st; post-stimulation period: post-st. Data is shown as means with error bars representing SEM. One-way repeated measures Anova. *p<0.05 between resting and stimulation period and between resting and post-stimulation period.

4.3.8 Participation in the study

Fifteen participants were contacted via e-mail, and the same number attended the first study session. Once enrolled in the study, continued participation was encouraged through electronic messaging, i.e. an email was sent to the participants three days before each study session to remind them about their attendance to the study. Of 15 participants enrolled in the study, one participant attended only two of the planned three sessions, so that 93% of the recruited participants completed all three sessions.

The participants of the study received a diary in the first and second sessions for completion over the following week. All participants completed the food diary and reported that they had consumed the product. None of the participants returned unused sachets. One participant recorded a potential minor effect of the prebiotic as “an upset stomach” during the first day of consumption but reported that he persisted with consuming the product daily on subsequent days without symptoms on subsequent days

4.3.8.1 Consumption of the prebiotic

The food diary collected after the second session (after seven days of consumption) was used to collect information from participants on the feasibility of taking the prebiotic in the way requested in the study. 93 % agreed that the product was easily mixed with food and drink. 93 % agreed that the product was tasteless. 86 % of participants agreed that consumption of the prebiotic did not represent a challenge in their everyday life.

The food diary was also used to record with which food or drink (coffee, tea, cereal or other) participants mixed the prebiotic. This approach to taking the Bimuno® product is that recommended by the manufacturer to obtain its maximum benefits. However, the company also states that the prebiotic can be mixed with any beverage, hot or cold and can be used in cooking or baking as well, as it is an insoluble fibre, it is heat and acid stable even at very high temperatures. It can also be used with normal water or even placed directly in the mouth (BIMUNO, 2017).

After seven days of consumption of the prebiotic, 54% of the participants reported that they had consumed the prebiotic mixed with tea, 26% with cereal, 13% with coffee and 7% with another food or drink. After 14 days of consumption of the prebiotic, tea remained the most common method of consumption (55%), followed by 15% in each of the coffee, cereal or another. The caffeine contained in the coffee and tea do not inhibit the power of the GOS prebiotic to increase good bacteria in the gut as well as SCFA, at least in animal models (Nakayama and Oishi, 2013).

4.4 Discussion

4.4.1 The effect of GOS prebiotic on BAT temperature

We have shown that the consumption of GOS prebiotic does not increase BAT temperature (measured as either T_{REL}, AUC, T_{SCR}) after 7 or 14 days and, on the contrary, the temperature of BAT assessed with different methods fell after the consumption of the prebiotic. These results contradict our initial hypothesis, and there are several possible explanations for this.

The first aspect involves the production of SCFA by the gut microbiota after the consumption of the prebiotic. Gut microbiota is considered a unique organ and differs from person to person (Moya and Ferrer, 2016). It is very much like a fingerprint, and its diversity and composition is influenced by many factors as mentioned in the introduction of this Chapter (i.e. diet, exercise, weight, health status, antibiotic usage, geographical location, stress, and age). Participants of the study were not separated according to those factors. However, before they were included in the study, we certified that they were not currently taking antibiotics and had not had antibiotic treatment in the month before starting the study. Antibiotics are considered one of the main disturbances to the structure of the microbial community (dysbiosis) in the gut microbiota (Petersen and Round, 2014). In addition to antibiotics, other medications, such as metformin (Rogers and Aronoff, 2016), proton pump inhibitors (Imhann et al., 2016), non-steroidal anti-inflammatory drugs (Rogers and Aronoff, 2016) and antipsychotics (Flowers et al., 2017) can also disturb the gut microbiota. However, during the length of this study, participants were asked in each session if they were taking any medication during the study (Appendix 4) and no participant reported medication use.

The second reason why the consumption of the prebiotic might not have have affected supraclavicular temperature is that it is possible that not all the participants involved in the study were BAT positive. Although our inclusion criteria was that all participants should have a normal BMI and be less than 30 years old (at the time of the study) as these two characteristics are important in the prevalence of BAT in adults (Cypess et al., 2009, Yoneshiro et al., 2011) there are other factors to be considered. The amount of fatty tissue

can directly affect the amount of visible BAT when assessed with TI, and the ageing process produces additional, notable changes in body composition. In this study, the body weight of the participants remained stable during the entire period of the trial. In the next chapter (PRE-BAT randomised controlled trial), we will assess the subject's body composition (specifically body fat percentage) in addition to body weight and BMI, as these two are less acceptable for measuring the fat percentage of an individual (Padwal et al., 2016).

The third reason was that objective measurements of compliance were not undertaken in this study, so it is not possible to be certain whether the participants consumed the entire prebiotic supplement provided. One method that reflects the gut microbial composition is the analysis of faecal samples. This method explains on average 67.7% (± 18.8%) the variance of gut microbiota's constitution (Zierer et al., 2018). However, measuring stool samples is expensive and volatile acids are hard to measure in free-living individuals, as measures have to be made on fresh samples (Torii et al., 2010). Asking for biological samples would have also decreased the participation in the study.

Although in animal studies the increase in BAT temperature and UCP1 can be observed after cold acclimatisation (Gao et al., 2009), in this particular study we did not observed that change after participants placed their hand in cold water (Figures 4.10, 4.11 and 4.12). However, the thermogenesis of BAT increased after the cold stimulus when assessed with AUC (figure 4.13). As previously explained in this chapter, AUC represents the average concentration of BAT thermogenesis over a time interval, or in this case, the stimulation and post-stimulation periods. Thus, it reflects the actual body exposure to the stimulus after its administration (Lappin et al., 2006). These findings support the idea of other researchers that have found that the exposure to mild cold increases the thermogenesis of BAT (Blondin et al., 2015, van der Lans et al., 2013). Nonetheless, the amount of exposure in those studies was longer (more than 60 minutes) and was assessed with PET-CT; this study proved that it is possible to see a difference in BAT temperature when assessed with TI and with shorter exposure to cold when compared with resting temperature.

The reasons why some of the participants' temperature dropped after the consumption of the prebiotic are mentioned above. However, the results obtained by our study agree with the findings of one specific trial performed by Thompson et al. (2016). The group of researchers found that the consumption of prebiotics, specifically GOS, decreased the diurnal temperature in mice due to the increase in the specific phylum of bacteria in the GI. They also found that GOS attenuated the impact of stress on diurnal physiology. Further research should be performed to investigate the effect of prebiotic consumption in the temperature of humans and if this decrease in temperature affects stress.

4.4.2. Conducting a study utilising a food supplement and thermal imaging

This study was our first one that assessed the influence of a dietary supplement on BAT assessed with TI. It was feasible for the reasons that it was acceptable to the participants, there was little dropout (one participant), I was able to recruit the participants in a less than estimated time, and the three visits of the study were acceptable to the participants. However, in the upcoming study (Chapter 5, PRE-BAT randomised controlled trial), an inconvenience allowance will be provided to encourage participation and facilitate the recruitment of an adequate number and type of subjects (Grady, 2005).

4.4.3 Methods of analysis of any BAT thermogenic responses measured on TI

In this study, T_{REL} and AUC were used to analyse the influence of the GOS prebiotic on BAT although previous studies (Symonds et al., 2012, Robinson et al., 2014) analysed the effect of a cold stimulus on the supraclavicular temperature of BAT (T_{SCR}) alone.

T_{REL} and AUC were chosen as absolute $T_{SCR,}$ and body surface temperature (here defined as T_{REF}) vary from one participant to another due to resting metabolic rate, energy exchange with the environment, self-heating, emissivity and reflection. T_{REL} (T_{SCR} and T_{REF}) allowed the researcher to control those differences by describing T_{SCR} relative to body surface temperature. The area under the time-temperature curve (AUC) was chosen to assess any effects of the food supplement or cold-water stimulation over a time interval (Chapter 2, materials and methods).

4.4.4 Differences in temperature between males and females

In our study, we found that the T_{REF} of the male participants was lower than that of the females, specifically when assessed during the first session and after seven days of consumption (Figure 4.4). However, the surface temperature of the female participants dropped after 14 days of consumption of the prebiotic compared with the males in whom it remained the same. According to published data, the highest body temperature in young men and women is found in the chest when assessed with a thermal camera (Chudecka and Lubkowska, 2015). Men, in general, have a greater muscle mass than women, helping them to generate heat; this is why they have higher surface temperatures than their female counterparts do. On the other hand, women in response to cold surroundings, reduce their peripheral blood flow to the skin and extremities more than men (Kaciuba-Uscilko and Grucza, 2001). In general, the data obtained didn't show any pattern between the results obtained for males and females.

In contrast to this, in our study, the outside (ambient) temperature did not influence the surface temperature of the participants. All the participants were assessed in autumn, winter and early spring in England when the atmospheric temperature is low and according to published data (Au-Yong et al., 2009, Wang, 2009, Wang et al., 2016), this influences BAT thermogenesis. In contrast, in this study, the atmospheric (ambient) temperature did not influence the T_{REL} of the participants. It will be interesting to perform a study in which participants are assessed in different seasons to verify if there is a variation in BAT thermogenesis measures as T_{REL} or AUC with TI as there is with PET-CT (Au-Yong et al., 2009).

4.4.5 Limitations of this study

Our study has several limitations. The first one was the number of participants. We calculated that we would need to recruit a sample size of 14 participants using previously published data from our department (Robinson et al., 2014) of a change in BAT temperature of 0.3°C. However, we observed no change in T_{REL} (0.0°C) with the same stimulation of one hand being placed in cold water. Indeed, we also observed a change of -0.08°C in T_{REL} in

response to 14 days consumption of the prebiotic. Therefore, thus, for this particular study, bigger sample size could have been more appropriate. In the upcoming chapter (Chapter 5, PRE-BAT randomised controlled trial), which compares the use of the prebiotic with a control group and a placebo group, our chosen sample size is of 63.

The reason why participants acted as their own controls was that I wanted to measure BAT temperature of the same participants at different time points (paired data) with the intervention of the GOS-prebiotic (Newcombe, 1998). Nevertheless, it was necessary to add a control group in the upcoming study, firstly because a control group is part of the scientific method and secondly because is essential to be used as a benchmark to measure the results of the experiment group (Pithon, 2013).

For the analysis of data, participants were not divided into responders (participants whose T_{REL} and AUC increased after the consumption of the prebiotic) and null responders (participants whose temperature dropped after the consumption of the prebiotic). Participants do were analysed according to their gender because some studies have demonstrated that women tend to have a higher quantity of brown fat than men (Haupt et al., 2010, Ludescher et al., 2007), although in this particular study the number of females exceeded the males.

4.5 Conclusion

In this study, the aim was to assess the possible increase in supraclavicular BAT temperature after the consumption of GOS prebiotic assessed with TI. The study's main finding was that there was no change in temperature of BAT supraclavicular when evaluated with T_{REL} and AUC. While our hypothesis was null, this was the first study to report the use of a GOS prebiotic and its influence on BAT supraclavicular temperature in healthy adults assessed with TI. Although the findings were limited by factors like sample size and study design, this pilot study recruited and retained participants who found the intervention acceptable and provided information to inform a further study (Chapter 5). A study with bigger sample size and a randomised double-blind controlled trial will be performed in the upcoming chapter of this thesis to demonstrate causality and reduce bias.

5 Does the consumption of a bifidogenic galactooligosaccharide (B-GOS) prebiotic modulate brown adipose tissue (BAT) thermogenesis in healthy adult volunteers? A Randomised control trial.

5.1 Introduction

This study is a continuation of the previous chapter (Chapter 4, pilot study). The main difference is in that this study, two study groups were added to the investigation, one that took a placebo and one that did not consume anything at all. It was a double-blinded randomised controlled trial (Pithon, 2013) and designed to be an improvement on the pilot study as well as to mitigate some gaps. It also aimed to include equal numbers of male and female participants and included a control group (Kinser and Robins, 2013) that was performed in the Academic Imaging Suite, which was thermally controlled (at 22°C). The continuation of the study was attainable because participants of the feasibility study mentioned in their compliance that the product was easy to take. Also, the consumption of the prebiotic did not represent an extra challenge regarding their daily routine and the taste of the product was imperceptible and easily mixed with their drinks or food. In this study, I provided an inconvenience allowance to the subjects involved in the research, which should encourage participation and facilitated the timely recruitment of an adequate number and type of subjects (Grady, 2005).

The placebo used was maltodextrin which was provided by the same company that provided us with the prebiotic, Clasado Ltd®. However, it is important to mention that the company did not fund this research.

5.1.1 Maltodextrin

Maltodextrin is a type of carbohydrate extracted from a wide range of natural sources, specifically from starch (Hofman et al., 2016), by partial hydrolysis. Once digested, its end product is glucose and provides the body with the same energy value as cane sugar (4 kcal/g) (Livesey, 2007).

Maltodextrin has been approved by the Food and Drug Administration (FDA) and the European Food Safety Agency (EFSA) as a safe food additive (EFSA, 2013). The regulation

made by the European Parliament (no.1169/2011) on the 25th of October of 2011, and published on Annex II, states that maltodextrin does not produce allergies and intolerances and that is a source of gluten-free carbohydrates (EUR-Lex, 2018). Usually, in the form of a white powder, maltodextrin is similar in appearance to Bimuno Nutri-Biotic Powder®, the prebiotic used in this research. The product was easily digestible, almost flavourless and commonly used in food products such as soft drinks, infant nutrition, clinical nutrition (applied in enteral and parenteral nutrition), oral rehydration drinks and sports drinks (Jennings, 1995).

Maltodextrin is a sugar (5.5 g of the product is the equivalent of 1 teaspoon of sugar) and while this is not harmful to healthy volunteers, people with diabetes and those who are glucose intolerant were excluded from the study. Maltodextrin is commonly used as a placebo for galacto-oligosaccharides studies (D. B. A. SILK, 2009, Vulevic et al., 2013, Vulevic et al., 2008) and therefore, has been selected for use in this randomised study in healthy volunteers. However, although maltodextrin was used as a placebo, its effects on BAT are unknown and, therefore an additional non-intervention group for comparisons with maltodextrin and prebiotic groups in this study was used.

5.2 Methods

The ethics committee reviewed and approved the study on the 3rd of March of 2016. The ethics reference number of the study was E11082015 SoM PhD. The University of Nottingham funded the study and participants received a small inconvenience allowance after their participation (second session of the study).

5.2.1 Study configuration

This was a randomised, double-blinded study of the consumption of prebiotic or maltodextrin compared with no supplement (as randomly assigned). A classic cross-over design was not selected as the duration of prebiotic effects on gut microbiota has not previously been studied in sufficient detail, and long-lasting effects on gut flora have been suggested by some authors (Slavin, 2013). At the first session, participants received 14 sachets of prebiotic (Bimuno Nutribiotic Powder®), maltodextrin or no supplement (as randomly assigned) and

went through the thermal imaging (TI) procedure. The sachets were given in a white sealed box and separated by maltodextrin or prebiotic by an independent researcher. Outside the box, the randomised number of each participant was printed so the main researcher could identify which box was given to each participant (Figure 5.1). The instructions on how to take the product were inside the box, although they were also given to each participant verbally.



Figure 5. 1 Box and sachet used in the study.

Each participant, according to the randomisation, received this box with 14 sachets inside. Participants were asked to take one sachet a day before repeating imaging on the 14th day of the study (second study session). The participants assigned to the “no supplement” group were just asked to return for repeat imaging on the 14th day of their second session. Each session in the study lasted no more than two hours and followed the overview as seen in Figure 5.2.

Participants were advised not to eat or drink, water was only allowed, and to refrain from exercise such as cycling and to walk briskly for the 8 hours before each study session as these may affect the reliability of thermal imaging measurements. The effects of the prebiotic/maltodextrin/no supplement were assessed by comparing the differences between

BAT between the first and second session (Schulz et al., 2010). All participants were asked to return unused sachets and to keep a diary of when they consumed the supplement along with which food or drink, if applicable (Appendix 8).

A further difference between this study and the feasibility one is that in this one, the body composition was measured in both sessions using a tetra polar bioelectrical impedance (BIA). BIA is a non-invasive, portable and low priced artefact which assess body composition (body fat percentage, free fat mass and body water) (Khalil et al., 2014). When compared with another method of assessment like body mass index (BMI), skin fold thickness, dilution method and dual-energy- energy x-ray (DEXA), BIA is more precise in determining fat mass (Heitmann, 1994) and does not require a trained practitioner to execute it, the person just needs to follow up the protocol and procedure of the machine. The protocol is explained extensively in Chapter 2 of this thesis (Materials and Methods), as well as the fundamentals of BIA measurement technique. In this study, the Maltron BF-906 Body Fat Analyser (Maltron International Ltd, 20 Sirdar Road, Rayleigh, Essex SS6 7XF UK) was used. This device has been utilised in some published articles (Doornweerd et al., 2016, Muskiet et al., 2016, ter Horst et al., 2016) and is conformed to ISO standards (ISO 9001-2000).

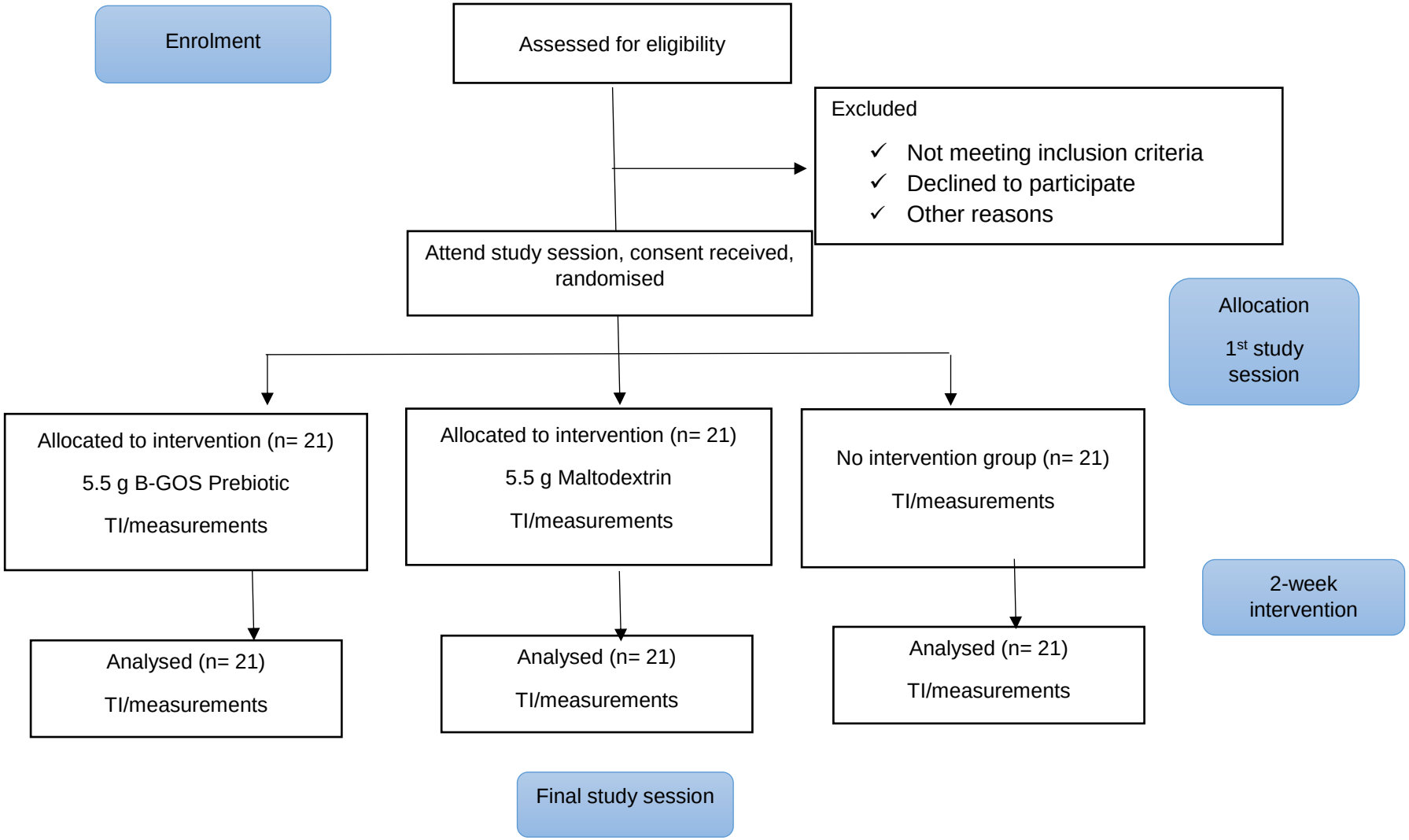


Figure 5. 2 Study overview PRE-BAT randomised controlled trial.

5.2.2. Sample size calculation

Using data previously published by the Division of Child Health, Obstetrics and Gynaecology (Symonds et al., 2012) which demonstrated an estimated standard deviation of 0.4 for an expected biologically significant change of 0.3°C, 21 participants were required in each group to reject the null hypothesis with probability (power) 0.9 with a Type 1 error probability of 0.05.

The reason why the sample size was calculated with previously published data from the department was that on the previous chapter, pilot, no change in temperature was observed after the consumption of the prebiotic on T_{REL} (0.0°C).

5.2.3 Randomisation of the sample size

To randomise the sample size, the package “blockrand” on R software was used. The program creates randomisations for block random clinical trials. The randomisation was performed within blocks because it creates a balance between treatments and, therefore, the blocks stay equal throughout the trial.

The components of the formula, for the determination of the randomisation, were:

- n: minimum number of subjects to randomise (21).
- Num .levels: the number of treatments or factors to randomise between (3: prebiotic, maltodextrin and no supplement).
- Id .prefix: string to prefix the identification of the column values. M for males and F for females were used.
- Stratum: a string that specifies the stratum being generated. “Male” and “Female” were used.
- block.size: 3 blocks were used in the formula.

The participants were stratified by males and females to avoid the problem faced during the pilot study and have a balanced number of both genders.

The formula used was this:

```
# stratified by sex, 63 in total, 3 treatments, 32 of each sex

male <- blockrand(n = 32, id.prefix = 'M', block.prefix = 'M', stratum = 'Male', num.levels = 3)

female <- blockrand(n = 32, id.prefix = 'F', block.prefix = 'F', stratum = 'Female', num.levels = 3).
```

The results were given in a list form as can be seen on Table 5.1.

Table 5. 1 Example of the randomisation of the sample size given by the R program after stratification.

	id	stratum	block.id	block.size	treatment
1	M01	Male	M1	6	A
2	M02	Male	M1	6	C
3	M03	Male	M1	6	C
4	M04	Male	M1	6	B
5	M05	Male	M1	6	B
6	M06	Male	M1	6	A
7	M07	Male	M2	12	B
8	M08	Male	M2	12	A
9	M09	Male	M2	12	A

id: identification; block.id: block identification

An external researcher defined the value of each letter (either maltodextrin, GOS-prebiotic or non-intervention) and kept that information secret until the data of all the participants was obtained, analysed and reviewed by the supervisor. The program automatically added more subject to the number specified in the formula in the case of a dropout.

5.2.4 Primary outcome

The primary outcome was the change in heat production in the tissue overlying the main depot of BAT in the supraclavicular region of the neck following the consumption of the prebiotic, maltodextrin or no supplement. BAT activation was calculated by measuring the change in the mean temperature of the upper percentiles of temperature points in the region of interest, the supraclavicular region, following stimulation with placing one hand in cold water using our previously established and published methodologies (Symonds et al., 2012, Robinson et al., 2014).

5.2.5 Secondary outcome

The secondary outcomes include:

- Associated changes in physical measurements like tympanic temperature, adiposity, weight, body mass index (BMI).
- Effects of influencing factor: environmental temperature.

5.2.6 Statistical analysis

The results are presented in temperature of the skin overlying the supraclavicular BAT (T_{SCR}), relative temperature (T_{REL}) and area under the curve (AUC) respectively. The temperature of the skin overlying the supraclavicular BAT measured using TI was defined as T_{SCR} and used as an indicator of BAT activity. Mean surface temperature (T_{REF}) was evaluated at the same time using the same TI camera, but its position was located 1 cm below the sternal notch. The T_{REL} was adjusted for the local of body surface temperature by calculating the difference between T_{SCR} and T_{REF}. This result was defined as the relative temperature (T_{REL}). AUC is the integrated measurement of a measurable effect. The result of AUC represented a cumulative measurement of the effect of cold in BAT thermogenesis and was obtained by performing a definite integral between two points; in this case, T_{REL} and the time of assessment.

In all methods, T_{SCR}, T_{REL} and AUC, three different times of assessment were considered:

- the resting period (five minutes before participants placed their hand in cold water)
- the stimulation period (five minutes when participants placed their hand in cold water).
- the post-stimulation period (five minutes after participants removed their hand of the water).

Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA).

Comparisons between groups were analysed using ANCOVA (analysis of covariance), one-way repeated measures ANOVA as well as linear regression analysis, where indicated. Data were assessed for normality using the Shapiro-Wilk Test. All data are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Baseline and environmental characteristics

Sixty-three participants undertook the study, male (31) and female (32) with 10-11 males and females per group. All participants were evaluated between June 2016 and July 2017 and had a mean age of 24.83 ± 0.31 years. The body composition characteristics of the participants, body mass index (BMI) and body fat percentage (assessed in both sessions), are described in Table 5.2 and 5.3 respectively.

Table 5. 2 BMI of the participants of the study.

	*BMI (kg/m^2)		p value
	0 days	**14 days**	
CG	22.65 ± 0.63	22.64 ± 0.63	0.15
IG	23.14 ± 0.56	23.17 ± 0.54	0.10
NI	22.89 ± 0.69	22.94 ± 0.69	0.16

CG: control group, IG: intervention group, NI: non-intervention group. All data are shown as mean ±SEM. There was no statistical difference between means of the 1st (0) and 2nd session (14 days) (p>0.05) — paired sample t-test. *BMI (body mass index) considered as normal from the range of 18.50 to 24.99 kg/m^2 (WHO, 2014).

Table 5. 3 Body fat percentage of the participants of the study.

	Males (%)			Females (%)		
	0 days	**14 days**	**p value**	**0 days**	**14 days**	**p value**
CG	22.5 ± 1.72	22.3 ± 1.80	0.16	25.2 ± 2.39	25.6 ± 2.41	0.27
IG	23.4 ± 1.23	22.7 ± 1.29	0.10	22.4 ± 1.80	22.2 ± 1.70	0.28
NI	22.6 ± 2.80	22.5 ± 2.92	0.79	25.2 ± 2.21	25.6 ± 2.24	0.28

CG: control group, IG: intervention group, NI: non-intervention group. All data are shown as mean ±SEM. There was no statistical difference between means of the 1st (0 days) and 2nd session (14 days) (p>0.05) — paired sample t-test.

The tympanic membrane temperature (core) of the participants remained unchanged in the non-intervention and intervention groups but declined in the control group after 14 days of consumption of the product (0: 36.88 ± 0.04; 14 days: 36.70 ± 0.05; p=0.003; Figure 5.3)

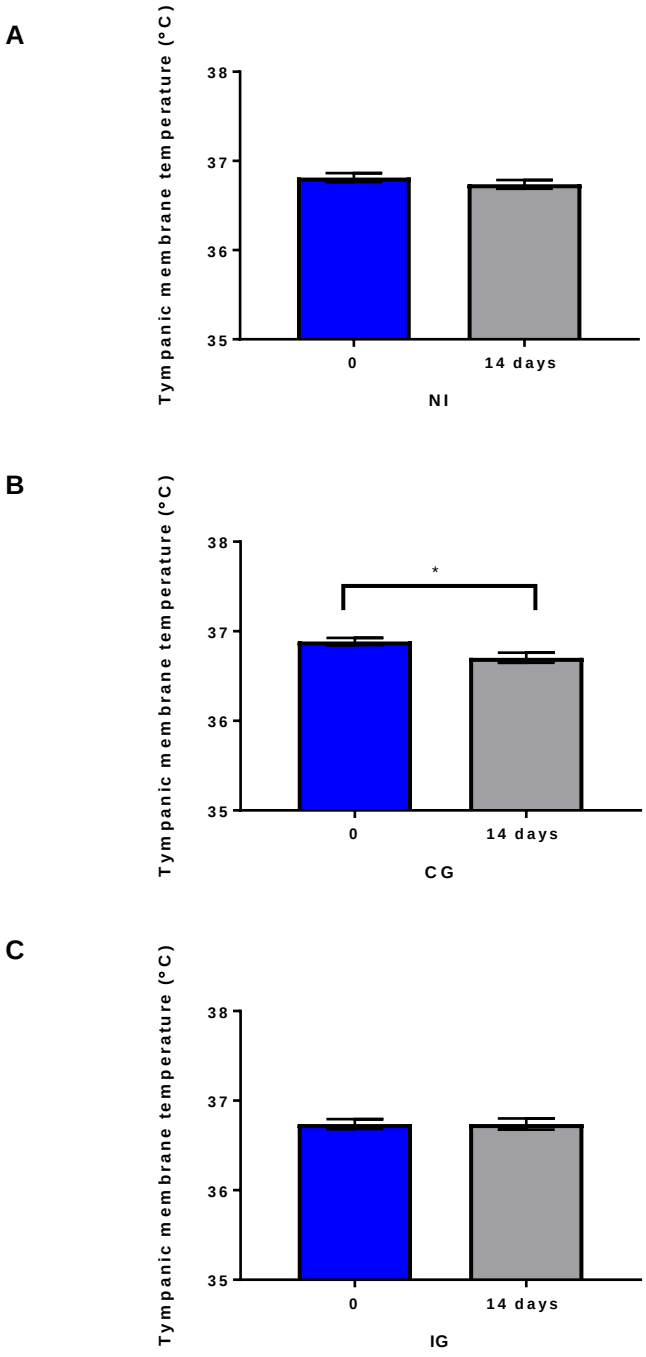
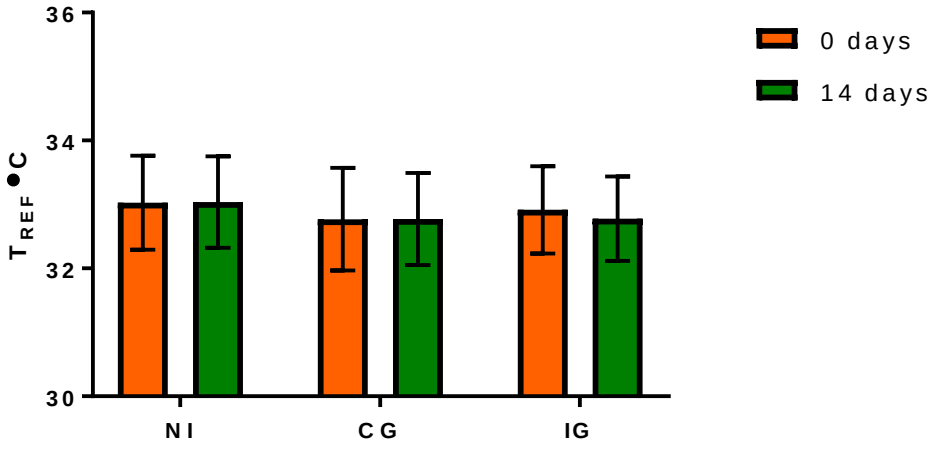


Fig 5.1 Tympanic membrane temperature of the participants during the two study sessions: before and after fourteen days of consumption/no consumption.

A) no intervention, B) control group, C) intervention group. 0: pre-consumption; 14: fourteen days of galacto-oligosaccharide (GOS) or maltodextrin consumption. Data are shown as means with error bars representing SEM. Paired sample t-test. *p<0.05

The temperature assessed at the sternal notch (reference temperature (body temperature), T_{REF}), which was located, 1 cm below the supraclavicular region of interest in between the neck and collarbones, was not altered in the three groups after fourteen days (Figure 5.4).

Figure 5.4 Reference temperature assessed in the three groups.



NI: non-intervention, CG: control group, IG: intervention group. Data are shown as means with error bars representing SEM. Analysis of covariance (ANCOVA). There was no significant difference between means of the first (0) and second (14 days) sessions (p=0.52).

The environmental conditions in which the study was performed did not vary between groups either concerning the outdoor temperature or within the study room (Table 5.4).

Table 5. 4 Environmental conditions assessed during the length of the PRE-BAT randomised controlled trial study.

	Environmental T. °C		**Room T. °C**		**Stimulus T. °C**	
	0 days	**14 days**	**0 days**	**14 days**	**0 days**	**14 days**
CG	14.75 ± 1.00	14.38 ± 1.18	22.00 ± 0.07	22.08 ± 0.11	16.66 ± 0.37	16.48 ± 0.32
IG	14.91 ± 1.16	14.53 ± 1.07	22.19 ± 0.09	22.19 ± 0.15	16.37 ± 0.28	16.38 ± 0.28
NI	13.31 ± 1.15	13.74 ± 1.16	21.97 ± 0.07	21.94 ± 0.09	16.53 ± 0.25	16.55 ± 0.34

NI: non-intervention, CG: control group, IG: intervention group, T: temperature. All data are shown as mean ±SEM. There was no statistical difference between means of the 1st session (0) and 2nd session (14 days) (p>0.05) — paired sample t-test.

As some studies have reported that ambient temperature influences tympanic temperature (Kakitsuba and White, 2014) and as on CG tympanic temperature dropped after 14 days of consumption of the product, a linear regression analysis was performed to determinate if there was a correlation between both temperatures, there was no significant relationship between environmental temperature and tympanic temperature in both times of assessment, first and second session, and in all groups, as can be seen in Table 5.5.

Table 5. 5 Linear regression analysis between environmental temperature and tympanic membrane temperature (TT).

Dependant variable		**Unstandardized coefficients**		**Standardized coefficients**	
Group	**Session**	**B**	**Std. Error**	**β**	**p value**
CG TT	**0 days**	0.003	0.009	0.070	0.76
	14 days	-0.011	0.010	-0.237	0.32
IG TT	**0 days**	-0.003	0.011	-0.065	0.78
	14 days	-0.010	0.013	-0.164	0.47
NI TT	**0 days**	0.007	0.010	0.152	0.51
	14 days	0.010	0.009	0.235	0.30

NI: non-intervention, CG: control group, IG: intervention group. TT: tympanic membrane temperature

5.3.2 Effect of GOS-prebiotic, maltodextrin or non-intervention on the temperature of the supraclavicular region (T_{SCR})

Supraclavicular temperature is the temperature of the skin overlying the supraclavicular BAT (T_{SCR}) and is used as an indicator of BAT activity. It is important to explain why there was a specific emphasis on the resting temperature. Resting was chosen because one of the aims of the study

was to determinate if the prebiotic/placebo could increase BAT supraclavicular thermogenesis without the need of a stimulus, in this case, placing the participant's hand in cold tap water. However, stimulation and post-stimulation periods were also assessed.

An analysis of covariance (ANCOVA) was conducted to adjust each participant's follow up temperature for his or her baseline temperatures (or first session results). Analysis of covariance is unaffected by baseline differences and has a greater statistical power to detect the effectivity of treatment than other methods (Vickers, 2001). There was no significant difference in T_{SCR} of the participants in the three groups of assessment after 14 days on the resting ($p=0.28$), stimulation ($p=0.61$) and post-stimulation ($p=0.56$) periods respectively (Figure 5.3).

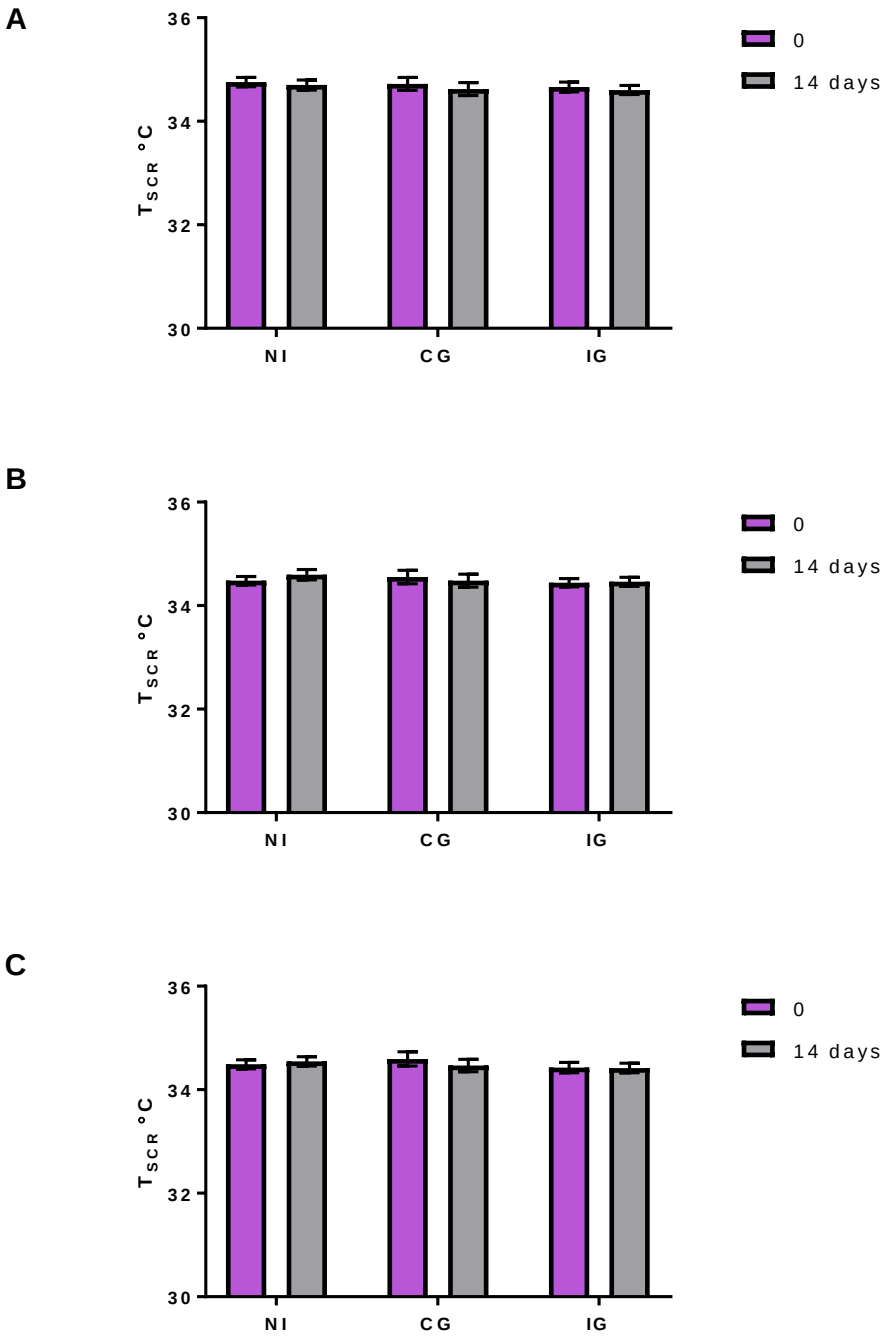


Figure 5. 3 T_{SCR} BAT temperature of the participants in the three groups of assessment.

A) resting period; B) stimulation period; C) post-stimulation period. NI: non-intervention, CG: control group, IG: intervention group. Data are shown as means with error bars representing SEM. Analysis of covariance (ANCOVA) (p=0.28).

Some authors have stated that outside/seasonal temperature (Au-Yong et al., 2009) can influence BAT supraclavicular thermogenesis. Therefore, a linear regression analysis was performed to determinate if the outside temperature influenced T_{SCR} during the two sessions of the assessment. The temperature considered for the statistical test was resting T_{SCR}. There was no significant relationship between environmental temperature and T_{SCR} in both times of assessment, first and second session, and in all groups, as it can be seen in Table 5.6

Table 5. 6 Linear regression analysis between environmental temperature and T_{SCR}

Dependant variable		**Unstandardized coefficients**		**Standardised coefficients**	
Group	**Session**	**B**	**Std. Error**	**β**	**p value**
CG T_{SCR}	**0 days**	-0.030	0.028	-0.244	0.28
	14 days	-0.013	0.024	-0.124	0.59
IG T_{SCR}	**0 days**	0.022	0.018	0.268	0.24
	14 days	0.008	0.019	0.097	0.67
NI T_{SCR}	**0 days**	-0.026	0.017	-0.329	0.14
	14 days	-0.009	0.019	-0.108	0.64

NI: non-intervention, CG: control group, IG: intervention group.

5.3.3 Effect of GOS-prebiotic, maltodextrin and non-intervention on T_{REL}

There was no statistically significant difference in post-intervention T_{REL} (after 14 days of consumption) between the groups as determined by analysis of covariance (ANCOVA) in the resting (p=0.89) and stimulation (p=0.30). However, there was a significant increase in mean T_{REL} temperatures between the three groups after 14 days (p=0.01) when assessed at post-stimulation. There was a significant increase in temperature between NI and IG group (NI: 1.053 ± 0.004; IG:

1.068 ± 0.004; p=0.004) and CG and IG group (CG: 1.057 ± 0.004; IG: 1.068 ± 0.004; p=0.004) respectively (Figure 5.4).

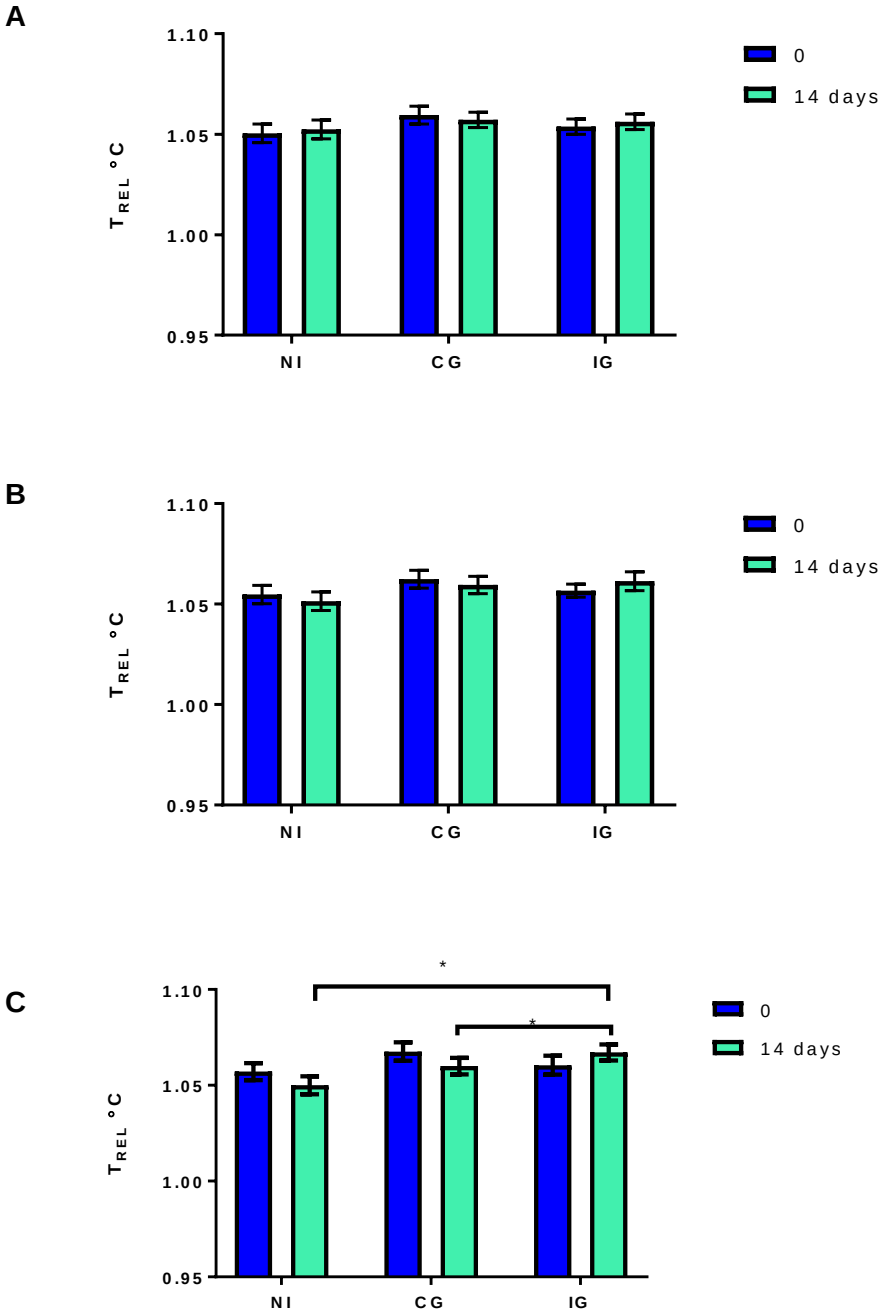


Figure 5. 4 Supraclavicular temperature relative to sternal (body surface) temperature (T_{REL}).

A) resting period; B) stimulation period; C) post-stimulation period. NI: non-intervention, CG: control group, IG: intervention group.0: Pre-consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) or maltodextrin consumption. Data are shown as means, and error bars represent SEM. Analysis of covariance. *p<0.05

To demonstrate if the outside temperature and body fat percentage had an influence on T_{REL} during the post-stimulation period, these two values were adjusted to the model using analysis of covariance (Pourhoseingholi et al., 2012).

Neither outside temperature (p=0.99) or body fat percentage (p=0.47) were statistically significant.

5.3.4 Effect of GOS-prebiotic, maltodextrin and non-intervention on the area under the curve (AUC)

There was no statistical significant difference in mean between the three groups after 14 days as determined by analysis of covariance (ANCOVA) in the resting (p=0.86), stimulation (p=0.75) and post-stimulation periods (p=0.10) respectively. However, there was a significant increase in temperature between NI and IG (NI: 313.42 ± 20.53; IG: 371.76 ± 20.29; p=0.04) during the post-stimulation period alone (Figure 5.5).

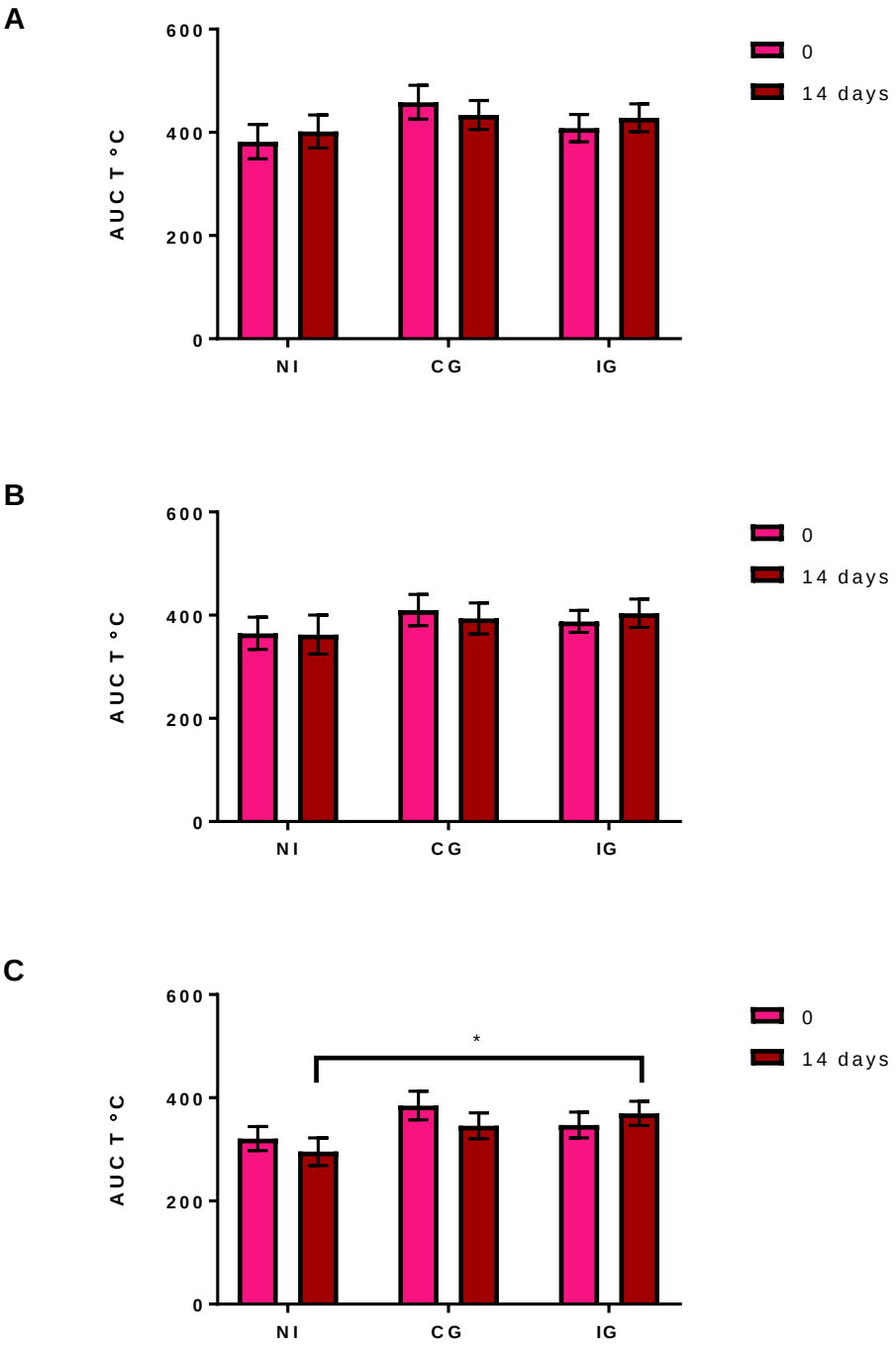


Figure 5. 5 AUC relative to sternal temperature.

A) resting period; B) stimulation period; C) post-stimulation period. NI: non-intervention, CG: control group, IG: intervention group. 0: Pre-consumption; 14 days: fourteen days of galacto-oligosaccharide prebiotic (GOS) or maltodextrin consumption. Data are shown as means, and error bars represent SEM. Analysis of covariance. *p<0.05

5.4 Activation of BAT thermogenesis from stimulation of hand in cool tap water.

5.4.1 Change in sternal (reference), T_{SCR}, T_{REL} and AUC temperature

A one-way repeated measure ANOVA were conducted to compare the effectiveness of the stimulus in increasing T_{REF}, T_{SCR}, T_{REL} and AUC at the stimulation and post-stimulation periods compared with resting. There was a significant drop in temperature after participants placed their hand in cool tap water for 20 minutes in all groups and both sessions when assessed as T_{REF} (Table 5.7) , T_{SCR} (except in NI group after 14 days, Table 5.8) and AUC (except in IG group after 14 days and NI during the first session, Table 5.9) . However, the temperature remained the same when assessed as T_{REL} in all groups (except group CG during the first session, Table 5.10). Nonetheless, there was a significant increase in temperature at the post-stimulation period when compared with resting after 14 days of consumption in group IG (Figure 5.6).

Table 5. 7 Activation of T_{REF} thermogenesis after incorporation of stimulus.

Group	Session	Resting	Stimulation	Post-stimulation	p value
CG	**0 days**	34.72 ± 0.12	32.53 ± 0.18	32.42 ± 0.19	<0.001
	14 days	34.62 ± 0.12	32.54 ± 0.15	32.50 ± 0.14	<0.001
IG	**0 days**	34.66 ± 0.09	32.55 ± 0.13	32.45 ± 0.17	<0.001
	14 days	34.60 ± 0.08	32.46 ± 0.19	32.32 ± 0.17	<0.001
NI	**0 days**	34.75 ± 0.09	32.70 ± 0.16	32.65 ± 0.15	<0.001
	14 days	34.70 ± 0.09	32.93 ± 0.16	32.89 ± 0.14	<0.001

CG: control group, IG: intervention group; NI: non-intervention. Data is shown as Means ± SEM. One-way repeated measures ANOVA.

Table 5. 8 Activation of T_{SCR} thermogenesis after incorporation of stimulus.

Group	Session	Resting	Stimulation	Post-stimulation	p value
CG	**0 days**	34.72 ± 0.12	34.55 ± 0.12	34.59 ± 0.13	<0.001
	14 days	34.62 ± 0.12	34.48 ± 0.12	34.46 ± 0.12	<0.001
IG	**0 days**	34.66 ± 0.09	34.44 ± 0.08	34.43 ± 0.09	<0.001
	14 days	34.60 ± 0.08	34.45 ± 0.09	34.42 ± 0.09	<0.001
NI	**0 days**	34.75 ± 0.09	34.48 ± 0.08	34.49 ± 0.08	<0.001
	14 days	34.70 ± 0.09	34.59 ± 0.09	34.54 ± 0.09	*0.152

NI: non-intervention, CG: control group, IG: intervention group. Data is shown as Means ± SEM. One-way repeated measures ANOVA. *p>0.05

Table 5. 9 Activation of AUC thermogenesis after incorporation of stimulus.

Group	Session	Resting	Stimulation	Post-stimulation	p value
CG	**0 days**	458.40 ± 32.89	410.00 ± 30.02	384.86 ± 27.83	0.002
	14 days	433.66 ± 28.07	393.83 ± 29.88	345.67 ± 25.01	<0.001
IG	**0 days**	462.49 ± 33.28	387.91 ± 21.09	347.22 ± 24.97	0.001
	14 days	438.96 ± 29.15	403.88 ± 27.51	369.52 ± 23.48	*0.102
NI	**0 days**	381.96 ± 33.42	364.86 ± 31.26	320.93 ± 23.39	*0.104
	14 days	401.78 ± 32.20	362.29 ± 37.77	295.56 ± 26.91	<0.001

NI: non-intervention, CG: control group, IG: intervention group. Data is shown as Means ± SEM. One-way repeated measures ANOVA. *p>0.05

Table 5. 10 Activation of T_{REL} thermogenesis after incorporation of stimulus.

Group	Session	Resting	Stimulation	Post-stimulation	p value
CG	**0 days**	1.060 ± 0.004	1.062 ± 0.004	1.068 ± 0.005	0.022
	14 days	1.059 ± 0.004	1.060 ± 0.004	1.060 ± 0.004	*0.612
IG	**0 days**	1.053 ± 0.004	1.057 ± 0.003	1.060 ± 0.0050	*0.056
NI	**0 days**	1.050 ± 0.005	1.055 ± 0.005	1.057 ± 0.004	*0.228
	14 days	1.052 ± 0.005	1.051 ± 0.005	1.050 ± 0.005	*0.576

NI: non-intervention, CG: control group, IG: intervention group. Data is shown as Means ± SEM. One-way repeated measures ANOVA. *p>0.05

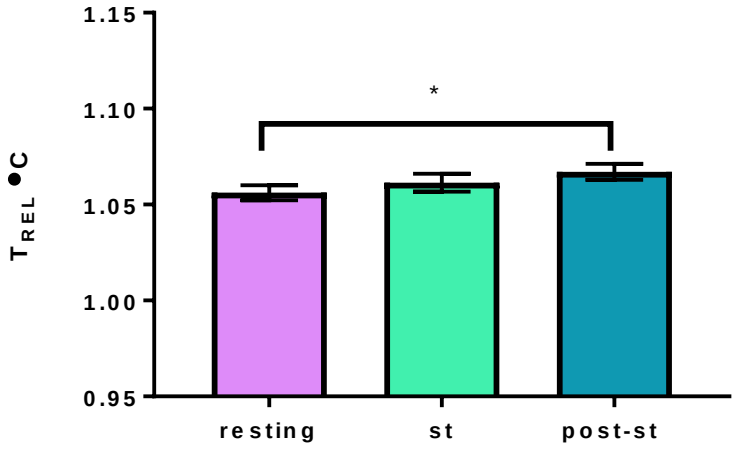


Figure 5. 6 Supraclavicular temperature relative to sternal (body surface) temperature (T_{REL}) after the introduction of the stimulus (hand in cold tap water), IG after 14 days (2nd session).

Resting (1.056 ± 0.004). Stimulation period: st (1.061 ± 0.005); post-stimulation period: post-st (1.067 ± 0.004). Data is shown as means with error bars representing SEM. One-way repeated measures Anova.*p=<0.001

5.5 Discussion

5.5.1 The effect of GOS prebiotic on BAT temperature

I have shown that the consumption of a GOS-prebiotic has the potential to increase BAT temperature as assessed by an increase in T_{REL} and AUC temperature after 14 days of consumption after a cold stimulation (i.e. hand in cool tap water, for 20 minutes) (Figure 5.6-C and 5.7-C). These results are comparable to those obtained by Gao et al. (2009), Hu et al. (2016b) and Sahuri-Arisoylu et al. (2016) in mouse models. In the Gao et al. study, when acetate was administrated (5 g per kg per day for 16 weeks), there was an increase in UCP1 after acclimatisation to 4°C for 90 minutes. Similarly, Hu et al. also used acetate to examine the effects of the SCFA on brown adipocytes. When BAT cells were treated with 10mM of acetate for seven days, there was an increase in the mRNA expression of UCP1. The main difference between Gao and Hu studies is that in the latter, the study was made *in vivo* and they did not use cold to stimulate UCP1 expression. In the Sahuri-Arisoylu et al. study, the researchers used a similar *in vivo* model, as Hu et al., to demonstrate the influence of acetate in BAT. They injected 52.9 µg of acetate into mice after they were fed with either a high-fat diet or a control diet. They also used magnetic resonance imagining to determine in which tissues the acetate reached. They demonstrated that in both dietary groups, after administration of acetate, there was an increase in heat production at the back of the neck of the animals ($p<0.01$) as well as an increase in UCP1 gene. Additionally, they found gene expression of peroxisome proliferator-activated receptor gamma coactivator one alpha (PGC1α) and PR domain containing 16 (PRDM16) were increased in BAT. Nonetheless, my study is the first one in showing an increase in BAT temperature when assessed with TI and in humans after just 14 days of consumption of GOS-prebiotic after cold acclimatisation of 20 minutes. However, these results must be interpreted with caution, as there are other factors that might have influenced BAT thermogenesis and not just the consumption of the GOS-prebiotic. Some of the factors that might have influenced the gut bacterial composition are:

- Genetics, the amount of bacteria in the gut is influenced by hereditary factors (Goodrich et al., 2014).
- Infections, gut microbiota is affected by viral and bacterial infections (Chou et al., 2015).
- Medications, the consumption of antibiotics and non-antibiotic drugs have an effect on the gut microbiota composition (Xu and Zhang, 2015, Devkota, 2016).
- Diet, a diet rich in plant-based foods like legumes, fruits and vegetables influences the number of beneficial bacteria in the gut microbiota (Koh et al., 2016, David et al., 2014)

Another factor that might have influenced BAT temperature was the environmental temperature. However, I have shown that environmental temperature did not influence T_{REL} temperature in all groups of the study (Table 5.7), these results contradict the findings obtained by Wang et al. (2016) which found that the fluctuation in environmental temperature has been shown to regulate BAT thermogenesis.

The tympanic temperature of the participants that consumed the maltodextrin dropped after 14 days, and this was not related to the environmental temperature (Table 5.5). There is not a specific study which has demonstrated that the consumption of maltodextrin reduces the tympanic temperature. One study showed a difference in physiological tests after the consumption of maltodextrin (Stevenson et al. (2017). In this study, the researchers compared the consumption of two drinks, one with isomaltulose, a carbohydrate composed of glucose and fructose, and the other a drink with maltodextrin in football players. The results demonstrated that maltodextrin does not increase blood glucose when compared with isomaltulose and placebo ($p=0.16$).

The main difference between this randomised controlled trial and the feasibility study (Chapter 4) was that, in the present study, I included a control group and a non-intervention group. These two groups, allowed me to analyse the results with an analysis of covariance. This analysis has a greater statistical power to detect the effectivity of treatment than other methods (Vickers, 2001) because it is unaffected by baseline differences.

It is important to recall that the only difference in BAT temperature, after the consumption of the prebiotic, was seen at the post-stimulation period (5 minutes after participants removed their hand

out of the water). The analysis of the stimulation period (average temperature of 5 minutes when participants placed their hand in the water) did not seem enough to show any difference in temperature. This timing was considered after an appropriate analysis (Chapter 2, materials and methods). However, for future studies stimulation temperature should be taken after more than the average of 5 minutes.

5.5.2 Influence of stimulus on BAT temperature

The T_{REF}, T_{SCR} and AUC temperatures decreased, or produced no change in temperature after participants placed their hand in the stimulus for 20 minutes (with certain exceptions, as shown in Tables 5.8, 5.9, 5.10 and 5.11). These changes are because cold exposure induces vasoconstriction and it has pronounced effects in extremities (i.e. fingers and toes) making them especially vulnerable to cold (Brajkovic et al., 1998). The use of cool tap water as stimuli of BAT thermogenesis, instead of other types of stimulus (i.e. exposing to cold air), make humans cool two to five times quicker (Hong, 1984) and hence, this results in a quick dissipation of the heat which is transported to the skin by blood circulation from deeper tissues (Castellani and Young, 2016). The temperature of the stimuli was on average of 16°C in all groups and both sessions (Table 5.4). When humans are immersed, the whole body, in temperatures of 14°C there is a reduction of rectal temperature and an increase of systolic and diastolic blood pressure (Šrámek et al., 2000). However, these results were seen after a cold immersion of one hour, and in my study, the immersion was for just 20 minutes.

It is important to mention that this study, in comparison with the cold challenges (Chapter 3) and feasibility (Chapter 4), was performed in a room where the temperature was regulated at 22°C (with small variations between sessions and groups but no statistically significant, Table 5.4). The participants wore only their trousers and a cotton vest inside the, already, thermally controlled room. The combination between the room temperature and the stimuli made the temperature of the body fall. This occurrence can be explained with a mathematical formula, S=M - (+Work) – E ± R ± C ± K. Where S=heat storage, M=metabolic heat production, E=evaporation, R=radiation, C=convection and K=conduction, when the room temperature is lower than the body temperature

the result is heat loss (Gagge, 1996). However, after 14 days of consumption of the prebiotic, there was an increase in T_{REL} temperature during the stimulation and post-stimulation periods compared with resting. This was the only group that showed an increase in temperature during the second session, or after the consumption of the GOS-prebiotic when the stimuli were applied. These results demonstrate that, in this specific group, BAT temperature was maintained after the stimuli, due to the heat production probably originated by the consumption of the GOS-prebiotic.

5.5.3 Clinical importance of the results obtained in the study

As mentioned in the introduction of this thesis (Chapter 1), by increasing the activity of BAT there can be a potential benefit on the metabolism of glucose and lipids (Labbé et al., 2015, Blondin et al., 2017). The regular consumption of prebiotics has been suggested to provide a wide range of health benefits. Some of these benefits include the reduction of symptoms of irritable bowel disease (Orel and Kamhi Trop, 2014), prevention of colon cancer (Markowiak and Śliżewska, 2017), increase in the uptake of minerals like calcium (Hicks et al., 2012) and the promotion of satiety and weight loss (Lambert et al., 2015) among others. So far, the end-up products of prebiotics, SCFAs, have shown to increase BAT temperature. In this study, I suggest that the consumption of a GOS-prebiotic for 14 days might have the potential to increase BAT thermogenesis if is combined with cold acclimatisation and therefore, provide a greater effect on health.

The consumption of prebiotics is a dietary strategy for modulating the gut microbiota composition by regulating the Bacteroidetes-to-Firmicute ratio (two major bacterial groups in the gut microbiota) (Sasaki et al., 2013). Obese adult population has a higher rate of Firmicutes than Bacteroidetes (Koliada et al., 2017). Bacteroidetes are negatively correlated with the percentage of body fat and body weight (Parnell and Reimer, 2012). Therefore, prebiotics may have a potential effect on the treatment of obesity. The way to assess the amount of Bacteroidetes and Firmicutes is with a faecal sample. In this study, however, we did not assess that because measuring stool samples is expensive and volatile acids are hard to measure in free-living individuals, as measures have to be made on fresh samples (Torii et al., 2010). Asking for biological samples would have also decreased the participation in the study.

A suggestion for future studies would be to include an analysis of blood glucose, triglycerides and stool sample, measuring a change in these parameters after the consumption of the prebiotic and the cold acclimatisation to evaluate biologically important adaptations.

5.5.4 Limitations of the study

The study has several limitations; the first one was that participants did not consume a standardised diet throughout the study. The studies that saw an increase in BAT after the consumption of SCFA and were performed with animal models that all had the same daily diet (Gao et al., 2009, Hu et al., 2016b). A standardised diet is a better way to control what the subjects of a study consume (Yao et al., 2013), however, it could have decreased the participation in the study and increased the recruiting time. Secondly, the sample size. Even though there was an increase in T_{REL} and AUC temperature in the group that consumed the prebiotic, it was only seen after the stimulation with cold. Therefore, different sample size was calculated using the difference in temperature between the NI group and the group that consumed the prebiotic. Thirty-four participants in each group will be needed to be able to reject the null hypothesis. Thirdly, in this study, there was no assessment of the diet prior to or during the length of the study. I could not verify if participants consumed another product that might contain prebiotics, for example, artichoke, garlic, onions, kimchi (fermented cabbage) or tempeh (fermented soybeans) (Douglas and Sanders, 2008). However, it is important to notice that not all whole foods should be considered as potential prebiotics. For example, fermentable fibres are good for overall health and well-being. Nonetheless, a prebiotic is a specialised product targeted to regulate the specific bacteria in the gut (Slavin, 2003). Fourthly, there was no assessment of the physical activity of the participants prior to and during the length of the study. Exercise can have a potential role in the activation of BAT in both animal and human studies (Sanchez-Delgado et al., 2015). Animal studies have been proved that endurance training, like running, has the potential to increase BAT thermogenesis (De Matteis et al., 2013). Human studies have demonstrated that the increase in physical activity is associated with a higher BAT activity when assessed with PET (Dinas et al.,

2015). A recommendation for future studies would be to assess exercise with a validated physical activity questionnaire (Adams et al., 2014).

5.6 Conclusion

The consumption of GOS-prebiotic for 14 days showed an increase of T_{REL} and AUC temperature after participants placed their hand in cold water for 20 minutes when compared with non-intervention and a control group (maltodextrin). However, results must be interpreted with caution as there are some other factors that might have influenced the increase in BAT thermogenesis. A study with a bigger sample size (34 participants per group), an analysis of blood glucose, triglycerides and stool sample, a dietary analysis before and after the study, as well as a validated physical activity questionnaire are suggested for future studies to demonstrate causality and reduce bias.

6. Does the consumption of extra virgin olive oil (EVOO) modulate brown adipose tissue (BAT) thermogenesis in healthy adult volunteers?

6.1 Introduction

The Mediterranean Diet (MD) has been recognised as Immaterial Human Heritage by the United Nations Education, Scientific & Cultural Organization (UNESCO) (UNESCO, 2013). EVOO is considered as fundamental part of the MD and the main responsible of the health benefits of the diet which include protection against cardiovascular diseases (Sofi et al., 2014), type 2 diabetes (Esposito et al., 2015) and even prevents alterations in the gut microbiota (Tomasello et al., 2016).

EVOO has a higher content of polyphenols compared with other types of oils, the most abundant being oleuropein aglycone (Leri et al., 2018). This compound has the potential to increase UCP1 expression and BAT function at least in animal models (Oi-Kano et al., 2007a, Oi-Kano et al., 2008a, Oi-Kano et al., 2017). However, no study has proven as yet if the same effect can be seen in humans.

When BAT function is stimulated, it can affect daily energy expenditure, making it a safe and rapid therapy to control obesity and cardiovascular diseases (Seale and Lazar, 2009).

Therefore, in this study, I hypothesised that BAT thermogenesis, as assessed with TI, would be activated by the consumption of 25 g of EVOO in healthy adult volunteers after 21 days.

6.2 Methods

The ethics committee reviewed and approved the study on the 21st of December of 2016. The ethics reference number of the study was B08122015 CHOG. The University of Nottingham funded the study and participants received a small inconvenience allowance after their participation (at the second session of the study).

6.2.1 Study configuration

The study was performed in collaboration with Dr Jose Muros from the University of Granada, Spain. He drafted the protocol of the study, while I collected the data from the participants and subsequently analysed the results.

This study was a stratified randomised controlled trial. Participants were randomly assigned to two groups: intervention and control. The intervention group (IG) received a daily dose of 25 ml of EVOO, and the control group (CG) maintained their dietary habits throughout the study. The randomisation was stratified according to their gender as the activation of BAT varied according to gender (van den Beukel et al., 2015) and was performed with an online generator of random numbers (Alazar, 2018).

The study consisted of two sessions. During the first session, thermal Images (TI) of the upper part of the body were taken in all participants. IG participants received eleven bottles of 50 ml of Fuenroble™ EVOO, CG participants did not receive any product at all and were just advised to maintain their usual dietary habits. IG participants were advised to take half the quantity of the bottle each day (25 ml) for 21 days; they were also provided with individual advice on how to incorporate the oil into their diet through the replacement of habitually-consumed other fats so as not to increase overall fat intake.

Furthermore, during the first session, all participants were invited to record their dietary intake over three different days of the week (two weekdays and one day of the weekend) for two different weeks with a 3-day food intake record (Appendix 9). Participants were encouraged to record a precise description of the amounts of food they ate and even write down the name of the brands of the food consumed. The advantage of a food record, compared with other types of dietary assessments like 24-hour dietary recall, is that they do not rely on memory and they have been used as a reference method to validate other dietary assessment methods (Yang et al., 2010). The main disadvantage is that, even though the researcher explains the correct way to fill the diaries, participants might over- or under-estimate the amount of food consumed. Total dietary fats, dietary

fibre and total calorie intake were estimated using a custom-made food composition database (Cronometer™) based on the USDA and Phenol-Explorer databases (Cronometer, 2018).

Ethnicity and age were also recorded in the first session. Participants were asked to self-report their 'ethnicity' using a standardised ethnicity classification. The standard classification used was the one utilised by the UK Home Office in collecting census data from the UK domiciled population. Participants were asked to choose which, from a list of standard ethnicities, they feel best describes their ethnicity (Appendix 10) Brown fat varies according to age (Symonds et al., 2012), so participants were asked to self-report their age range in the age bands 18-21 years, 22-25 years and 26-29 years (Appendix 10).

Each participant was asked to return after 21 days for the second study session. In this, thermal imaging was repeated and completed food records returned along with and the empty, or remaining, bottles of EVOO to determine its likely consumption.

Each session in the study lasted no more than two hours and followed the overview as seen in Figure 6.1. In both sessions, body composition, resting metabolic rate and heart rate variability were measured using standardised protocols and ISO standard equipment (Chapter 2, Materials and Methods). Participants were also advised not to eat, to refrain from exercise such as cycling and walking briskly and to drink only water for the 8 hours before each study session, as these aspects may affect the reliability of thermal imaging measurements. The effects of the EVOO were assessed by comparing the differences between brown adipose tissue temperature between the first and second session.

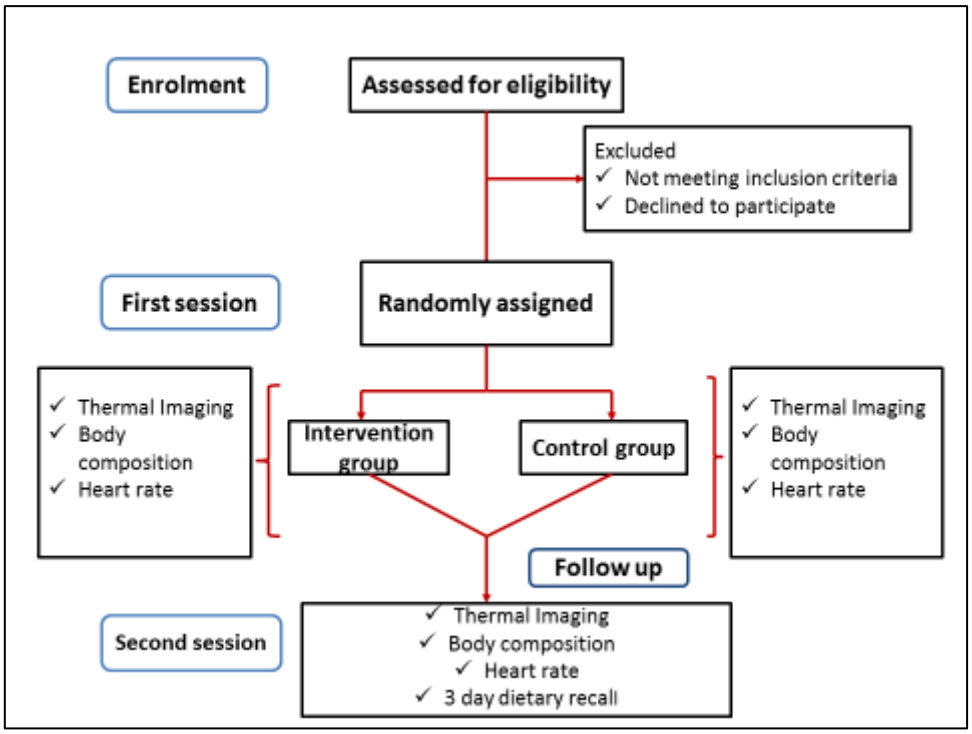


Figure 6. 1Study overview of the EVOO-BAT study.

This figure briefly explains the recruitment and study regime criteria followed by the researcher during the length of the study.

6.2.2 Fuenroble™ EVOO

The EVOO chosen for the study was of the brand Fuenroble™. This oil has been used in previous studies (Oliveras-Lopez et al., 2013, Fernandez-Castillejo et al., 2017) and has received numerous international awards. The oil is produced in Andalucía Spain, and its levels of acidity are of 0.4% per 500 ml. For the study, the company individually packed the oil in bottles of 50 ml each. Intervention group participants were encouraged to take half the bottle, approximately 25 ml, per day as substitution of their commonly consumed fats in their diet. This amount was determined by the published data regarding the amount of oil considered able to increase BAT thermogenesis on the supraclavicular area (Oi-Kano et al., 2007a). The polyphenol content of the oil used in the study is shown in Table 6.1 (featured as mg/l).



Figure 6. 2 Bottle of 50 ml of Fuenroble™ EVOO used in the study.

Each participant on the intervention group received 11 bottles to consume in the intervening 21 days between the first and second study visits.

Table 6. 1Polyphenol composition of Fuenroble™ EVOO.

Polyphenol	mg/l
Hidroximetil-furan-carbaldehyde	0.054
3,4-Dihydroxybenzoic	1.125
3,5- Dihydroxybenzoic	54.007
3,4-Dihydroxyphenylacetic	0.245
3,4-Dihydroxybenzaldehyde	0.110
Chlorogenic acid	0.017
Vanillin	0.194
4-O-Caffeoylquinic acid	0.178
Epicatechin	0.030
Epigallocatechin gallate	0.044
Ferulic	0.045
3,4-Dimethoxybenzaldehyde	0.017
Coumaric acid	0.016
Resveratrol	0.027
Daidzein	0.010
Genistein	0.321
Formonotein	0.398
Oleuropein aglycone	0.0915

The data was obtained from an analysis performed at the University of Granada (Spain) in June 2018.

6.2.3 Sample size calculation

Using data previously published by the Division of Child Health, Obstetrics and Gynaecology

(Symonds et al., 2012), which demonstrated an estimated standard deviation of 0.368, 30

participants per group (15 male and 15 female), 60 in total; were required to give an 80% power to detect an expected biologically significant change of 0.3°C with a significance level of 5%.

6.2.4 Primary outcome

The primary outcome was the change in temperature in the tissue overlying the main depot of BAT (ΔT) in the supraclavicular area, following the consumption of EVOO for 21 days. BAT activation was calculated by measuring the change in the median temperature of the upper percentiles of temperature points in the region of interest, the supraclavicular region, following stimulation with placing one hand in cold water.

6.2.5 Secondary outcomes

The secondary outcomes included:

· associated changes in physical measurements: tympanic temperature, body weight, body fat, heart rate.

· effect of influencing factors: environmental temperature, diet.

6.2.6 Statistical analysis

The results are presented as the temperature of the skin overlying the supraclavicular BAT (T_{SCR}), relative temperature (T_{REL}) and area under the curve (AUC) respectively.

Supraclavicular temperature is the temperature of the skin overlying the supraclavicular BAT (T_{SCR}) and is used as an indicator of underlying BAT activity. The relative temperature was defined as the temperature obtained from the difference between the supraclavicular area of BAT (T_{SCR}) and the surface temperature (T_{REF}) of the sternal notch.

AUC is the integrated measurement of a measurable effect. The result of AUC represented a cumulative measurement of the effect of cold in BAT thermogenesis and was obtained by performing a definite integral between two points; in this case, T_{REL} and the time of assessment.

In all methods, T_{SCR}, T_{REF}, T_{REL} and AUC, two different times of assessment were considered:

- the resting period (five minutes before participants placed their hand in cold water)
- the stimulation period (five minutes when participants placed their hand in cold water).

Only these two periods of assessment were considered for this study, in comparison with the previous chapters that also included the post-stimulation period. This decision was made in discussion with Dr Muros to increase participant the acceptability to attend both study visits by decreasing their length. Images were taken every 20 seconds, previously every 30 seconds, and the total study length lasted 40 minutes: 20 minutes of resting and 20 minutes of stimulation.

Data were analysed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA).
Comparisons between groups were analysed using an independent t-test, paired sample t-test and simple linear regression analysis where indicated. Data were assessed for normality using the Shapiro-Wilk Test. All data are presented as median ± SEM. P <0.05 was considered statistically significant.

6.3 Results

6.3.1 Baseline characteristics

Fifty-eight participants undertook the study: male (28) and female (30), two participants were excluded from the study due to the quality of their thermal images and the impossibility to analyse them. Participants had a mean age of 24.42 ± 0.31 years and were evaluated between October 2016 and October 2017. The anthropometric characteristics of the participants are summarised in tables 6.2 and 6.3.

Table 6. 2 BMI of the participants.

	***BMI (kg/m^2)**		**p value**
	0 days	**21 days**	
Control	23.53 ± 0.51	23.65 ± 0.52	0.58
Intervention	21.62 ± 0.47	21.64 ± 0.48	0.47

Data are shown as means (±SEM). There was no significant difference between the two times of assessment (p>0.05: paired sample t-test). 0 days: first session, 21 days: second session of the study. *Body Mass Index (BMI) considered as normal from the range of 18.50 to 24.99 kg/m^2 (WHO, 2014).

Table 6. 3 Body fat percentage of the participants.

	Males (%)			Females (%)		
	0 days	**21 days**	**p value**	**0 days**	**21 days**	**p value**
Control	22.7 ± 2.09	22.5 ± 2.05	0.58	28.6 ± 1.56	28.7 ± 1.44	0.69
Intervention	25.2 ± 2.11	25.2 ± 2.07	0.81	24.80± 1.07	24.9 ± 1.15	0.67

Data are shown as means (±SEM). There was no significant difference between the two times of assessment (p>0.05: paired sample t-test). 0 days: first session, 21 days: second session of the study.

The ethnicity of the participants is summarised In Table 6.4 according to their group: control and intervention.

Table 6. 4 Ethnicity of the participants according to their randomisation.

Ethnicity	Control (number of participants)	Intervention (number of participants)
Indian	2	2
Chinese	7	4
Pakistani	-	1
Other Asian	5	4
British	4	5
Irish	2	-
Other white	3	6
African	1	-
Other Black	-	1
Other mixed	1	6
Not stated	3	1

The core temperature (tympanic), sympathetic and parasympathetic activity indicator (LF and HF respectively) did not significantly change in either the intervention or the control group after 21 days (Table 6.5).

Table 6. 5 Core temperature, LF and HF of the participants during the length of the study.

	Core temperature (°C)		LF (%)		HF (%)	
	0 days	**21 days**	**0 days**	**21 days**	**0 days**	**21 days**
Control	36.6 3 ± 0.06	36.57 ± 0.07	7.19 ± 0.19	6.87 ± 0.20	6.98 ± 0.21	6.58 ± 0.23
Intervention	36.59 ± 0.06	36.55 ± 0.07	7.39 ± 0.22	7.28 ± 0.18	7.11 ± 0.25	7.03 ± 0.20

Data are shown as means (±SEM). There was no significant difference between the two times of assessment of each characteristic (p>0.05: paired sample t-test). LF: sympathetic nervous system activity indicator. HF: parasympathetic nervous system activity indicator.

As diet-induced thermogenesis has been shown to be higher in BAT positive subjects (Hibi et al., 2016a), the total energy intake of the participants was assessed with a self-reported 3-day food record. There was no statistical difference in the energy intake (total calories, fatty acids, dietary fibre) between groups, control and intervention (Table 6.6).

Table 6. 6 Characteristics of the diet of the participants according to their randomisation after 21 days.

	Total calorie intake (kcal)	Dietary Fibre (g)	Monounsaturated fats (g)	Polyunsaturated fats (g)
Control	1719.69 ± 91.02	19.11 ± 2.00	19.66 ± 1.47	12.87 ± 1.15
Intervention	1773.14 ± 91.70	19.70 ± 1.34	20.71 ± 1.70	14.31 ± 1.74

Data are shown as means (±SEM). There was no significant difference between the two groups on each characteristic ($p>0.05$: paired sample t-test).

6.3.2 Environmental conditions

The participants were evaluated in different seasons, and therefore daily ambient temperatures were recorded. Even though the study was performed in a thermally controlled room, still there was a small variation in it from one participant to another and from session to session. However, the variations were not statistically significant as it can be seen in Table 6.7

Table 6. 7 Environmental conditions assessed during the length of the EVOO-BAT study.

	External environmental temperature (°C)		Ambient (room) temperature (°C)	
	0 days	**21 days**	**0 days**	**21 days**
Control	10.58 ± 0.87	12.26 ± 0.87	21.91 ± 0.06	22.13 ± 0.03
Intervention	12.63 ± 0.81	12.38 ± 0.80	22.26 ± 0.10	22.12 ± 0.06

Data are shown as means (±SEM). There was no significant difference between the two times of assessment of each characteristic ($p>0.05$: paired sample t-test). 1st: first session, 2nd: second session of the study.

6.3.3 Effect of EVOO on the temperature of the supraclavicular region (T_{SCR})

The resting supraclavicular BAT temperature corresponds to the average temperature of the last five minutes before the participants placed their hand in cool water. There were no significant differences in resting T_{SCR} temperature in both sessions and between groups (Figure 6.3). It is important to explain why there was a specific emphasis on the resting temperature. Resting was chosen because one of the aims of the study was to determinate if EVOO could increase BAT supraclavicular thermogenesis without the need of a stimulus, in this case, placing the participant's hand in cold tap water.

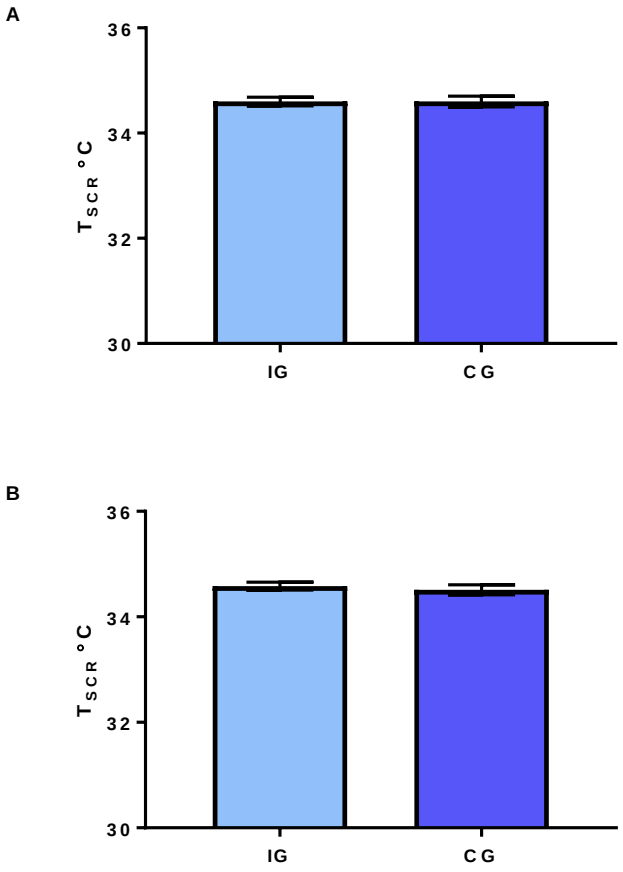


Figure 6. 3 Resting supraclavicular BAT temperature (T_{SCR}).

A) First session (p=0.99); B) After 21 days (p=0.55); IG: Intervention group; CG: control group. Data are shown as means, and error bars represent SEM. Independent t-test.

6.3.4 Effect of EVOO on T_{REF}

There were no significant differences in resting T_{REF} temperature in both sessions and between groups (Figure 6.4).

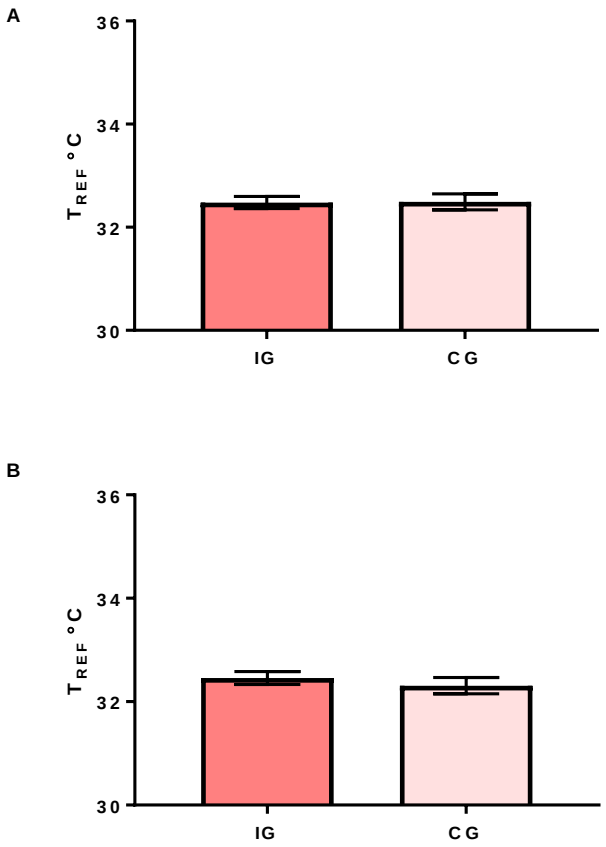


Figure 6. 4 Resting reference temperature (T_{REF}).

A) First session (p=0.94); B) After 21 days (p=0.45); IG: Intervention group; CG: control group. Data are shown as means, and error bars represent SEM. Independent t-test.

6.3.5 Effect of EVOO on T_{REL}

Relative supraclavicular temperature (T_{REL}), i.e. temperature of the supraclavicular region relative to the sternal temperature was calculated as the difference between the supraclavicular temperature (T_{SCR}) and reference temperature (T_{REF}). There were no statistically significant differences in T_{REL} in both sessions and between groups (Figure 6.5).

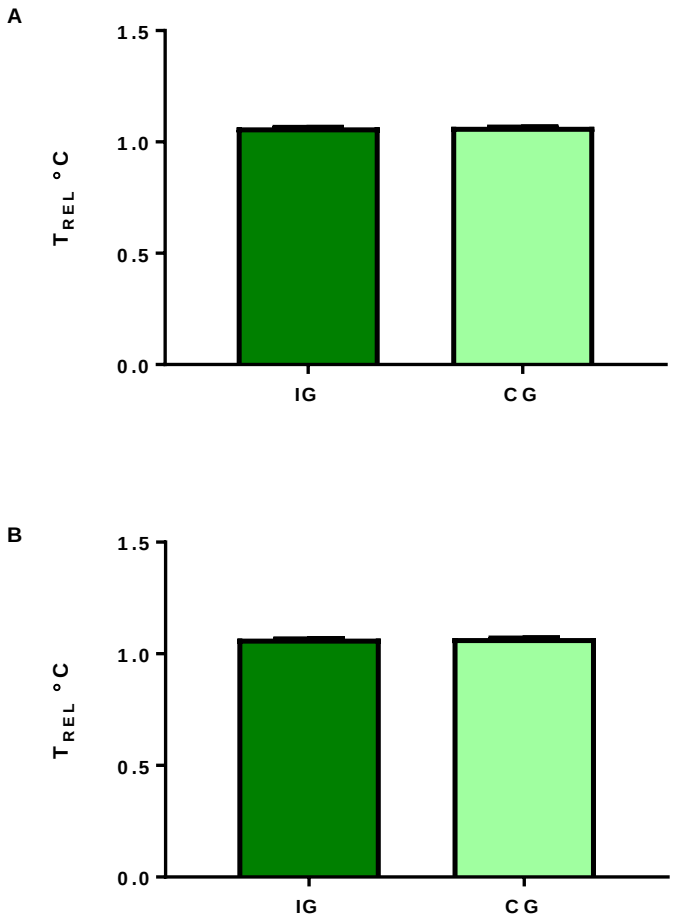


Figure 6. 5 Resting supraclavicular temperature relative to sternal (body surface) temperature (T_{REL}).

A) First session (p=0.74); B) After 21 days (p=0.47); IG: Intervention group; CG: control group. Data are shown as means, and error bars represent SEM. Independent t-test.

As the fluctuation in environmental temperature has been shown to regulate brown adipose tissue thermogenesis (Wang et al., 2016), a simple linear regression analysis was calculated to predict

whether the external environmental temperature influenced resting supraclavicular temperature (T_{SCR}) and relative temperature (T_{REL}). There was no significant relationship between environmental temperature and T_{SCR} and T_{REL} in both times of assessment, first and second session, and in both groups, IG and CG, as it can be seen in Table 6.8 and Table 6.9 respectively.

Table 6. 8 Linear regression analysis between environmental temperature and T_{SCR}.

Dependant variable		**Unstandardized coefficients**		**Standardized coefficients**	
Group	**Session**	**B**	**Std. Error**	**β**	**p value**
IG T_{SCR}	**0 days**	0.004	0.016	0.042	0.82
	21 days	-0.001	0.015	-0.013	0.94
CG T_{SCR}	**0 days**	-0.004	0.20	-0.042	0.83
	21 days	0.006	0.017	0.067	0.73

IG= intervention group, CG= control group.

Table 6. 9 Linear regression analysis between environmental temperature and T_{REL}.

Dependant variable		**Unstandardized coefficients**		**Standardized coefficients**	
Group	**Session**	**B**	**Std. Error**	**β**	**p value**
IG T_{REL}	**0 days**	0.000	0.001	0.105	0.56
	21 days	0.000	0.001	0.144	0.43
CG T_{REL}	**0 days**	0.000	0.001	0.095	0.63
	21 days	-0.001	0.001	-0.229	0.24

IG= intervention group, CG= control group.

6.3.6 Effect of EVOO on the area under the curve (AUC)

There were no statistically significant differences in AUC in both sessions and between groups (Figure 6.6).

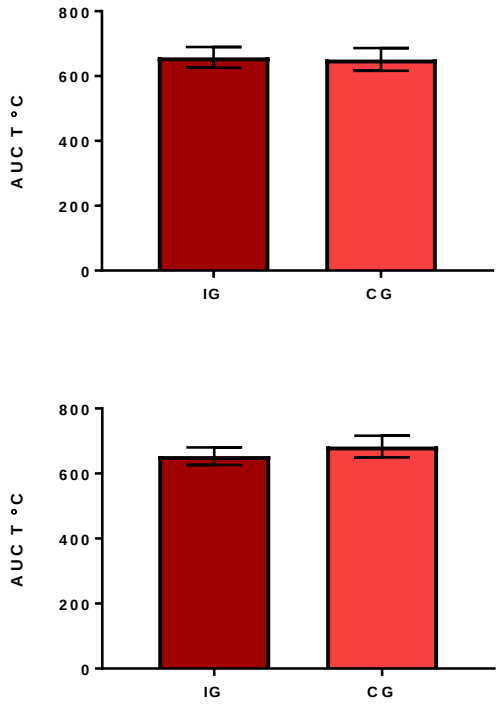


Figure 6. 6 Resting AUC relative to sternal temperature.

A) First session (p=0.89); B) After 21 days (p=0.48); IG: Intervention group; CG: control group. Data are shown as means, and error bars represent SEM. Independent t-test.

6.3.7 Activation of BAT thermogenesis from stimulation of hand in cool tap water.

6.3.7.1 Change in supraclavicular, reference, relative temperature and Area under the T_{REL} curve

The stimulus used to activate supraclavicular BAT, placement of one hand in cold water for 20 minutes, did not significantly increase the participant's T_{SCR} during the stimulation period, in either group or during the two study sessions (Table 6.10).

Table 6. 10 Activation of T_{SCR} thermogenesis after incorporation of stimulus.

Group	Session	Resting	Stimulation	p value
IG	**0 days**	34.58 ± 0.08	34.60 ± 0.08	0.73
	21 days	34.58 ± 0.07	34.59 ± 0.07	0.91
CG	**0 days**	34.60 ± 0.10	34.53 ± 0.09	0.55
	21 days	34.53 ± 0.09	34.47 ± 0.09	0.68

IG: intervention group; CG: control group. Data is shown as Means ± SEM. Paired sample t-test.

There was no difference in T_{REF} after participants placed their hand in the cold-water, during the first session (Figure 6.7). After 21 days, in the control group, T_{REF} remained unchanged during the second session while it significantly dropped in the intervention group, as can be seen in Figure 6.8.

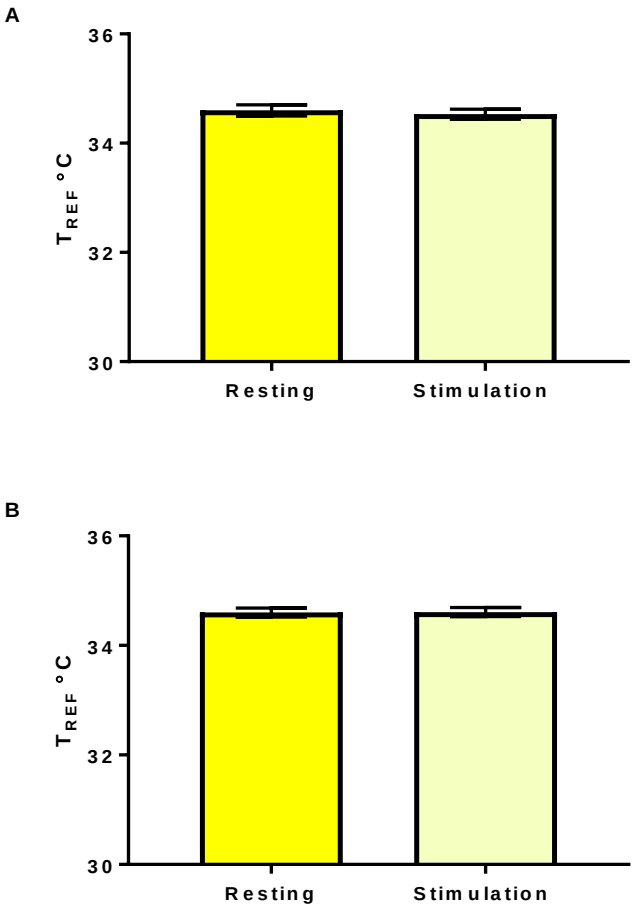


Figure 6. 7 Sternal (surface) temperature (T_{REF}) after the introduction of the stimulus (hand in cold tap water), first session.

A) Control group (p=0.55); B) Intervention group (p=0.73). Data are shown as means with error bars representing SEM. Paired sample t-test. *p<0.05

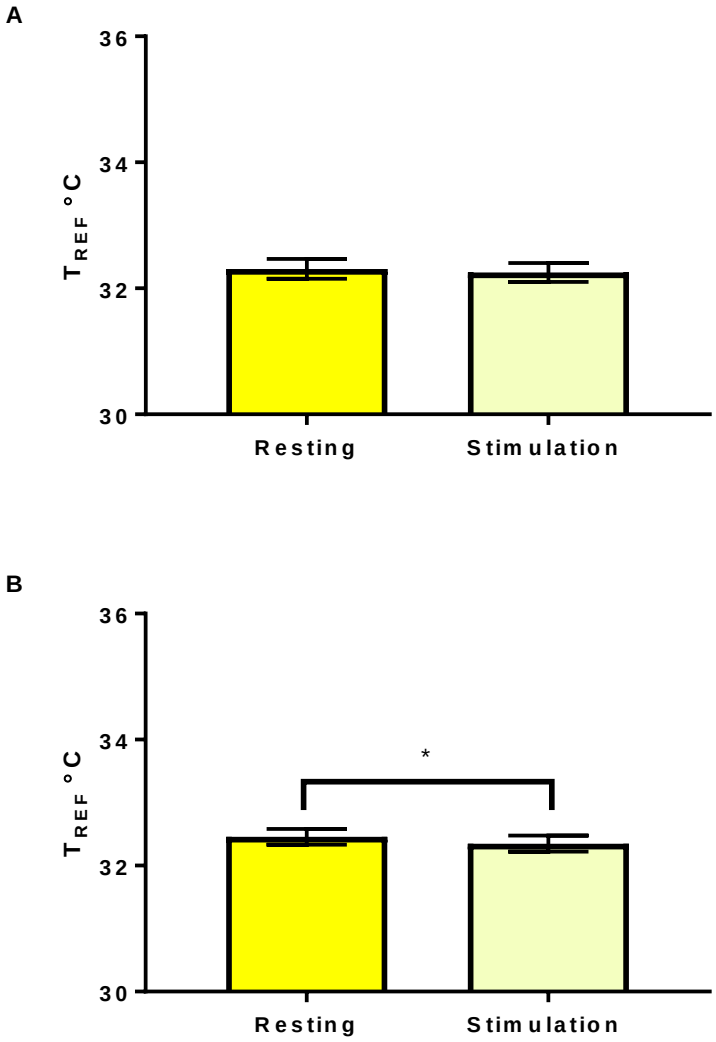


Figure 6. 8 Sternal (surface) temperature (T_{REF}) after the introduction of the stimulus (hand in cold tap water), second session.

A) Control group (p=0.15); B) Intervention group (resting: 32.45 ± 0.70; stimulation: 32.34 ± 0.12; p=0.000). Data is shown as means with error bars representing SEM. paired sample t-test. *p<0.05.

The stimulus used to activate supraclavicular BAT did not significantly increase the participant's relative temperature (T_{REL}) during the stimulation period, in either group or during the two study sessions (Table 6.11).

Table 6. 11 Activation of T_{REL} after incorporation of stimulus.

Group	Session	Resting	Stimulation	p value
IG	**0 days**	1.06 ± 0.00	1.06 ± 0.00	0.40
	21 days	1.06 ± 0.00	1.06 ± 0.00	0.40
CG	**0 days**	1.06 ± 0.00	1.06 ± 0.00	0.11
	21 days	1.07 ± 0.00	1.06 ± 0.00	0.25

IG: intervention group; CG: control group. Data is shown as Means ± SEM. Paired sample t-test.

However, the stimulus (hand in cold water) significantly decreased the area under the time-T_{REL} curve between the resting and the stimulation period at all two-study visits: the first session and after 21 days (Figure 6.9 and 6.10).

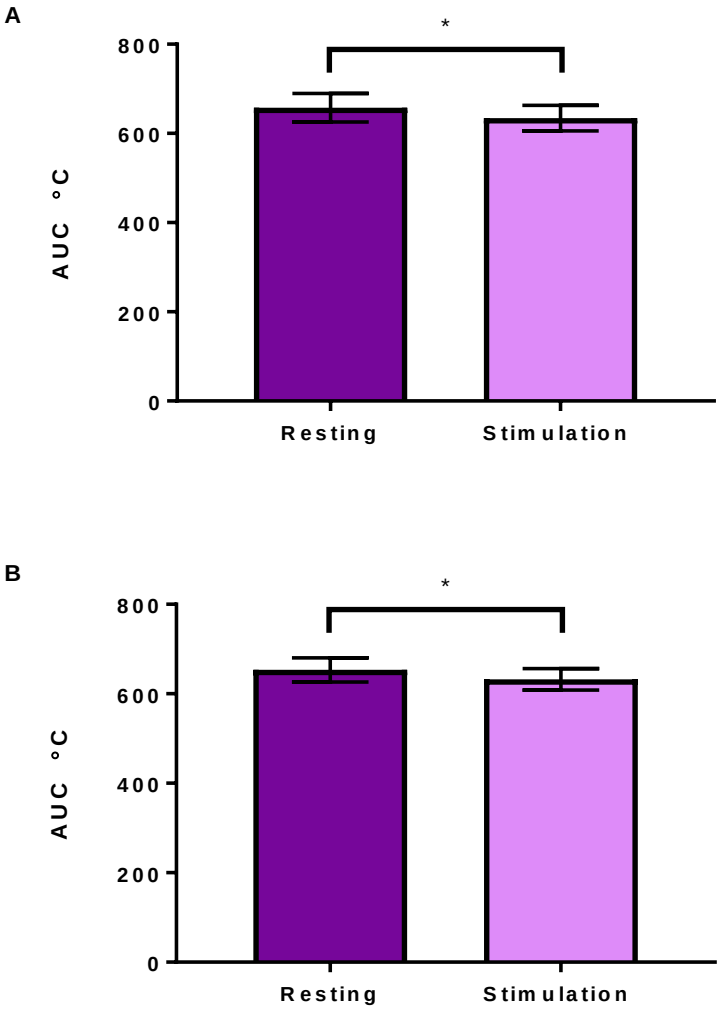


Figure 6. 9 Area under the time-T_{REL} curve after the introduction of the stimulus (hand in cold tap water), the intervention group

A) First session (resting: 657.77 ± 32.08; stimulation: 634.20 ± 28.63; p=0.04); B) after 21 days (resting: 653.36 ± 26.71; stimulation: 632.10 ± 23.93; p=0.03). Data are shown as means with error bars representing SEM. Paired sample t-test.

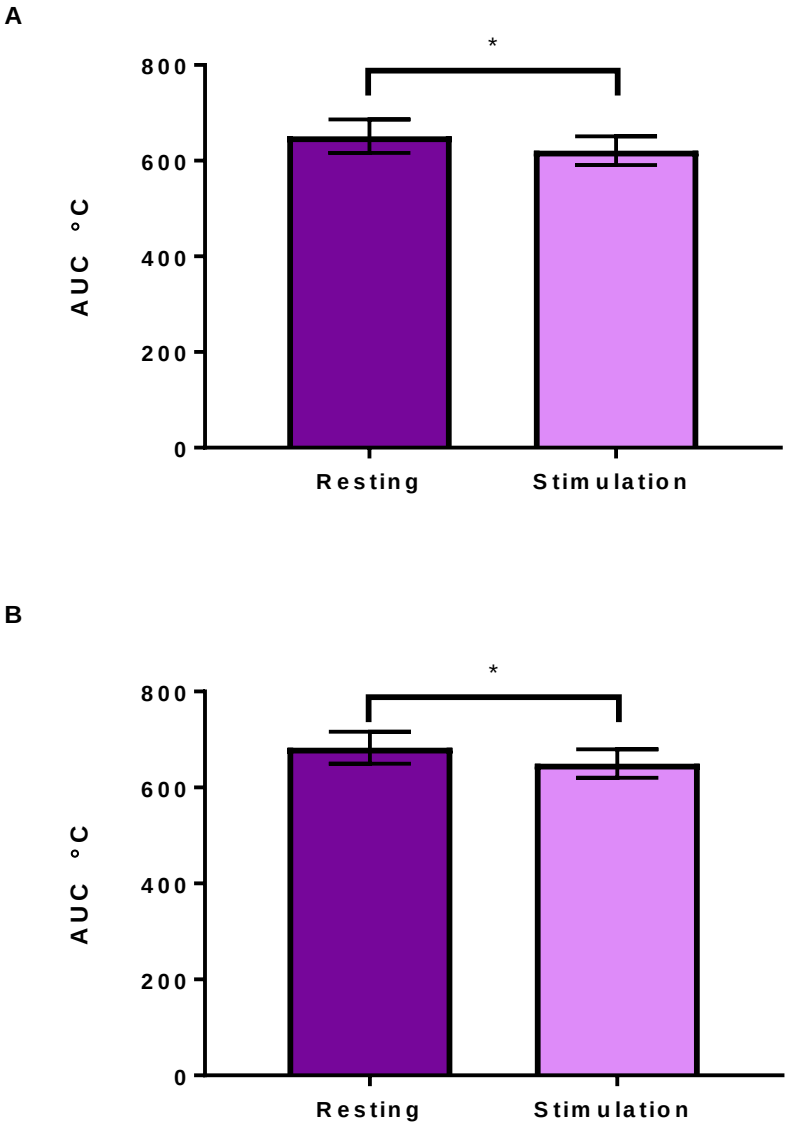


Figure 6. 10 Area under the time-T_{REL} curve after the introduction of the stimulus (hand in cold tap water), the control group

A) First session (resting: 651.31 ± 31; stimulation: 620.99 ± 30.03; p=0.005); B) after 21 days (resting: 683.31 ± 33.32; stimulation: 649.98 ± 29.55; p=0.01). Data are shown as means with error bars representing SEM. Paired sample t-test.

6.4 Discussion

6.4.1 The effect of EVOO on BAT temperature

This study has shown that the consumption of 25 ml of EVOO per day did not increase BAT temperature (measured as either T_{REL}, AUC or T_{SCR}) compared with a control group and assessed with TI. These results contradict my initial hypothesis, and there are several possible explanations for this.

The first explanation involves the fact that the previous results that showed the increase in BAT after the consumption of oleuropein aglycone were performed in animals and not in humans (Oi-Kano et al., 2017, Oi-Kano et al., 2008b). The animals used in these studies were obese rats, compared with normal weight volunteers used in my study. In the same studies, BAT amount, UCP1 abundance and gene expression were assessed with histological analysis, mRNA, compared with TI used in this study. The main difference between animal and human studies is that animal models are not ideal predictors of what can happen in humans (Shanks et al., 2009) as well as the fact that the methods of assessment were different. The reason why they are not good predictors is due to the differences in both species which vary from gene regulation and gene expression to epigenetics and genomics, as well as a different variations of metabolic pathways (Bracken, 2009).

The second aspect involves the fact that in the animal studies oleuropein aglycone was directly administrated to the animals, the animals were fed with 1 gram of oleuropein aglycone per kg of weight (rats weighed between 80 to 90 grams) (Oi-Kano et al., 2017). While in our study, the participants consumed EVOO with their diet and then obtained the phenols directly from its degradation. The phenolic compounds of EVOO, oleuropein aglycone included, are more feasible when the oil is consumed raw (García-Martínez et al., 2016). This is why participants were encouraged to take the oil in this form since the first session. However, according to their 3-day food records, many participants used the oil to cook their food. Participants reflected that it was easier for them to consume the oil in that way and because, in this form, the taste was imperceptible.

Nevertheless, when EVOO is cooked, i.e. domestic frying or any type of cooking that uses temperatures higher than 180°C, it suffers phenol oxidation (Koidis and Boskou, 2015) and has less ability to increase BAT thermogenesis. High-fat diets can produce an upregulation of brown markers in BAT and WAT, at least in animal models (Garcia-Ruiz et al., 2015). However, in my study, the consumption of fats either as monounsaturated or as polyunsaturated remained low in comparison with the daily recommendations. Monounsaturated fats daily consumption recommendation should be up to 22 to 55 grams per day (Ebbeling et al., 2018), compared with the average consumed by the participants which were 19 grams. Polyunsaturated fats recommended dietary amount should be between 40 to 62 grams per day (Van Horn et al., 2016), participants consumed an estimated amount of 12 to 14 grams. Also, the intervention group switched their usual cooking oils and fats for EVOO as my initial hypothesis stated that it would have a potential effect on BAT thermogenesis. However, the participants on that group might have excluded another fat source that also have the potential to regulate BAT like eicosapentaenoic acid (fish oil) (Pahlavani et al., 2017).

Furthermore, participants in both the control and intervention group consumed on average the same amount of calories during the length of the study. When there is an increase in the calories consumed through the diet, there is a marked increase in heat production of BAT in animal studies (Bytautiene Prewit et al., 2018). However, in my study, all diets remained the same in calorie intake. The amount of fibre consumed in the diets of the participants was low, on average of 19 grams, when the recommendation for adults is of 30 grams per day (Nutrition, 2015). Diets with high consumption of fibre have shown to promote WAT browning by activation of UCP1 (Han et al., 2017) in animal models. The results of the analysis of the diet of this study concur with the results obtained from Tanton et al. (2015), the researchers found that students from a British University had a high prevalence of unhealthy eating behaviours ($p<0.05$) and only 18.6% of their sample size consumed what was considered a balanced and healthy diet.

The third explanation includes the difference in ethnicity of the participants recruited to the study. In this study, the number of participants from Asian backgrounds surpassed those from a White

background (Asians: 25; White ethnicity: 20), 13 participants split across other ethnicities. The ethnic variation of BAT volume is usually higher in white Caucasians than in Asians (Bakker et al., 2014). However, another study that assessed BAT using PET-CT did not find any difference when it comes to ethnicity in a sample size of 38 patients (Perkins et al., 2013), the main difference was observed in seasonal temperatures. Nevertheless, my study demonstrated that the outside temperature did not influence BAT, measured as either T_{REL} or T_{SCR}.

Fourthly, in this study, it was decided to do not assess the post-stimulation period of the participants. Post-stimulation period corresponds to the average temperature after participants remove their hand out of the cool tap water. In the previous chapter, Chapter 4, I found an increase in T_{REL} and AUC temperature after participants were stimulated with the cold water in the intervention group. For future references in thermal imaging studies, it is convenient to assess three different times of assessment: resting, stimulation and post-stimulation.

6.4.2 Influence of stimulus on BAT temperature

As was previously seen in preceding chapters of this thesis, T_{REF}, after 21 days of consumption of EVOO, and AUC significantly decreased after the participants placed their hand in cool tap water for 20 minutes regardless of intervention or control group. When a participant is exposed to cold acclimatisation, there is a reduction in peripheral blood flow and stimulation of the sympathetic nervous system, and consequently, a decrease in the body's skin temperature (Marriott BM, 1996, Rintamaki, 2007) such as may have occurred in the case of the participants in the study. Cold exposure, along with the consumption of EVOO, did not significantly increased the T_{SCR} and T_{REL} of the participants; on the contrary, BAT temperature remained the same or dropped.

6.4.3 Limitations of this study

My study has several limitations; the first one was that participants did not have a standardised diet throughout the study. The studies that saw an increase in BAT after the consumption of oleuropein aglycone, and were performed with animal models, all had a standardised diet (Oi-Kano et al., 2007b, Oi-Kano et al., 2017). A standardised diet is a better way to control what the subjects of a study consume (Yao et al., 2013) and therefore, to see a possible difference in BAT temperature

after the consumption of EVOO. However, a standardised diet could have decreased the participation in the study and increased the recruiting time.

Secondly, I did not study a group that consumed a different type of oil from EVOO, and this could have helped us to do a comparison between different types of fats. In our research group, canola oil has been proved to increase UCP1 mRNA expression in animal models (Woods et al., 2015), we could have made a comparison between canola, EVOO and the control group to obtain a more robust set of results. Thirdly, my study was not blinded; when a study is blinded, there are fewer possibilities to generate bias both from the participants and from the researcher (Akobeng, 2005).

Lastly, even though intervention group participants were asked to bring the empty bottles of the oil on the second study session to verify if they consumed the product during the length of the study; naturally, I could not personally verify if they consumed the whole amount. Many of them verbally, but not in a written dietary record of compliance, mentioned that the amount was too much to eat in just one day. Participants also expressed that eating the oil in a raw form was challenging, as they are not used to consuming olive oil in that way. As mentioned in the baseline characteristics, most of the participants of the study are from different ethnic backgrounds, other than Mediterranean countries where olive oil consumption can reach up to 8.6 kilograms of oil per year (Guasch-Ferré et al., 2014), and therefore their dietary and culinary habits are different.

A suggestion for future studies would be to use a food supplement that contains oleuropein aglycone, like olive leaf supplements, instead of the EVOO. Olive oil leaf supplement contains a diverse variety of phenolic compounds and capsules of 400 mg of this product can contain an estimated amount of 250 mg of oleuropein aglycone, compared with the 3.61 mg contained in 50 ml of EVOO (Talhaoui et al., 2015). It has been demonstrated that the absorption of oleuropein aglycone in humans is increased in plasma and urine after the consumption of olive leaf extract in the form of capsule or liquid (de Bock et al., 2013). Capsules are easier to consume in everyday life, thus, I can assure that participants would not have a problem in taking them. For the study, the suggestion of taking a pill instead of the oil seems more viable. However, in everyday life

recommendations, is better to follow a balanced diet that includes healthy fats like EVOO due to the properties contained by the oil and that is briefly described in the introduction of this chapter.

Another suggestion would be to increase the sample size of the population. Although the calculation of this study was based on a standard deviation of 0.36 to see a change in BAT temperature of 0.3°C, as previously published data from my department (Robinson et al., 2014, Symonds et al., 2012), I could not see any significant difference in T_{REL} temperature between groups. The reason why there was no difference was that the change in temperature used to calculate the sample size was used for studies that looked at the influence of cold in BAT thermogenesis, while in our study, we assessed the influence of a dietary compound.

Therefore, to calculate a new sample size for future studies according to the results obtained from this particular one, I took the change in temperature observed in T_{SCR} of 0.07 (SD 0.4) after the consumption of EVOO for 21 days. With these data, another number was power calculated, giving us the sample size of 514 experimental subjects and 514 control subjects to be able to reject the null hypothesis (Dixon Wilfred J.; Massey, 1983). The reason why the sample size number is big, compared with our first power calculation, was that there was a small difference in temperature in our results (Faber and Fonseca, 2014).

6.5 Conclusion

In this study, the aim was to assess the possible increase in supraclavicular BAT temperature after the consumption of EVOO assessed with TI. The study's main finding was that there was no change in temperature of BAT supraclavicular when evaluated with $T_{REL,}$ AUC and $T_{SCR.}$ While our hypothesis was null, it is important to recall that this was the first study that has reported the use of EVOO and its influence on BAT supraclavicular temperature in healthy adults assessed with TI. However, my findings were limited by factors like sample size, the way that EVOO was consumed by the participants and the amount of oleuropein aglycone contained in the oil.

A study with a bigger sample size (514 experimental subjects and 514 control subjects), a randomised double-blind controlled trial, assessment of three periods (resting, stimulation and

post-stimulation) and the use of a supplement of oleuropein aglycone, instead of the EVOO, are suggested for future studies in order to demonstrate causality and reduce bias.

7. General Conclusions

7.1 General Aims

The thesis aimed to investigate the influence of dietary (GOS-prebiotic and EVOO) and non-dietary interventions (cold challenges) on the thermogenesis of BAT when assessed with TI in four different studies. The first one investigated the influence of seven days of cold challenges in BAT thermogenesis in one participant. The second and third studies investigated the influence of a GOS-prebiotic on BAT thermogenesis. Of these studies, the second one was a feasibility study, to determinate whether the GOS-prebiotic was easily accepted by the participants. While the third and fourth studies were randomised controlled trials to assess the effect of the GOS-prebiotic and EVOO, respectively on BAT thermogenesis.

7.2 Summary of findings and limitations

7.2.1 Findings

From the three studies performed, the one which showed a persistent increase in BAT thermogenesis after the stimuli were the cold study. This study showed that, after seven days of taking cold challenges, it is possible to increase BAT thermogenesis when assessed at all different times: resting, stimulation and post-stimulation. Most importantly, this study also helped me to identify if it was possible to reproducibly measure BAT temperatures.

The GOS-prebiotic pilot study helped me to recognise if the prebiotic could increase BAT thermogenesis and if it was well accepted by the participants. The product was well accepted; however, even when the hypothesis was null, I could manage to identify the problems faced during the length of the study and, therefore, create a better-planned study for the upcoming chapter, a randomised controlled trial.

The randomised controlled trial using a GOS-prebiotic, its main founding was that BAT thermogenesis, measured as T_{REL}, can increase after the consumption of the prebiotic and after a cold stimulation when compared with participants who received a placebo and who did not

consume anything at all. However, this increase in temperature was not seen at the resting or stimulation periods.

In the EVOO-BAT study, the initial hypothesis was null. EVOO cannot increase the amount of thermogenesis in BAT in healthy adult volunteers.

In all studies, no biological changes were seen. This means that there was no change in body weight, body fat (randomised controlled trial and EVOO studies) or sympathetic and parasympathetic activity (EVOO). Only in the cold challenges study, there was an increase in body weight of the participant. However, is unknown if it was due to the effect of the cold in her body, which triggers appetite, or hormonal changes.

7.2.2 Limitations

The main limitation of the cold study was the low number of participants; a wider sample size could have been more appropriate as well as a validated method to assess the cold challenges.

In the GOS-prebiotic studies, the main limitation was the amount of prebiotic given to the participants and the length of the time that the product was given.

The EVOO study main limitation was the lack of comparison with another oil and not just with a non-intervention group, the incorrect use of the oil from the participants and the low amount of oleuropein aglycone in the EVOO used for the study.

7.3 Clinical implications

BAT is a tissue specially designed to maintain the temperature of the body high through heat production, primarily by non-shivering thermogenesis. The results from these studies proved that, unlike cold, diet-induced thermogenesis, specifically using GOS-prebiotic and EVOO, are not as effective in increasing BAT thermogenesis at least in humans and when assessed with TI. However, by increasing BAT activity, there can be a direct benefit on glucose and lipid metabolism, thereby having a promising a therapeutic effect on metabolic disorders related to obesity like diabetes, dyslipidaemia and stroke (Cypess and Kahn, 2010b).

The consumption of prebiotics is a dietary strategy for modulating the gut microbiota composition by regulating the Bacteroidetes-to-Firmicute ratio (two major bacterial groups in the gut microbiota) (Sasaki et al., 2013). Obese adult populations have a higher rate of Firmicutes than Bacteroidetes (Koliada et al., 2017). Bacteroidetes are negatively correlated with the percentage of body fat and body weight (Parnell and Reimer, 2012). Therefore, prebiotics may have a potential effect on the treatment for obesity. Thermal Imaging is a novel technique to measure BAT activation, can monitor BAT within minutes or hours, is not an invasive tool as it is PET-CT (Lee et al., 2011) and it has the capability to assess heat directly from the supraclavicular area of the subjects (Ang et al., 2017). TI has been shown to detect areas if BAT activation in a comparable way with PET-CT (Law et al., 2018b). Using prebiotics to promote BAT remains a novel approach to contribute in the management of cardiovascular diseases and obesity, but needs further refinement.

7.4 Future work

It is important to notice, that these were the first set of studies that reported the use of EVOO and GOS-prebiotic and its influence on BAT supraclavicular temperature in healthy adults assessed with TI. However, my findings were limited by factors like sample size. Further investigation should be made to support a definitive role of diet in BAT thermogenesis in humans. These should use a larger sample size. For cold challenges three participants, for GOS-prebiotic 34 subjects per group and EVOO, 514 experimental subjects and 514 control subjects. Another recommendation is to give standardised diets to all participants, and all studies should be randomised double-blind controlled trials. It would then be less likely to generate bias both from either the participants or the researcher (Akobeng, 2005).

For future prebiotic studies, is recommended to use a higher amount of the product per day and for a longer period, specifically in the randomised-controlled trial where the increase in temperature after the consumption of the GOS-prebiotic was seen.

In the case of the EVOO study, is necessary to use an olive oil leaf supplement instead of the oil as the latter has a significantly higher number of oleuropein aglycone (Talhaoui et al., 2015), which

is the phenolic acid that has the potential to increase BAT thermogenesis. For GOS-prebiotic, it is recommended to undertake an animal study and see if the intervention promotes BAT directly.

Another recommendation for the future is to assess biological markers before and after each study. These markers should be blood glucose, blood triglycerides and for the GOS-prebiotic study short-chain fatty acids in urine and blood.

In conclusion, adding potential thermogenic compounds to the diet appear to not affect BAT thermogenesis as determined by TI. However, the consumption of GOS-prebiotic for 14 days combined with a cold stimulus for 20 minutes seems to increase BAT thermogenesis. Nonetheless, results must be interpreted with caution as there are some other factors that might have influenced the increase in BAT thermogenesis. Future studies should examine the effect of longer-term use of other dietary compounds, e.g. tea catechin or caffeine, and their impact on BAT thermogenesis.

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Appendix 1 PRE-BAT feasibility study, participant information leaflet.

What are the possible benefits of taking part?

Your involvement will help us to ultimately uncover better ways to understand human metabolism, in this case how brown adipose tissue is controlled. No one will be paid to participate in the study.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database.

Any information about you which leaves the institution will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it. If you give us permission we may contact you in the future to ask if you would like to take part in further imaging or brown fat studies.

What will happen if I don't want to carry on with the study?

It is up to you to decide whether or not to take part. If you decide to take part, you are still free to withdraw

What will happen to the results of the research study?

The results of the study will be published in academic journals so that others can see them. You will not be identified in anyway.

Your data will remain on a database of thermal imaging participants. This will be stored securely by the University of Nottingham in order to allow comparisons to be made with future subjects and studies

Who is organising and funding the research?

This research is being organised and funded by the University of Nottingham

Who has reviewed the study?

All research in the University of Nottingham is looked at by independent group of people, called a Research Ethics Committee, to protect your interests.

This study has been reviewed and given favourable opinion by the Faculty of Medicine & Health Sciences (FMHS) Research Ethics Committee

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researcher (Lucia Vazquez) who will do her best to answer your questions, or you can speak to the Chief Investigator, Prof Helen Budge on helen.budge@nottingham.ac.uk or by calling 01158230611.

If you are still unhappy, you should then contact FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, School of Medicine Education Centre, Medical School, University Hospital, Derby Road Nottingham, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk

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The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

The PRE-BAT Study (Prebiotic effects on Brown Adipose Tissue)



What is the purpose of the study?

The study aims to increase our knowledge about how brown fat is regulated. Brown fat is a special tissue in the body which produces heat and may help people avoid becoming overweight. We are interested in how the consumption of a prebiotic (Galacto-oligosaccharides (B-GOS) affects the activity of this tissue.

Why I have been invited?

You are being invited to take part because your responses on the screening questionnaire implied that you are eligible to participate in the study. We are inviting 36 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. Any information already collected will be used in the final analysis but you will not be identified from it.

What are the possible disadvantages and risks of taking part?

We do not expect there to be any risks of taking part. You will be able to have your usual foods and take any usual medications. If you have a significant medical condition, if you are taking certain medications, or if you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation, you may not be able to take part in the study to avoid it affecting the results. The investigator will discuss this with you when you first speak to them.

What will happen to me if I take part?

We would like you to meet you on three occasions . When we meet you for the first time, we will confirm that you still want to take part and ask you to sign a consent form. You will be given a copy to keep.

If you consent to take pat in the study, we will take some basic details like your date of birth, height , weight and tympanic (ear) temperature, then we will take a series of thermal (heat) images over around an hour.

Part way through this first study visit, we will ask you to do something which will stimulate brown fat. This may be putting your hand in cold water or drinking a cool drink. You will know exactly what you will be asked to do before we start. We may also ask to measure things like your heart rate and skin temperature during this time.

After the first session, you will receive 7 sachets of Bimuno® Nutri-Biotic Powder and you'll be asked to take one sachet a day for the next seven days; we will show you how to take the product by mixing it with a standard beverage (such as coffee or tea). You'll be asked to come back after seven days for the second study session and we will perform the same process we did the first time.

After the second session you will receive another 7 sachets to take one each day over that week, we will ask you to come back from the last time after seven days and we will perform the same process.

So, this will be the last part of the study. We will have asked you to take the sachets for 14 days and come for 3 thermal imaging sessions in total).

If you are happy to take part in the study, we will ask you to avoid caffeine, alcohol or recreational drugs from midnight before each study visit and any exercise for one hour prior imaging.

What is Bimuno® Nutri-Biotic Powder?

Bimuno® Nutri-Biotic Powder is a prebiotic containing Galacto-oligosaccharides (B-GOS).

Prebiotics are carbohydrates that cannot be digested by the human body. Bimuno® Nutri-Biotic Powder has previously been tested and is a food supplement not a drug. The primary benefit of prebiotics appears to be helping maintain a healthy digestive system.

This prebiotic belongs to a group of nutrient fibres (oligosaccharides) also found in milk which feed and encourage certain types of bacteria including bifidobacteria, naturally present in the gut.

The product is free from artificial colours, flavours and preservatives and is gluten free.

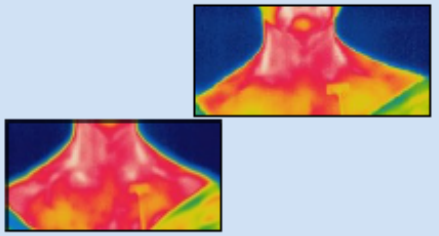
You can consume it by adding it to your everyday food and drinks (cereal, coffee, tea).

How a thermal camera works?

The thermal camera takes heat images in a similar way to a normal digital camera but shows hot and cold emissions rather than details of the way we look.

The pictures we take are of the neck and shoulders.

Thermal imaging of brown adipose tissue in the neck is a safe and simple technique developed, tested and routinely used at the University of Nottingham.



Appendix 2 PRE-BAT randomised controlled trial study, participant information leaflet.

What are the possible benefits of taking part?

Your involvement will help us to ultimately uncover better ways to understand human metabolism, in this case how brown adipose tissue is regulated.

At the end of the study, a £20 pound voucher will be offered to you for your participation.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database.

Any information about you which leaves the institution will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it. If you give us permission we may contact you in the future to ask if you would like to take part in further imaging or brown fat studies.

What will happen if I don't want to carry on with the study?

Is up to you to decide whether or not to take part. If you decide to take part you are, still free to withdraw

What will happen to the results of the research study?

The results of the study will be published in academic journals so that others can see them. You will not be identified in anyway.

Your data will remain on a database of thermal imaging participants. This will be stored securely by the University of Nottingham in order to allow comparisons to be made with future subjects and studies

Who is organising and funding the research?

This research is being organised and funded by the University of Nottingham

Who has reviewed the study?

All research in the University of Nottingham is looked at by independent group of people, called a Research Ethics Committee, to protect your interests.

This study has been reviewed and given favourable opinion by the Faculty of Medicine & Health Sciences (FMHS) Research Ethics Committee (FMHS REC reference: v1.0 E11082015 SoM CHOG PhD)

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researcher (Lucia Vazquez) who will do her best to answer your questions, or you can speak to the Chief Investigator, Prof Helen Budge on helen.budge@nottingham.ac.uk or by calling 01158230611.

If you are still unhappy, you should then contact FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, School of Medicine Education Centre, Medical School, University Hospital, Derby Road Nottingham, NG7 2UH. E-mail: louise.sabir@nottingham.ac.uk

Further information and contact details

Lucia Vazquez

Telephone: 0115(82)30613
Email: msxldv@nottingham.ac.uk

The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

The PRE-BAT Study (Prebiotic effects on Brown Adipose Tissue)



What is the purpose of the study?

The study aims to increase our knowledge about how brown fat is regulated. Brown fat is a special tissue in the body which produces heat and may help people avoid becoming overweight. We are interested in how the consumption of a prebiotic (Galacto-oligosaccharides (B-GOS), maltodextrin or no supplement over a 14 day period affects the activity of this tissue.

Why I have been invited?

You are being invited to take part because your responses on the screening questionnaire implied that you are eligible to participate in the study. We are inviting 63 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. Any information already collected will be used in the final analysis but you will not be identified from it.

What are the possible disadvantages and risks of taking part?

We do not expect there to be any risks of taking part. You will be able to have your usual foods and take any usual medications. If you have a significant medical condition, if you are taking certain medications, or if you have been a subject in any other research study in the last three months which involved taking a drug, being paid a disturbance allowance, having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation, you may not be able to take part in the study to avoid it affecting the results. The investigator will discuss this with you when you first speak to them.

What will happen to me if I take part?

We would like you to meet you on two occasions.

When we meet you for the first time, we will confirm that you still want to take part and ask you to sign a consent form. You will be given a copy to keep. If you consent, we will take some basic details like your date of birth, height, body composition, weight and tympanic temperature.

We will ask you to lie down for a few minutes and measure 'body fatness' (body composition) by bioelectrical impedance. This the flow of a small, harmless electrical signal between small sensors which will touch your hands and feet and calculates 'body fatness'.

Then we will take a series of thermal (heat) images over up to 1 hour whilst you sit down.

Part way through this first study visit, we will ask you to do something which will stimulates brown fat. This may be putting your hand in cold water or drinking a cool drink. You will know exactly what you will be asked to do before we start. We may also ask to measure things like your heart rate and skin temperature during this time.

After the first session, you will receive 14 sachets of Bimuno® Nutri-Biotic Powder or maltodextrin or no supplement. Whether or not you will receive a supplement, or which supplement you will receive will be determined randomly (like tossing a coin). Each participant will have 1/3 chance of receiving a prebiotic, 1/3 chance of receiving maltodextrin and a 1/3 chance of receiving no supplement. You will not be able to choose.

You'll be asked to take one sachet a day for the next 14 days; we will show you how to take the product by mixing it with a standard beverage (tea). We ask that you come back after 14 days for the second study session when we will perform the same process we did in the first session.

This will be the last part of the study. We will have asked you to take the sachets for 14 days, or not to take supplements and come for 2 study sessions in total.

If you are happy to take part in the study, we will ask you to avoid caffeine, alcohol, recreational drugs from midnight before each study visit and to refrain from eating and drinking and taking any exercise imaging

What are the prebiotic and the placebo?

Bimuno® Nutri-Biotic Powder is a prebiotic containing unique patented Galacto-oligosaccharides (B-GOS) developed by scientists. Prebiotics are carbohydrates that cannot be digested by the human body. The primary benefit of prebiotics appears to be to help maintain a healthy digestive system.

B-GOS belongs to a special group of beneficial nutrient fibres (oligosaccharides) (also found in maternal milk) that feed and encourage certain types of bacteria including bifidobacteria, naturally present in the gut. Bimuno® Nutri-Biotic Powder is free from artificial colours, flavours and preservatives and is gluten free.

The other powder in the study is a placebo used to compare against the prebiotic Bimuno®. The placebo is maltodextrin which is a carbohydrate used as a food supplement and in soft drinks and some foods. The Food and Drug Administration (FDA) and the European Food Safety Agency (EFSA) have approved maltodextrin as a safe food additive.

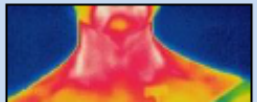
You can consume Bimuno® and maltodextrin in your everyday food and drinks (cereal, coffee, tea).

How a thermal camera works?

The thermal camera takes heat images in a similar way to a normal digital camera but shows hot and cold emissions rather than details of the way we look.

The pictures are centred on the neck and shoulders.

Thermal imaging is a safe and simple technique developed by the University of Nottingham to assess brown adipose tissue.



Appendix 3 EVOO-BAT study, participant information leaflet.

What are the possible benefits of taking part?

Your involvement will help us to understand human metabolism, and the function of brown adipose tissue. Participants who complete the study will receive a £20 'Amazon' voucher for the inconvenience of completing the study if they attend both session. All participants who choose to complete the food diaries will have the option of receiving information about their dietary record by email if they wish to do so.

What are the possible disadvantages and risks of taking part?

We do not expect there to be any risk in taking part. You will be able to have your usual food and take any usual medications. If you have a significant medical condition, a pacemaker or epilepsy, are taking certain medications, or if you have been a subject in any other research studies in last three months which involved: taking a drug; being paid; having an invasive procedure (e.g. blood sample >50ml) or exposure to ionising radiation, you may not be able to take part in the study. The Investigator will discuss this with you when you first speak to them.

What my taking part in the study be kept confidential?

We will follow ethical and legal practices and all information about you will be treated as confidential so that you cannot be identified from it. However, whilst your study data will be confidential, we do have a legal duty to report any criminal activity or information indicating possible risk to you or others if it is disclosed to us during the study.

The University of Nottingham

Who is organising and funding the research?

This research is being organised and funded by the University of Nottingham U.K. and University of Granada, Spain.

Who has reviewed the study?

All research in the University of Nottingham is looked at by an independent Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the University of Nottingham Faculty of Medicine & Health Sciences (FMHS) Research Ethics Committee [*insert REC number*].

What if there is a problem?

If you have concerns about any aspect of this study, you should ask to speak to the researcher (Dr. José Muros) who will do his best to answer your questions, or you can speak to the Chief Investigator Prof. Helen Budge - email: helen.budge@nottingham.ac.uk.

If you are still unhappy, you should then contact FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, School of Medicine Education Centre, University Hospital, Derby Road Nottingham, NG72UH - E-mail: louise.sabir@nottingham.ac.uk

FURTHER INFORMATION /CONTACT:

Dr. JOSÉ MUROS

jose.muros-molina@nottingham.ac.uk

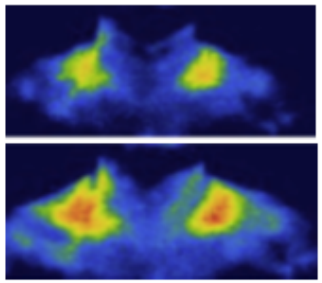
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The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

THE EVOO-BAT STUDY

The effects of Extra Virgin Olive Oil (EVOO) on Brown Adipose Tissue (BAT)





What is the purpose of the study?

The study aims to increase our knowledge about how brown fat is controlled. Brown fat is a special tissue in the body which produces heat and may help people avoid becoming overweight. We are interested in whether Extra Virgin Olive Oil in the diet affects how active BAT is.

Why I have been invited?

You are being invited to take part because your responses on the screening questionnaire implied that you are eligible to participate in the study. We are inviting 60 participants to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw at any time and will not need to provide any reason. Any information already collected may be used in the final analysis but you will not be identified from it.

If you decide to take part in the study, we will ask you to avoid caffeine, alcohol or recreational drugs from midnight before you come for a study visit and to avoid any exercise and drinking any liquids for one hour before.

What will happen to me if I take part?

We would like to meet with you on two occasions. When we meet you for the first time, we will confirm that you still want to take part and ask you to sign a consent form. You will also be given a copy to keep.

If you consent to take part in the study, we will ask some question such as your age, gender, age and race as brown fat can be more active in people of different ages, ethnicities and genders. We will take some measurements such as your temperature, heart rate and how much light is reflected from the skin on your finger using a small light. We will ask you to lie down for a few minutes and measure 'body fatness' (body composition) using a small, harmless electrical signal between small sensors which will touch your hands and feet. We will ask you about your physical activity and usual diet. We will take a series of thermal (heat) images over about forty minutes.

Part way through this first study visit, we will ask you to do something to turn on brown fat. This may be putting your hand in cold water, on a cool blanket or drinking a cool drink. You will know exactly what you will be asked to do before we start. We may also ask to measure things such as your heart rate during this time.

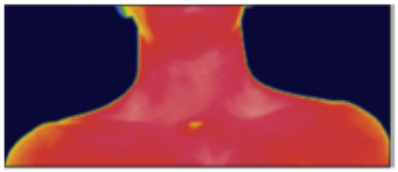
At the first session, the computer will assign you to consuming some Extra Virgin Olive Oil (EVOO) each day or just to continuing your usual diet. This will be done randomly (by chance) and you will not be able to choose. If you are in the half of study participants who are asked to add EVOO to their food, we will provide you with the oil. You will be asked to take around 2 tablespoons of the olive oil each day in your diet such as, as a dressing for salad or pasta. A nutritionist will advise you on the nicest ways to do this with your usual diet. We will also ask if you will keep a diary of the foods you eat at the start and end of the study but this will be optional. We will ask all those participating in the study to return after 3 weeks for the second study session during which the same measurements and thermal images will be repeated.

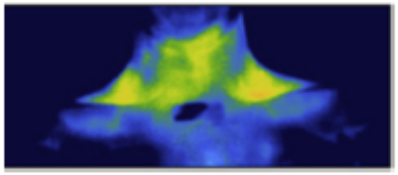
What is Extra Virgin Olive Oil?

Olive oil is the source of most of the fat in the Mediterranean diet and has already been shown to have several health benefits. Extra Virgin Olive Oil is the highest quality olive oil and meets food standards.

What is thermal heat imaging?

The thermal (heat-seeking) camera takes heat images in a similar way to a normal digital camera but shows hot and cold rather than details of the way we look. The pictures we will take will be of the neck and shoulders. Thermal imaging of the brown adipose tissue in these areas is a safe and simple technique developed, tested and routinely used at the University of Nottingham. We will ask that the thermal images remain on our database in order to build our experience of BAT using this technique. If you give your permission, the images will be stored securely by the University of Nottingham and you will not be identified from them.





Appendix 4 PRE-BAT studies registration sheet.

Subject ID		Gender		Humidity					
Height		Age		R.Temperature					
Weight		Adiposity		Room Temperature					
BMI		Fasting		Date/Season					
Are you currently taking any medicine?		Y/N	Which one (name/dose)						
Notes:		Skin Temperature		Water temperature					

Pre-Stimulation Period (10 minutes)				
	Picture Number		Time	CT
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Stimulation Period (20 minutres)				
	Picture Number		Time	CT
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				

PRE-BAT study

19				
20				

Post-stimulation (20 minutes)				
	Picture Number		Time	CT
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

Appendix 5 EVOO-BAT study registration sheet.

The University of Nottingham

EVOO-BAT Study

DATE		DAY	

Subject ID	Tympanic temperature			
	Room temperature			
	Humedity			
	Reflected temp. (camera)			
Current medication:	Water temperature			
Medical problems:	Notes:			

iButton

	Pre-Stimulation Period (20 minutes)			
	Pic ref(:00)	Pic ref(:20)	Pic ref(:40)	PC time
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

EVOO-BAT Study

	Stimulation period (20 minutes)			
	Pic ref(:00)	Pic ref(:20)	Pic ref(:40)	PC time
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

Appendix 6 PRE-BAT feasibility study, questions.

The University of Nottingham

The PRE-BAT study

Please answer the following questions once you have completed the intake of the prebiotic for 7 days. Bring this document to the following study session. Thank you.

Version 1.0, 28.07.2015

1. Was the product easy to mix with your daily food and drinks?

__Yes

__ No

2. Did you think the product had a flavour?

__Yes

__ No

3. The product represented a challenge to consume in your everyday life?

__Yes

__ No

Thank you

Appendix 7 PRE-BAT feasibility study, food diary.

The University of Nottingham

The PRE-BAT study

Write at what time of the day, and hour, you mix the sachet and thick next to the beverage or food you mix it with. If you experience something related with the consumption of the content in the sachet, please write on the notes section. Thank you

Version 1.0, 28.07.2015

Day 1

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 2

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 3

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 4 Date:		
Time	**Food/Beverage**	**Notes**
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 5 Date:		
Time	**Food/Beverage**	**Notes**
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 6 Date:		
Time	**Food/Beverage**	**Notes**
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 7 Date:		
Time	**Food/Beverage**	**Notes**
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Appendix 8 PRE-BAT randomised controlled trial, food diary.

The University of Nottingham

The PRE-BAT study

Write at what time of the day, and hour, you mix the sachet and thick next to the beverage or food you mix it with. If you experience something related with the consumption of the content in the sachet, please write on the notes section. Thank you

Version 1.1, 24.01.2016

Day 1

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 2

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 3

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 4

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 5

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 6

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Day 7

Date:

Time	Food/Beverage	Notes
Breakfast _____ am	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Lunch _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	
Dinner _____ pm	Tea ☐ Coffee ☐ Cereal ☐ Other ☐	

Appendix 9 EVOO-BAT study, 3 day food record.

Three-Day Food Record

Name: _____

Dates of recorded intake: _____

Instructions for Keeping Your Three-Day Food Record

- Please keep your three-day food record for three consecutive days.
- The days should include two weekdays and one weekend day.
- Select days that closely resemble your usual eating habits.
- Each time you eat or drink anything (meals, snacks, etc.) during the three days, write down what and how much it was served and what and how much was eaten.
- To measure how much was eaten, use a set of **measuring cups and spoons** to help estimate amounts. Also see the examples below to estimate portion sizes.
- Note if food choices are homemade or purchased. Please include brand names whenever possible.

Amounts and Conversions

1/4 cup = 50 ml or 4 Tablespoons

1/3 cup = 75 ml or 5 1/2 Tablespoons

1/2 cup = 125 ml or 8 Tablespoons

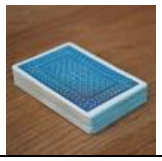
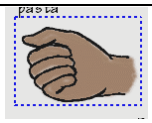
2/3 cup = 150 ml or 10 1/2 Tablespoons

3/4 cup = 175 ml or 12 Tablespoons

1 cup = 250 ml or 16 Tablespoons

1 oz = 1 slice of processed cheese or lunchmeal

How o Estimate Your Portion Size

Meat Three (3) ounces of meat are about the size and thickness of a deck of playing cards or an audiotape cassette.	
Fruit A medium apple or peach is about the size of a tennis ball.	
Grains One cup of rice or pasta is about the size of your fist.	
Cheese One ounce of cheese is about the size of four dice.	

Three-Day Food Record Checklist

Beverages	What kind of milk? Homo, 2%, 1%, skim, other. Was it fruit juice or fruit beverage or drink?
Breads	Did you spread on butter or margarine?
Cereal	Did you add milk? Did you add sugar or fruit?
Dairy	What brand or kind of yogurt? What brand or kind of cheese?
Vegetables	Was it raw or cooked? Was it fresh, frozen or canned? Did you add any butter, margarine or sauce?
Fruit	Was it a small, medium or large fruit? Was it fresh, frozen or canned?
Grains	Did you add any butter, margarine, peanut butter, jam or honey? Was it a half or whole sandwich? Was it a small or large muffin or bagel?

Fish	Was your canned fish packed in water or oil How did you cook your fish?
Meats	How did you cook your meat? What kind of cut was it e.g. chicken leg or chicken breast?
Soups	Was your soup prepared with milk, water or cream?
Restaurants	What restaurant was it?
Packaged food	What brand was it?

Sample Menu

Day 1: Tuesday, May 14, 2005				
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast	Cereal	½ cup	Honey Nut Cheerios	
	Milk 2%	½ cup		On cereal
	Banana	½ med		
AM Snack	Animal Crackers	10	Christie	
	Apple juice	4 oz	Allen's pure apple juice-canned	
Lunch	Grilled cheese sandwich			
	Whole wheat bread	1 slice	Dempsters	No crusts
	Cheese slice	1 slice	Kraft slices	
	Butter on bread	1 Tbsp		
	Yogurt – strawberry	75 ml	Mini-go	
	Milk	½ cup	2%	
PM Snack	Granola bar	1 bar – 35 g	Quaker Chewy, Trail Mix – tropical fruit	Ate half of it
Dinner	Chicken fingers	1 ½	President's Choice	
	French fries	10	McCain regular	
	Honey	2 Tbsp		For dipping
	Ketchup	2 Tbsp	Heinz	
	Carrots	½ medium	Raw, cut in sticks	
	Milk	½ cup	2%	

Evening Snack	Ice cream	1 cup	Chocolate Nestle	

DAY 1 (……)

Food Item (List of food and beverages. List separately main ingredient in mixed dishes)	**Amount Homemade**	**Amount (gr)**
MORNING		
MIDMORNING		
NOON		

AFTERNOOM		
EVENING/LATE EVENING		

TBSP: tablespoon; tsp: teaspoon; OZ: ounce;

DAY 2 (……**)**

Food Item (List of food and beverages. List separately main ingredient in mixed dishes)	**Amount Homemade**	**Amount (gr)**
MORNING		

MIDMORNING		
NOON		
AFTERNOON		
EVENING/LATE EVENING		

TBSP: tablespoon; tsp: teaspoon; OZ: ounce;

DAY 3 (……**)**

Food Item (List of food and beverages. List separately main ingredient in mixed dishes)	**Amount Homemade**	**Amount (gr)**
MORNING		
MIDMORNING		
NOON		

AFTERNOOM		
EVENING/LATE EVENING		

TBSP: tablespoon; tsp: teaspoon; OZ: ounce;

Appendix 10 EVOO-BAT study, demographics and age sheet.

Participant demographics

(For use at the first study visit after informed consent, on has been received)

We are asking for the following information because the activity of brown fat can be different in people of different ethnicity and of different ages.

1. Which of the following do you feel best describes your ethnicity:

	Please tick
Asian or Asian British	
Indian	
Pakistani	
Bangladeshi	
Any other Asian ethnic background	
Black or Black British	
Caribbean	
African	
Any other Black ethnic background	
Mixed	
White and Black Caribbean	
White and Black African	
White and Asian	
Any other Mixed ethnic background	
Chinese or other ethnic group	
Chinese	
Any other ethnic group	
White	
British	
Irish	
Any other White ethnic background	
Not stated	
Not Stated	

2. Which of the following age ranges does your current age fit?

	Please tick
Aged 18-21 years	
Aged 22-25 years	
Aged 26-29 years	