Effects of a novel combination of prebiotics and polyphenols on gut microbiota and stress, with a focus on active military personnel.

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Research suggests that the intake of certain foods – often referred to as nutraceuticals, may affect both gastrointestinal health and wellbeing through manipulation of the gut microbiota. This may be particularly beneficial for those who are experiencing physiological or psychological stressors – which can have a noticeable effect on health and wellbeing. A growing body of research suggests that the use of prebiotic supplementation and high fibre diets may modulate the microbiota and attenuate stress responses, whilst also improving cognitive function. Polyphenols, a putative prebiotic also, have demonstrated similar action in vitro and in animal studies. By combining the two supplements it may be possible to garner the benefits of both, whilst reducing the need for costly pharmaceutical treatments when it comes to reducing the physiological effects of stress. Though stress is ubiquitous in daily life, a subset of the population who are often subject to extreme stressors are active military personnel. Such stressors often result in soldiers having severe gastrointestinal diseases and cognitive perturbations such as Post Traumatic Stress Disorder (PTSD). By exploring the potential of beneficial nutritional interventions, it may be possible to establish whether the increased intake of certain nutraceuticals (such as this novel polyphenols and prebiotics) could improve psychological and gut health in combat soldiers and in the general 'stressed' population.

This work uses both *in vitro* fermentation work, and *in vivo* studies to explore whether a combination of these health positive supplements will have superior efficacy than either supplement alone on both the gut microbiome, and the perceived wellbeing of both healthy general populations, and active military personnel under artificial physical stressors. The *in vitro* fermentation work within this thesis demonstrated positive evidence for the novel combination to be more efficacious than either supplement alone, and when compared to a negative control, with improvements such as increased serotonin, elevated SCFAs such as acetate, and lactate, and positive changes within the gut

microbiota. In both human trials, positive changes within the faecal microbiota occurred, as well as potential reductions in pathogenic bacteria that were more acutely obvious in the presence of the novel combination. As well as this, changes in health-related metabolites such as hippurate were evident in those groups that had consumed the novel combination – as well as potential attenuation of stress related blood pressure increases. Wellbeing scores stayed relatively similar throughout the supplementation period, though more research is needed to confirm the effects on perceived mood. The novel combination, throughout both *in vitro* and *in vivo* did seem to present superior efficacy, suggesting that this would be a preferential option to either supplement alone, in both populations.

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Chapter 1 - Exploring the potential of prebiotic and polyphenol-based dietary interventions for the alleviation of cognitive and gastrointestinal perturbations associated with military specific stressors

1.1 Abstract

Active military personnel are often subject to extreme stressors, whether psychological or physical. Such stressors often result in soldiers having severe gastrointestinal diseases and cognitive perturbations such as Post Traumatic Stress Disorder (PTSD). Whilst pharmaceutical treatments are available, they are not always the most viable option, either because of poor efficacy, side effects, availability or economic detriment. By exploring the potential of beneficial nutritional interventions, it may be possible to establish whether the increased intake of certain nutraceuticals (such as polyphenols and prebiotics) could improve psychological and gut health in combat soldiers, and reduce the effects that PTSD and related gastrointestinal issues have on health and wellbeing. This report investigates the link between prebiotics, polyphenols and cognitive and gastrointestinal health.

1.2. General introduction

The human intestinal commensal microbiota and its metabolic products are regarded as important contributors to host health (Cani, 2018). This mixed community of microorganisms, and its resulting functionality, contributes to complex biological processes within the mammalian system, and is instrumental in metabolic crosstalk which occurs between the host and microbiome (Burcelin, 2016, Qin and Wade, 2017).

There is a need to discover the cause of gut disorders and develop effective new therapies or prevention strategies. This has resulted in a drive to expand research into treatment to proactively address issues and control symptoms. Although there is a lack of mechanistic evidence, there is acceptance that gut dysbiosis is involved in the pathogenesis of many digestive disorders (Wang et al., 2017) such as Irritable Bowel Syndrome (IBS), Inflammatory Bowel Disease (IBD, Gastroenteritis,

Diarrhoea. Though dysbiosis is a generalised term, for the purpose of this chapter, I will use it to refer to an imbalance, or change, from the homeostatic balance of microbiota in the gut that had been associated with different disease conditions (DeGruttola et al., 2016).

Although it is recognised that diet is one of the most modifiable indicators of human health (Leeming et al., 2019), the human gut microbiome is still fairly under-explored as a functional ecosystem (Arumugam et al., 2011, Kho and Lal, 2018, Vrancken et al., 2019). This is despite it providing an extraordinary opportunity to reduce the impact of common gastrointestinal diseases such as IBS and gastroenteritis (El-Salhy et al., 2019), via dietary intervention to modify bacterial communities (Staudacher et al., 2017).

Recent research shows that there is potential for the use of gut mediated therapies to treat or at least control symptoms of psychological disorders, such as Post Traumatic Stress Disorder (PTSD) (Bersani et al., 2020, Leclercq et al., 2016). The ability of the gut microbiota to influence the biological state of an individual has led to an acknowledgement that research into the microbiome is an essential part of current and future healthcare strategies (Hadrich, 2018).

Not only is diet one of the most important modifying factors of the gut microbiota, but it is also instrumental in regulating stress related responses (Shively et al., 2020). This is because diet has an impact on the microbiota-gut-brain axis, especially in situations perpetuated by homeostatic challenge (Foster et al., 2017).

Routes of communication between the microbiota and brain are of growing interest and, whilst more information is needed, key components have now been identified: the vagus nerve; gut hormone signalling; the immune system; tryptophan metabolism; and microbial metabolites such as short chain fatty acids (SCFA) (Carabotti et al., 2015). Although it may initially seem incongruous that the gut can influence the brain, common phrases in most languages such as gut wrenching, gut feeling – even butterflies in the stomach - suggest an intuitive understanding of such a link between these organs.

Grasping the importance of the gut microbiome in both neurological and gastrointestinal pathologies and developing treatments is a major challenge for 21st century medicine. Nowhere is that need greater than in the military. Gastrointestinal illness is an extremely common reason for sick visits

within military personnel (Riddle et al., 2015) and PTSD is present in (71%) of veterans (Armenta et al., 2018). These issues not only affect active duty performance but cause detriment to the economy of the military, and clearly damage the general well-being of soldiers.

By studying nutritional interventions such as pro- and prebiotics it could be possible, through exploitation of gut microbial communities, to develop safe and effective interventions. If this positively influences both gut health and the mental state of individual war fighters, this can mitigate many military-specific issues that are currently compounded by military-relevant physical, physiological and psychological stressors. Although a lot of research has been carried out on non-war fighters gut and brain health and their interactions, the role of the gut microbiota (and how it is affected by stress) is under explored.

This review will focus on polyphenols and prebiotics; outlining their mechanisms of effect, identifying any crosstalk between different combinations and applying this to warfighter-specific problems. It will examine the potential for novel nutritional mixes (of prebiotics and polyphenols) to directly affect specific bacteria in order to attenuate or prevent gut dysbiosis and associated cognitive perturbations during stress exposure. By looking at interactive mechanisms, it may also be possible to discover whether or not current military MREs (meals ready to eat) could have effects on the potential benefits of prebiotics and polyphenols added to the military diet. By expanding knowledge on this subject and improving understanding of combinatorial nutritional supplementation, it may be possible to establish whether or not it is appropriate to treat certain warfighter associated physical and cognitive conditions through gut microbiota targeted dietary interventions. By examining both gastroenteritis and the neurobiology of cognition, the findings of this chapter will be relevant to not only warfighter specific cases but also general health and wellbeing.

1.3. Military specific stressors

One primary reason to focus on war fighters is because they are in highly stressful situations. This review will attempt to evaluate the potential for polyphenols and prebiotics to mediate stress response, whether cognitive or physical. It is important to note that, as there is a lack of warfighter specific research, civilian and (where available) athlete research will be used. Athletes may be appropriate

representatives for war fighters as stressors commonly occurring in those groups are similar, i.e. strenuous exercise leading to injuries.

Here, stress is defined as 'a disruption in homeostasis due to environmental, physical, or psychological stimuli (i.e., stressors) that elicits adaptive physiological and behavioural responses to restore homeostasis (i.e., the stress response)' (Glaser & Kiecolt-Glaser, 2005). Although, as mentioned, this paper will categorise research on non-military personnel, it is still possible to examine stressors unique to the military situation. As such, stressors can be separated into physical (strenuous exercise, undernutrition, etc.), psychological (anxiety and consequent cognitive demands, etc.) and environmental (pathogens, high altitude, etc.) (Weeks et al., 2010).

Research has correlated these stressors with detriments to health such as nutrient deficiencies, hormone disruption, injury or impairments (musculoskeletal and cognitive), inflammation and immune suppression, as well as general illness and infection (Karl et al., 2018).

Despite the belief that people can build tolerance, or resilience to stress (Dienstbier, 1989), it has been established that highly stressful situations can affect performance, cognitive abilities, illness and recovery time (Wu et al., 2013). It is, therefore, both interesting and reassuring that growing evidence has linked many stress related health conditions to dysbiosis of the gut, as it might prove to be the case that, by manipulating the gut microbiota through nutrition, we may mediate such responses in military personnel.

1.4. Gut-brain axis

Though a link between the gut microbiota and the brain has long been suspected, it is only in recent years that we have seen evidence of causal links between changes in the gut microbiota and brain function and behaviour. *In vitro* research has also revealed the potential molecular mechanisms involved in communication between the gut and the brain – the gut-brain axis, as illustrated in Fig. 1.

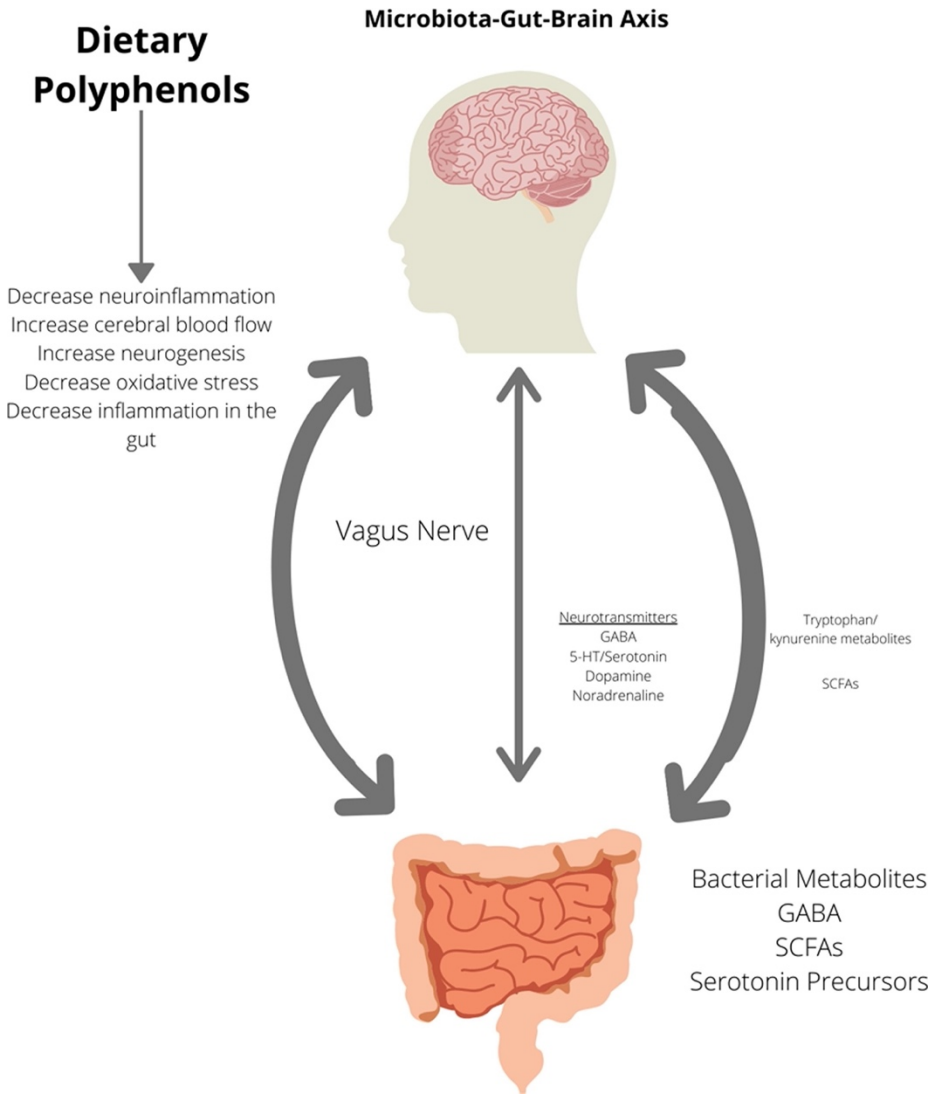

Fig. 1. Illustration of the gut-brain axis in the human superorganism. The putative effects of dietary polyphenols and the bidirectional communication of the microbiota-gut-brain axis are indicated. GABA: gamma-Aminobutyric acid; SCFA: Short Chain Fatty Acid; 5-HT, 5-hydroxytryptamine.

Interactions between peripheral intestinal function and cognitive and emotional centres of the brain appear to be bidirectional (Sun et al., 2020), and much research shows that communication is highly associated with signalling from the gut microbiota (Carabotti et al., 2015). Communication within both the central and enteric nervous systems (signalling from the gut microbiota to the brain and from the brain to the gut microbiota) involves endocrine, immune and neural mechanisms (Carabotti et al., 2015, Ma et al., 2019). Though much recent research has been in animal models (Park et al., 2013),

links between the microbiota and the gut-brain axis have been demonstrated by correlations between gastrointestinal disorders such as IBS (Mayer, 2011), and cognitive disorders like anxiety or depression (Zheng et al., 2016).

GABA is an inhibitory neurotransmitter, and dysregulation of GABA is evident in conditions such as anxiety and PTSD (Huang et al., 2023). Regulation of GABA, the GABAergic system, has been shown to be highly related to the gut microbiota, and to the pathophysiological process of PTSD. Briefly, gut microbes such as *Lactobacillus* and *Bacteroides* can convert glutamate to GABA (Hemarajata et al., 2013), as some enteric bacteria encode for the glutamic acid decarboxylase (GAD) gene – meaning that the glutamate is decarboxylated into GABA (Yogeswara et al., 2020). Microbially produced GABA, or even the consumption of GABA enriched foods, has been associated with the attenuation of many conditions, such as depression, insomnia, and anxiety (Yu et al., 2020), as well as the production of GABA stimulating the immune system, through interactions with the GABA receptors in the intestinal tract (Zhao et al., 2020).

Serotonin is another microbially derived neurotransmitter. Most of the serotonin within the body is produced in the gastrointestinal tract by enterochromaffin cells, and serotonin has many functions within the body – not just in the gastrointestinal tract (Kitson et al., 2007) An important note is that a significant amount of serotonin in the body is synthesised by the central nervous system, and this centrally derived serotonin plays an important role in many functions, such as regulation of appetite and mood (Jenkins et al., 2016).

Decreased levels of serotonin are highly related to cognitive function decreases, and to conditions like depression. The enterochromaffin cells in the gut interact with the gut microbiota to influence the production of serotonin (Appleton et al., 2018; Carabotti et al., 2015). This can be seen in studies where gnotobiotic, or germ-free mice demonstrate low concentrations of serotonin, which is then rectified by the addition of commensal microbiota (Hata et al, 2017).

The precursor for serotonin, tryptophan, is provided entirely from the diet. Though it is metabolised into serotonin and melatonin, 95% of tryptophan is metabolised along the kynurenine pathway (Muneer et al., 2020). This pathway is regulated by the gut microbiota – some bacteria within the gut can metabolise tryptophan, producing bioactive compounds such as indole derivatives that can modulate the activity of enzymes involved in the kynurenine pathway. The gut microbiota can also influence these pathways more indirectly, potentially through the production of SCFAs. Overall, the gut regulation of the tryptophan-kynurenine pathway has implications for host health, including immune response and neuroinflammation (Gao et al., 2020; Hou et al., 2023).

Research has shown that the brain can affect structure and function of the gut microbiota through modulation of gut motility and gut permeability. It has also been shown that through this bidirectional mechanism, direct secretion of hormones may directly affect microbial gene expression (Martin, Osadchiy, Kalani, & Mayer, 2018). These interactions are thought to be a circular communications loop and any disturbance within the loop can result in dysregulation. One example of this is where secretion of hormones such as 5-HT from enterochromaffin cells are seen to travel towards the gut lumen, potentially resulting in microbial alterations (Lund et al., 2018). This is likely a bidirectional relationship, where secondary bile acids and short chain fatty acids derived from gut bacteria are responsible for the regulation of enterochromaffin cell derived 5-HT synthesis (Mandić et al., 2019). Hormones can affect microbial gene expression in other ways, such as in the case of the increased virulence of pseudomonas aeruginosa, by norepinephrine (Hegde et al., 2009). Though the mechanisms of this are not fully understood, it is thought that direct affectation of norepinephrine on the virulence of bacteria is through enhancement of bacterial attachment to host tissue (Freestone, 2013).

Not only is IBS associated with general detriment to cognitive function, but research has directly linked the hyper-arousal and hyper-vigilant state of PTSD to IBS as a result of the bidirectional signalling of the GBA (gut-brain axis) (Ng et al., 2019). Traditional diagnoses of PTSD rely on examination of behavioural symptoms (Spoont et al., 2010) but more recent evidence (as mentioned) has shown PTSD to be linked with immune system and inflammatory changes. IBS has been independently associated with PTSD (Iorio et al., 2014). One study showed that 36% of patients with

IBS met behavioural and psychological criteria for diagnosis of PTSD (Irwin et al., 1996). It has also been reported that, specifically in female veterans, there was an increase of IBS in those diagnosed with PTSD (Savas et al., 2009).

Psychiatric illness is highly debilitating to some and often one of the most dangerous aspects is the risk of relapse. By taking a more holistic approach to the treatment of cognitive perturbations, such as exploring the potential for modulation of the gut microbiota to reduce symptoms or lessen these, recovery may be improved.

Associations have also been made between microbiota and stress-related changes in behaviour and brain function. For example, one study explored whether postnatal microbial colonisation affected neuroplasticity and biological systems response. By using germ-free, specific pathogen-free and gnotobiotic mice, this study explored the hypothalamic–pituitary–adrenal (HPA) reaction to stress. It found that germ free mice had a substantially higher hypothalamic-pituitary axis response to stress, and that this exaggerated response could be reversed through colonisation by a probiotic *Bifidobacterium infantis* (Sudo et al., 2004). This suggested that commensal microbiota can strongly affect the development of the stress response, that changes were not permanent and that the introduction of specific bacteria can alter stress responses. Though the idea of psychobiotics has been around since 2013 (Dinan et al., 2013), the use of bacterial and nutritional intervention has not been greatly explored in human studies. When it has been tested on humans it has often been on healthy volunteers rather than exploring psychobiotics as a treatment for those in a disease state.

By looking at inflammatory biomarkers and hormonal levels associated with disease states, researchers have been able to associate dysregulation of immune function and the HPA axis with an individual response to stress and therefore, likelihood to develop PTSD (Neigh & Ali, 2016).

PTSD is often characterised by high pro-inflammatory cytokines and low cortisol responses (Gill et al., 2008, Kim et al., 2020, Kim et al., 2020, Speer et al., 2018, Speer et al., 2019). Analyses have primarily shown increases in levels of pro-inflammatory cytokine interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ (Kim et al., 2020, Kim et al., 2020). Research has shown that dysbiosis of the gut may increase susceptibility to PTSD after traumatic or high stress events (Leclercq et al., 2016). Furthermore, when stress alters the microbiota early in life, it can shape

immune homeostasis and nervous system for the host (Borre et al., 2014), and increase the risk of developing PTSD later in life (Leclercq et al., 2016). It may therefore be possible, by targeting this dysbiosis, to manipulate the gut-brain axis with nutritional intervention or supplementation in order to reduce likelihood of the occurrence of PTSD.

Research in mouse models, using intruder stressors, has shown that Firmicutes and Bacteroidetes are vulnerable to stress that can cause PTSD, and the ratio between these increases with increasing stress (Gautam et al., 2018).

By studying such links and how alterations affect different parts of this system, it may be possible to identify novel therapeutic targets that address cognitive disorders that have so far been poorly understood. Case study research has suggested that treatment of PTSD symptoms may alleviate symptoms of gastrointestinal illnesses, like IBS (Weaver et al., 1998).

There is a link between dysbiosis of the gut and cognition, as illustrated in a study that involved antibiotic disruption of colonic bacteria in adult mice (Fröhlich et al., 2016). The study found that, by treating these mice with antibiotics, significant changes occurred in metabolite levels, changing expression of certain molecules and hindering specific brain functions such as memory. It is posited that cognitive impairment correlating with dysbiosis, is related to HPA axis activity and changes in the expression of certain tight junction proteins (Fröhlich et al., 2016). This direct correlation of dysbiosis to cognitive impairments and biochemical alterations may be relevant to PTSD.

Cirrhosis in veterans can be directly linked to PTSD with changes in the gut-liver-brain axis being observed. Research has shown a lower microbial diversity in PTSD, with higher levels of pathogenic bacteria. Studies have correlated the increase of some pathobionts such as *Enterococcus* spp. with general poor cognition and have specifically linked *Shigella* spp. with PTSD patients (Bajaj et al., 2019). Interestingly, when combat-exposed veterans with PTSD were directly compared to combat-exposed patients with no PTSD, functionality was seen to differ in the gut-brain axis between the groups, demonstrating that PTSD was directly linked to differences in microbial diversity (Bajaj et al., 2019).

Of further relevance to military personnel is their diet, as an inadequate diet has been shown to have a deleterious impact on cognitive performance (Gómez-Pinilla, 2008, Lu et al., 2016). In a study during

which young people were put into a military training environment with multiple stressors, increased intestinal permeability was exhibited as a response to the stress, and concentrations of microbial metabolites in faecal samples were also altered (such as p-cresol which increased, and benzoate metabolites which decreased), with decreases in Bacteroidetes and increases in Firmicutes also being observed (Karl et al., 2017).

1.5. The gut microbiota

The human gut microbiome, which is comprised of various organisms such as bacteria, viruses, parasites and other microbes, has an enormous effect on health and disease outcomes (Clemente et al., 2012). This can be as a result of contributions to metabolic function which enhance resistance to disease by both improving immunity and protecting against pathogens. Through this metabolic action, the gut microbiome affects many human physiological functions. The majority of gut microbes are beneficial (or harmless) but dysbiosis is associated with diseases such as IBD, IBS, psychological/neurological disorders, certain cancers and obesity (Zhang et al., 2015). Dysbiosis can be defined as any change to the composition of resident commensal communities relative to the community found in healthy individuals (Petersen & Round, 2014). This discovery has improved understanding of how the microbiota may be modulated as a response to human health and has shown that the gut microbial community should be considered as a whole, rather than focussing on individual bacteria (Thursby & Juge, 2017).

Because gut microbiome profiles vary from individual to individual, specific characteristics of a healthy gut microbiome cannot be narrowly defined (Bäckhed et al., 2012, Conlon and Bird, 2014, Human Microbiome Project, 2012). Over 1000 phylotypes exist in the human gut but most of these belong to a few phyla: Bacteroidetes and Firmicutes are predominant, with other more minor constituents also commonly present (Rinninella et al., 2019). Examples of healthy adult microbiota have some gut bacterial species in common and, through culture-based studies, this has come to be considered as a 'core microbiota' (Guinane and Cotter, 2013, Ursell et al., 2012). However sequencing research has demonstrated that microbiota are highly temporally and spatially variable in the colon, which calls into question validity of the idea of a 'core' microbiota (Parfrey and Knight,

2012, Ursell et al., 2012). Although the idea of this 'core' microbiota may no longer be universally accepted, one review paper has suggested that, alternatively, a core microbiome is shared by healthy gastrointestinal tracts (Lozupone et al., 2012). Though the terms are often used interchangeably, usually, microbiota refers to the actual bacteria, whereas the term microbiome often is used in a more functional capacity, describing microbes and their genes. The gut microbiome may be functionally highly similar, whilst hosting many different microbiota species due to varying environmental influences.

It is important that we understand effects that such environmental variations have on human health, as this may account for individuals' differing responses to drugs or dietary components. Host-specific responses to certain foods may involve pathways outside common functional metabolism (i.e. differential microbial metabolism). This is illustrated by a study which focused on potential health benefits of consuming soy products. Many health benefits associated with soy consumption have been shown to be associated with the bacterial metabolism of soy isoflavone daidzein to S-(-) equol (Mayo et al., 2019). However, production of this beneficial compound seems to be reliant on habitual consumption of soy as shown by Rowland et al. (2000). This was further demonstrated by a comparison of studies on equol production in western countries versus those in Japan, Korea or China where soy was consumed as part of a habitual diet. It was shown that, while a non-western diet had a 50–60% occurrence of S-(-)equol upon dietary soy intake, the adult population of western countries produced only around 25–30% (Miura et al., 2016). This is interesting, not only because it demonstrates ways in which habitual diet can affect the processing of food components, but also because it has potential ramifications in medicine as key microbiota differences may affect drug metabolism in different populations. Another study that looked at the effects of of microbially derived eqol (Hazim et al., 2016) which suggested that differing existing phenotype groups – in this case equol producers (EP) and non-Eps – was the primary indicator for whether responders would benefit from soy equols. The study demonstrated that only the EP group showed cardiovascular benefits after intake. This study demonstrated that it is not necessarily solely habitual diet that regulates how equols are processed, rather this is governed phenotypically. It is also important to note that, though some of the above studies showed buimarkers of the consumption of these isoflavanols – these are not

necessarily corresponding to biomarkers indicating improved health, such as the cardiovascular markers in the Hazim study.

Having established that habitual diet affects how dietary components are metabolised, it is also important to understand and explore resilience of the gut microbiota – in short – how much can the response to certain functional foods be changed by diet, for how long and how quickly? This is important when considering therapeutic diets such as introducing prebiotics, probiotics or polyphenol compounds. Human studies have usually demonstrated statistically significant changes to the microbiome when diet is changed over a period of time, for example, differences have been seen after 10 days of a high fibre diet (Wu et al., 2011). However, it is important to note that some changes in microbiome composition in one study were actually detectable within 24 h of controlling the diet (Wu et al., 2011). This was further confirmed in studies on gnotobiotic mice which demonstrated that switching from a low fat, plant polysaccharide rich diet to a high fat, high sugar western diet caused structural changes in the microbiota within a day. Not only was structure altered, but also gene expression and metabolic pathways in the microbiome (Turnbaugh et al., 2009). It is important however to note that, although these changes are observed and may have effects on potential health benefits conferred by eating certain foods (as in the case of soy), differences are still minimal compared to simple interpersonal variation.

The gut microbiota affects digestion and host nutrition by breaking down non-digestible substrates. This symbiotic relationship provides strong evidence of the importance of the gut microbiota for host health (Makki et al., 2018). One way that digestion of substrates contributes to host health is by releasing short chain fatty acids (SCFA) from indigestible fibres. SCFA may help modulate both the immune response and tumorigenesis in the gut (Bishehsari et al., 2018, Chambers et al., 2018).

The abundance of many bacteria may be inversely correlated to several disease states (Arboleya et al., 2016; Heiman and Greenway, 2016). It may therefore be possible to utilise dietary components to selectively enhance the growth of beneficial bacteria that improve host health (Zhang et al., 2015).

While there is considerable research on the use of probiotics to improve gut microbiome health, prebiotics can also be used to maintain and improve health through nutritional interventions that

increase the activity of bacterial groups such as *Bifidobacterium* and *Lactobacillus* spp. (Singh et al., 2017).

1.6. Prebiotics

The inclusion of probiotics, prebiotics or synbiotics into the human diet can favourably alter the intestinal microbiota. According to the latest definition by the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Probiotics have been shown in clinical studies to have a positive effect on gastrointestinal diseases (Allen et al., 2004, Dale et al., 2019, McFarland, 2006) as well as disorders such as diabetes (Tao et al., 2020). Research has also shown that probiotics can aid the body's immunity (Vanderpool et al., 2008) and be used prophylactically to attempt to prevent certain cancers (Kim and Jin, 2001, Lidbeck et al., 1991).

A prebiotic is defined by ISAPP as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Prebiotics stimulate the growth of different gut bacteria and can have a large effect on the modulation of the gut microbiota (Chung et al., 2016). Health benefits conferred from the intake of prebiotics can vary, but research has shown that prebiotics can aid in metabolic health (Kellow et al., 2014), allergic outcomes (Brosseau et al., 2019) and gastrointestinal disorder (Lindsay et al., 2006, Welters et al., 2002). Prebiotics are found in fruit, vegetables, fermented foods, and can also be ingested through supplementation, as can probiotics (Markowiak & Śliżewska, 2017).

The term synbiotic was first introduced by Gibson & Roberfroid (1995), and was described as a combination of synergistic probiotics and prebiotics, but in 2019 the definition was updated to " a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson et al., 2020). Synbiotics aim to aid the survival of probiotics in the gastrointestinal tract thereby, theoretically, improving their efficacy (Peña, 2007).

Plant polyphenols are compounds that may also meet the criteria of prebiotics (Gibson et al., 2017) and, whilst more evidence is needed, the health benefits linked to polyphenol consumption are

associated with metabolites produced after microbial metabolism (Dueñas et al., 2015). Polyphenols are a large group of phytochemicals, with enormous variation in both structure, function and metabolite production (Tsao, 2010).

The primary bacteria 'targeted' by prebiotics are *Bifidobacterium* and *Lactobacillus*, as these have evidence of health promoting effects (Manning & Gibson, 2004). Established prebiotics (through *in vivo* studies) include inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), although there are other compounds that also meet the criteria of this definition (Davani-Davari et al., 2019). There are other oligosaccharides, algae, resistant starches, and polyphenols, which may positively affect bacteria within the gastrointestinal environment although there is less *in vivo* evidence than for fructans or galactans (Gibson et al., 2017)

By selectively modifying the gut microbiota through prebiotics, we may not only induce beneficial effects in the colon and surrounding digestive tract, but also potentially benefit other areas of the body. Research has shown that the prebiotic effect is associated with improvements in the activities of the immune system, and biomarker levels such as blood lipids (Markowiak & Śliżewska, 2017).

One of the primary mechanisms by which dietary fibre and other prebiotics change the gut microbiota is through fermentation in the colon (Slavin, 2013). The majority of bacteria in humans are in the large intestine and this is the most diverse and metabolically productive area of the body (Louis et al., 2016). Due to a slow transit time, anaerobic conditions, favourable pH and readily available nutrients, bacterial growth is extensive therein. Bacteria that are potentially beneficial are often those with a solely saccharolytic metabolism, such as the lactobacilli and bifidobacteria mentioned previously (Ouwehand et al., 2005). The primary fermentation pathway generates pyruvate from hexoses in undigested carbohydrates (Oliphant & Allen-Vercoe, 2019). Then, colonic bacteria hydrolyse many of these to produce CO_2, SCFA and other compounds, some generating energy from the fermentation (Slavin, 2013). SCFAs can be absorbed into the bloodstream and some, like acetate, are metabolised systematically (Hernández, Canfora, Jocken, & Blaak, 2019). Production of SCFAs through fermentation in the colon is also thought to repress pathogen growth by reducing the intestinal pH

(den Besten et al., 2013). One positive aspect of the introduction of prebiotics to improve host health is that prebiotics occur naturally in many foods, such as garlic, onions, soybeans, wheat, banana, asparagus, artichoke and oats (Slavin, 2013).

Although fibre and thereby, some prebiotics, are recommended in nutritional guidance, their intake is small in western diets (Holscher, 2017). It may be possible however, to confer health benefits to those unable to consume the necessary amounts by nutritional interventions such as fortification of foods with prebiotics. If we consider polyphenols to have a prebiotic effect, highly concentrated derivatives of plants containing these, as well as other prebiotic supplements, could be added to foodstuffs.

One often overlooked group of people unlikely to consume enough prebiotics (to counteract the harmful effects of physical and mental stress) is combat soldiers. Military personnel have to operate under conditions that civilians would not usually be subjected to, for example: poor sleep; less than ideal nutrition; extreme environments e.g. altitude, all of which can lead to elevated stress (Hill et al., 2011, Karl et al., 2018). Due to the nature of their job, combat soldiers are often required to perform their roles despite these suboptimal conditions, which has the potential to lead to poor health outcomes including cognition. 39% of military personnel report feeling a great deal of stress in their work and it is possible that these stressors and associated issues could dictate mission success or failure (Bray et al., 2001). It is therefore in the best interests of the military to ensure that everything possible is done to ensure optimum cognitive and physical performance of military personnel. Studies have demonstrated that, although the gut microbiome does generally display some stability, it is possible for stressors to alter gut microbiome composition (Karl et al., 2018). Gut microbiota could, therefore, be manipulated to modulate the human stress response to improve host health, and growing evidence does show that a healthy gut microbiota has positive effects on military performance (Arcidiacono et al., 2018).

Not only could acute stress hamper performance (Bray et al., 2001) but continued and chronic stress and trauma experienced by some military personnel may result in cognitive perturbations such as PTSD (Iribarren et al., 2005). The prevalence of PTSD in military personnel may be twice as high as in civilian populations (Spottswood et al., 2017).

The gut microbiome is known to have a critical role in the brain-gut axis, and in regulation of intestinal permeability, (Carabotti et al., 2015, Kelly et al., 2015), so nutritional supplementation to improve function of the gut microbiome may be fundamental when considering the treatment and prevention of PTSD. Because of its association with low grade inflammation, PTSD may result in deficits in intestinal permeability (Bersani et al., 2020, Kim et al., 2020, Kim et al., 2020, Leclercq et al., 2016). Therefore, it is feasible that treatment (curative and proactive) through manipulation of the gut bacteria by nutritional intervention, could benefit military personnel. It is also important to note that, while a diet of USA military food rations alters faecal microbial composition, it does not directly increase intestinal permeability (Karl et al., 2019).

It has been postulated that prebiotics provide a protective effect on cognition through aiding of production of Brain-derived neurotrophic factor (BDNF) (Burokas et al., 2017, Franco-Robles and López, 2016).

BDNF supports neuron differentiation and survival. It has been shown to have a neuroprotective effect under stress to the brain and stimulates the growth of new neurons (Maisonpierre et al., 1991, Huang et al., 2001). BDNF is found to be decreased in certain neurodegenerative diseases, like multiple sclerosis (Sohrabji et al., 2006). BDNF regulates synaptic plasticity, and is regulated by the gut microbiota. In brief, BDNF is released from the neuron in response to stimuli such as synaptic activity. It then binds to receptors - TrkB receptor (tyrosine receptor kinase B) and triggers signalling cascades within the neuron.

There are three distinct cascades, involving different proteins. The first of these engages insulin receptor substrate-1/2 (IRS-1/2), phosphatidylinositol-3-kinase (PI-3K), and protein kinase B (Akt). The second activates Shc/Grb2, Ras, Raf, mitogen-activated protein kinase kinases (MEKs), and extracellular signal-regulated kinases (ERKs). The third cascade includes phospholipase C (PLC), inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3], diacylglycerol (DAG), and protein kinase C (PKC). These then activate transcription factors like cAMP-response-element-binding protein (CREB) and CREB-binding protein (CBP) to regulate gene expression tied to neural plasticity, stress resilience and cellular health and survival (Bathina et al.,2015, Schiro et al., 2022).

This is particularly relevant to those in high stress situations, as BDNF has been shown to decrease when acute stress occurs. In brief, supplementation with prebiotics such as inulin, will possibly modulate the gut-brain axis, by increasing BDNF levels and reducing pro inflammatory cytokine concentrations. One study (Romo-Araiza et al., 2018) using a mouse model suggested that prebiotics increased the effects of beneficial bacteria, or probiotics, thus increasing butyrate production, which results in these positive changes. By increasing the intake of prebiotics, and therefore promoting an increase in microbial activity, cognition may be protected. A study has further supported the association between prebiotics and BDNF by supplementing rats with a prebiotic, showing that the levels of BDNF were elevated in the prebiotic group, compared to a control (Williams et al., 2016). A 2023 study (Church et al) highlighted the beneficial impact of the intake of fibre, which increased BDNF. Most interestingly within this study, they were able to correlate the serum changes of acetic acid with the reduced neuroinflammation and the increased in BDNF. This further supports the use of fibre supplementation to influence cognition. Acetate exists, in a healthy population, at an approximate ratio of 80:10:10 (acetate:butyrate:propionate) (Ktsoyan et al., 2016). It is likely that acetate and butyrate differ in terms of mechanism and function. Whilst acetate is able to cross the blood-brain barrier and act as an energy source – it's primary function is to service the peripheral tissues (O'Riordan et al., 2022). However, it has been shown to regulate levels of GABA in the hypothalamus (Hernandez et al., 2019). Butyrate is thought to stimulate the production of GABA and decrease pro-inflammatory cytokine secretions. It is a primary energy source for colonic cells and some research suggests that (though in much lower peripheral circulating quantities) butyrate may have a more direct impact on systemic function and cognition (Siddiqui et al., 2021).

A 2020 study by Blaak et al suggests that around 99% of SCFAs are used by the gut mucosal environment, by examining the concentration difference rom the colon to the portal vein. This study also suggested that, after the portal vein, propionate and butyrate may be extracted by the liver. The table below illustrates the proportions and quantities of the three primary SCFAs in the colon to the peripheral blood.

	Colon		Portal Vein		Hepatic Vein		Periphery	
	Ratio	µmol/kg	Ratio	µmol/L	Ratio	µmol/L	Ratio	µmol/L
Acetate	60	43,500-63,400	71	182-263	81	71-220	90	5-220
Propionate	20	14,200-26,700	21	18-30	12	0.8-7	5	3-8
Butyrate	20	14,700-24,400	8	15-30	7	0.5-12	5	7-10

Table 1, adapted from Blaak et al., 2020.

Although probiotic supplements have been studied in rela tion to improving individual performance (Agans et al., 2020), the use of prebiotics is relatively under explored in this population. Research has shown that prebiotic supplementation can alter cognitive states in some individuals, including increased attention to positive emotional cues and improved mood (Schmidt et al., 2015). Other studies have shown that consumption of inulin resulted in better accuracy in recognition memory tasks, and improved recall performance (Smith et al., 2015).

1.7. Polyphenols

Polyphenols are ubiquitous plant chemicals. They are structurally categorised by the presence of large multiples of phenol structural units and have, in recent years, become a focus of nutritional research. Due to their abundance in plants, they naturally form a part of the human diet and evidence suggests that consumption of these molecules is a key modulator of human health. Though previously thought to be due to direct antioxidant effects, beneficial modulation of both physical and cognitive health by polyphenols is now widely accepted to be due to interactions with the gut microbiota, as metabolites of these interactions may provide beneficial effects throughout the host system (Kennedy, 2014). There are over 8000 types of polyphenol (currently identified) but they are broadly categorised (as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another) into flavonoids (which account for roughly 60% of all polyphenols), phenolic acids, stilbenes and lignans (Pandey and Rizvi, 2009, Tsao, 2010). Polyphenols can be generally divided into two main groups – Flavonoids and non-flavonoids. The flavonoids are split into six groups: flavonols, flavones, flavanols, flavanones, isoflavones, and anthocyanins (Abbas et al., 2017), and non-flavonoids include stilbenes, lignans and phenolic acids (Pandey & Rizvi, 2009).

Polyphenols may initially be absorbed in the small intestine, often, though not always, conjugated with sugars or organic acids (although this usually only occurs with those structures that are mono or dimeric (D'Archivio et al., 2010)). This releases aglycones which enter the intestinal cell lining and undergo biotransformation, after which metabolic products are spread round the body or excreted (D'Archivio et al., 2010). Other, more complex structures will reach the colon intact, and can be metabolised therein by the gut microbiota. This transformation is mediated by microbial enzymes, and may include demethylation and decarboxylation, amongst other processes (Chen et al., 2018). It is important that we understand biotransformations mediated by phase I and II reactions in polyphenols, as it is these phases of metabolism that cause low bioavailability (in contrast with high bioactivity) (Luca et al., 2020). Poor absorption of dietary polyphenols results in extensive metabolism within enterocytes and the liver by phase I and II enzymatic reactions, followed by biotransformation by the gut microbiota into varying structures that can be circulated in the blood (Luca et al., 2020). One study estimated that less than 5% of dietary polyphenol intake is absorbed and reaches plasma unchanged (Faria et al., 2014). As mentioned above, low bioavailability/high bioactivity paradox means that metabolites, whether through enzymatic transformation or microbial degradation, are of great interest to the scientific community as they demonstrate significant mechanistic effects (Luca et al., 2020).

Known to be secondary metabolites in plants, dietary polyphenols are primarily involved in defence against oxidative damage (ultraviolet radiation) or damage caused by pathogen aggression. In humans, these protective effects seem to be transferred, and long-term consumption of diets high in plant polyphenols may protect against: cancer development and progression (green tea) (Yuan et al., 2018); cardiovascular disease (Khurana et al., 2013); neurodegenerative diseases (Mandel & Youdim, 2004)), and other chronic diseases (Hogervorst et al., 2018, Pandey and Rizvi, 2009). Polyphenol consumption has also been associated with modulation of human health through anti-inflammatory properties (Zhang & Tsao, 2016). There is much evidence that supports associations= between the consumption of polyphenols and a reduced risk of chronic disease, and many reviews and research have stated that although specific classes of compounds are yet to be quantified and explored

sufficiently to give specific recommendations, a diet high in polyphenol containing foods should be encouraged (Del Bo et al., 2019, Knekt et al., 2002).

One of the reasons that it is difficult to establish specific recommendations when exploring the potential protective effects of polyphenols is large methodological variation when collecting data (Del Bo et al., 2019). Furthermore, many intervention studies see a much higher dose of polyphenol content being administered than is realistic for a human to consume in a 'normal' healthy diet. (Williamson, 2017). There may also be different mechanistic actions of polyphenol isolates versus wholefood consumption – we see in broccoli, for example, that supplementation does not have the same beneficial effects as consumption of the whole food (thought to be due to a lack of the enzyme Myrosinase in supplements (Clarke et al., 2011, Gautam et al., 2018), so this should also be taken into consideration. Another difficulty when evaluating the efficacy of polyphenols in protecting human health is their myriad structures, each of which has a different metabolic pathway and physiological roles, which means that each individual compound's health effects should be explored – both long and short term (Carbonell-Capella et al., 2014). Whilst it is reasonable to recommend polyphenol intake, guidelines for supplementation need to be established.

One benefit of polyphenols is that they are often found in foods already associated with a healthy diet. Whole plant foods, such as fruits and vegetables, are high in polyphenols and consumption of these foods is known to be safe and beneficial. Some specific foods that are high in polyphenols include green tea, cocoa, blueberry and cranberry, coffee, cereals, as well as nuts, seeds and vegetables such as artichoke (Pérez-Jiménez et al., 2010). We should note that, because deficiencies in polyphenol intake do not result in deficiency diseases (except in the case of general malnourishment), it is difficult to define appropriate reference intake values for such food components (Fraga et al., 2019). Though many plant foods contain polyphenols, this chapter will primarily focus on those that exist in tea, cocoa and berries (blueberry and cranberry). Before discussing these specific foods, however, it is important to consider the types of polyphenol structures and differential bacterial metabolism. Considering the differing structures is important as variations will cause differences in physicochemical factors, such as digestibility. In order to assess bioavailability and metabolic influence of polyphenolic compounds, one must first, therefore, explore bio-accessibility.

1.7.1 Bioactivity of dietary polyphenols

Inter-individual variation of the human gut microbiome means that there are many possible metabolic pathways that could be used by microbiota to contribute to bioavailability of dietary polyphenols (Manach et al., 2004). Due to this differential processing amongst humans, it is difficult to characterise specific mechanisms by which polyphenols are considered bioactive, and by which specific microbial species they are transformed (Cardona et al., 2013). Fig. 3 illustrates this.

As previously mentioned, there is diversity within the structure and function of dietary polyphenols. lower molecular weight polyphenols are likely to be immediately absorbed into the small intestine, whereas more complex polyphenols may reach the colon unchanged.

These larger weight polyphenols undergo enzymatic (α-rhamnosidase, β-glucosidase, and β-glucuronidase) transformation by the gut microbial community, breaking these structures into metabolites that can be absorbed, and are likely to be responsible for the health benefits correlated (Fig. 2) with the consumption of polyphenol rich foods (Duda-Chodak et al., 2015).

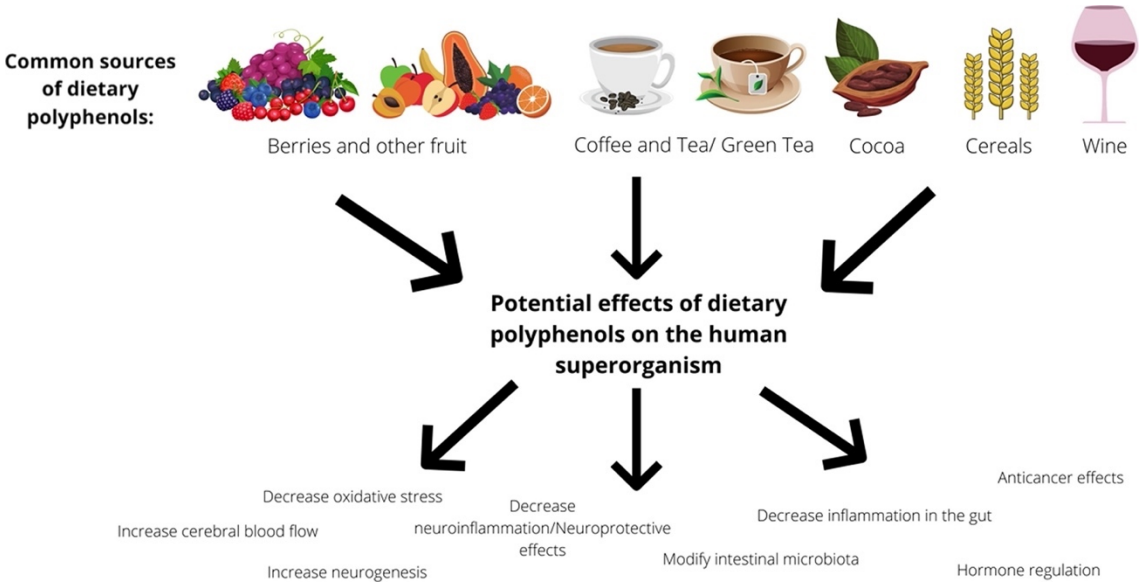

Fig. 2. Potential gut microbial associated effects of common dietary polyphenols on the human superorganism.

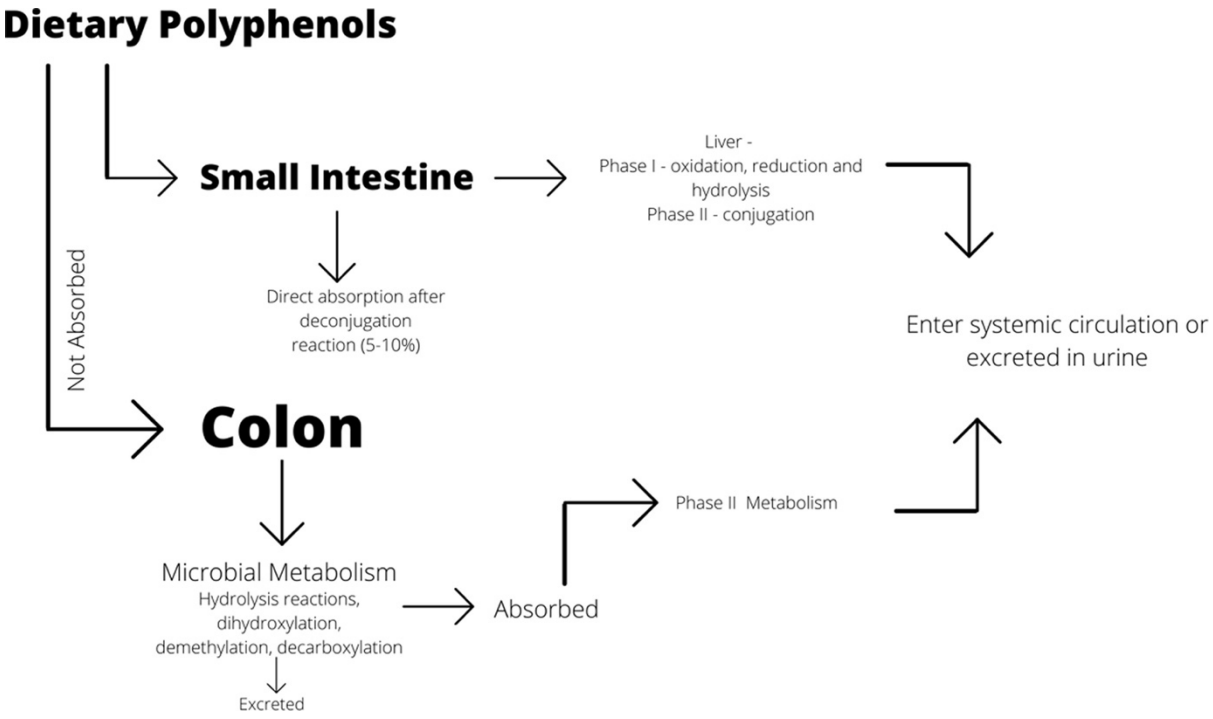

Fig. 3. Schematic illustration of the metabolic fate of dietary polyphenols in the human intestinal system. The routes shown include Phase I and Phase II metabolism, direct absorption into the small intestine, microbial metabolism, and entering into systemic circulation or excretion into urine.

It is important to note that whilst polyphenols are present in food in the free form, they are also found bound to other compounds, like within a dietary fibre matrix (Jackson and Jewell, 2017, Pandey and Rizvi, 2009). This makes them an interesting focus as a combinatorial product with prebiotics, many of which are dietary fibre. Stimulation of the gut microbiota by dietary fibre to produce specific microbial metabolites makes understanding interactions between these compounds crucial (Kardum & Glibetic, 2018). It is plausible that polyphenols may also have a prebiotic effect (Alves-Santos et al., 2020), and that dietary fibres present in the plant compounds might facilitate transport of polyphenols to the colon.

The use of cereals as a food that contains both polyphenols and dietary fibre is one option, wherein bioavailability is extended and thereby the putative health benefits of polyphenol consumption is improved.

However, there is dietary fibre in many other foods, such as berries (Dreher, 2018), so this beneficial effect is not likely limited to just cereals.

There is an established, general, pattern of polyphenol metabolism, whereby natural polyphenols are transformed via a few general processes, such as deglycosylation, dehydroxylation and demethylation. Microbially modified phenolic metabolites will either be absorbed into the body or excreted in urine and faecal matter. Those that are absorbed may undergo Phase II metabolism before being circulated around the body (Mosele et al., 2015). Research has shown that the main genera involved with phenolic degradation are *Clostridium* and *Eubacterium,* spp. which differs from primary genera associated with intake of prebiotics (Selma et al., 2009).

It is also important to consider that the action of polyphenols on bacterial cells can differ – mechanisms will change depending on bacterial wall composition. The action may inhibit or encourage bacterial growth (Puupponen-Pimia et al., 2005). Compounds from green tea extracts have been seen to modulate certain bacteria (Jung et al., 2019), and blueberry extract has shown to increase bifidobacteria in the gut (Vendrame et al., 2011).

After consumption of flavonoids, sugar moieties may be removed and absorbed in the small intestine. Hydrolysis will occur in those flavonoids that are glycosylated, by action of β-glucosidase (amongst others), and these aglycones will then passively diffuse into epithelial cells (D'Archivio, 2010). It is important to note that, where rhamnose moieties exist, these flavonoids can reach the colon and may be hydrolysed by *Bifidobacterium* spp. through α-rhamnosidases (Bang et al., 2015).

Whilst, as mentioned, anthocyanins are one of the flavonoid groups, one of the ways they can be metabolised is by transformation into a non-flavonoid, specifically phenolic acids (Keppler & Humpf, 2005). There are not many free circulating anthocyanins, and this is largely due to metabolism by the gut microflora into phenolic acids (Han et al., 2021).

One of these phenolic acids, protocatechuic acid, is especially potent in its beneficial effects towards host heath. Though some research suggests that the primary precursor for the dihydroxybenzoic acids is the catechin group, protocatechuic acid has been consistently identified as a major metabolite of the anthocyanins (Wang et al., 2010). It has many putative health benefits, including tumoricidal properties, such as the induction of apoptosis in human leukaemia cells. It has also been shown to have significant neuroprotective effects, more specifically, protective against oxidative stress, and nitrosative stress (Winter et al., 2017). The bioactivity of the anthocyanins is thought to be largely

due to these circulating microbial metabolites, which also remain longer in relevant tissues than the anthocyanins themselves (Tsuda et al., 1999). Protocatechuic acid is also more stable against metabolism by microflora than anthocyanins. (Fleschhut et al., 2006, Woodward et al., 2011). Finally, the interaction between gut microflora and anthocyanin consumption is further solidified by in vitro studies suggesting that anthocyanins enhance the growth of *Bifidobacterium* spp. and *Lactobacillus-Enterococcus* spp (Hidalgo et al., 2012).

1.7.2. Molecular mechanisms related to polyphenol metabolites

As discussed, while there is variation in both the polyphenol metabolism and inter-individual microbial metabolism, most, if not all, polyphenols must reach the colon and undergo microbial or enzymatic transformations to ensure bioactivity and produce beneficial effects (Marín et al., 2015, Pandey and Rizvi, 2009).

One study looking at quinine metabolites directly linked the intake of polyphenol and metabolites to the stress response and specific gene regulation. Research showed that, by activating transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an adaptive stress response could be induced (Lee-Hilz et al., 2006). NRF2 has been highly associated with antioxidant effect genes that encode for antioxidant proteins and detoxification enzymes (Eggler et al., 2008)). This association occurs because Nrf2 is regulated by a cysteine-rich protein, and quinones are able to act as acceptors that modify cysteine residues, leading to nrf2 activation and antioxidant response gene production (Eggler et al., 2009).

Many other polyphenols have been shown to potentially activate Nrf2, providing further evidence of the antioxidant and anti-inflammatory properties of polyphenols (Hussain et al., 2016). Because there is a two-way relationship between the gut microbiota and polyphenols (Ozdal et al., 2016), it is important that specific mechanisms of metabolite action are further explored so that people in a disease state can benefit as effectively as possible from intaking of polyphenol rich foods.

Several studies have suggested certain polyphenolic compounds can benefit athletic performance (Myburgh, 2014) but literature has not, to date, provided a comprehensive review on benefits to military personnel.

Research consistently demonstrates the successful use of bioactive plant compounds, such as polyphenols, in reducing oxidative damage by reducing inflammation and influencing the immune response (Hussain et al., 2016). When experiencing extreme physical conditions such as endurance, fatigue or stress, one of the most damaging effects on the body and brain is from increased oxidative damage caused by excess release of ROS (He et al., 2016, Hussain et al., 2016) As previously mentioned, polyphenols protect plants from oxidative damage and it is believed that these phytochemicals will have a similar effect in humans, albeit through different mechanisms (Pandey & Rizvi, 2009). Though artificial antioxidant supplementation may have some benefits, optimal doses of polyphenols have not been identified, making the likely success of supplements difficult to assess (Myburgh, 2014). This is because mechanisms and bioavailability of all polyphenols have not been accurately or completely described, partly due to variable interaction with the gut microbiota. Consequently, supplementation may be slightly less desirable (Cory et al., 2018, Myburgh, 2014) than consumption of whole plant foods. Furthermore, research also suggests that one of the benefits of consuming polyphenols in plant foods derives from their interaction with other nutrients; for instance the presence of other foodstuffs lessens post-prandial glucose spikes because the polyphenols interfere with carbohydrate digestion rates (Williamson, 2013). It would be useful to identify specific bacterial gene expression associated with polyphenol intake and metabolism, but there has not been enough research in human studies to confirm this.

In such a substrate rich environment as the gut, it is difficult to identify specific bacterial expression because the gut microbiome is a complex ecosystem, and it is known that cross-feeding occurs. It may, however, be possible to determine which substrates are metabolised first and which would be especially useful in terms of identifying interactions between carbohydrates and polyphenols. Pure culture studies have shown this in the case of NRF2, as mentioned above. However, this is difficult to extrapolate to the gut microbiota as we cannot separate out individual compounds and the experiments would also need to be repeated in a mixed community of microbes.

1.7.3. Specific foods with high polyphenol concentration

1.7.3.1. Blueberries

Studies have found that blueberries can reduce oxidative stress in athletes, possibly due to their antioxidant effect. Although this particular study was on athletes under heat stress (hyperthermic environments), athletes or military personnel under other stressful conditions such as high altitude may also benefit from a reduction in oxidative stress. This would suggest that blueberry supplements could be a useful addition to their diet. (McAnulty et al., 2004) Research has also demonstrated the effects of blueberries on metabolic diseases and suggests that the polyphenol content is responsible for the prevention of metabolic disease through modulation of the gut microbiota (Curtis et al., 2019). Not only do blueberries seem to have an effect on prevention of metabolic disease, they are also associated with improved cognitive processing. A study showed that, in cognitively impaired adults, performance was altered after blueberry supplementation, with semantic access, memory and processing speed all improving (Krikorian et al., 2020). It is important to note that it is difficult to assess the absorption of blueberry polyphenols in studies such as this because, due to phase II metabolites, there was no difference in urinary excretion of anthocyanins (Krikorian et al., 2020). This study also showed that those older adults who had ongoing blueberry intake before developing dementia maintained better cognition (Krikorian et al., 2020). As dementia is considered to be at least in part due to inflammation (Peila & Launer, 2006), it is likely that blueberries may have a similar impact on other inflammatory cognitive disorders, such as PTSD.

Supplementation of polyphenols in young people has also been shown to be beneficial; participants given an extract of grape and blueberry in one trial showed an improvement in cognition (Philip et al., 2019). This provides justification for further research into the use of polyphenol rich supplements to improve memory and attention, which would be of use to military personnel.

Not only do blueberries appear to play a role in the amelioration of cognitive impairments, but they also seem to improve ‘healthy’ cognition (Whyte et al., 2020). As it is well known that polyphenols rely heavily on the gut microbiota for bioavailability, it is likely that metabolism through the microbiota will be instrumental in this. Blueberry extracts have also been demonstrated to increase *Bifidobacterium* and *Lactobacillus* (Molan et al., 2009).

1.7.3.2. Green tea

Results from several studies into green tea suggest that it would be an advantageous addition to a military diet. For example: green tea extract (GTE) may be beneficial for reducing the impact cumulative fatigue has on athletic performance, through lessened muscle damaged and lower magnitudes of oxidative stress. Military personnel are often subjected to cumulative fatigue (Machado et al., 2018). This study also showed that GTE supplementation confers positive effects on neuromuscular function as a response to cumulative fatigue.

A 2018 study showed that, when green tea polyphenols were introduced into mice with high fat induced obesity, there were significant differences in differentially expressed genes (through KEGG pathway analysis) in ABC transporters and amino acid biosynthesis (Zhang et al., 2018). This suggests that intake of green tea polyphenols did have an effect on metabolic pathways and consequent gene expression. Though this was an artificial/environmentally induced microbial imbalance (through the high fat diet imposed on the mice) it provides a good basis for further human studies in the exploration of green tea polyphenols and metabolic pathways affected, especially when considering that bioactivity of green tea polyphenols is made possible by transformation of compounds in the gut. This study was able to demonstrate that dysbiosis seen in the mouse gut after the high fat diet was mitigated by the intake of green tea polyphenols. Firmicutes were found to be less abundant and Bacteriodetes more abundant in faecal samples post intervention. However, there was much individual variation, probably as a result of gut microbial variability between individuals. This symbiotic relationship between the gut microbiome and polyphenols needs clarification and further study (Zhang et al., 2018).

Other studies have demonstrated that the metabolites of green tea (for example the polyphenols catechins) are likely to be responsible for the beneficial health effects and may be biotransformed then metabolised further by the gut microbiota into phenolic acids (Higdon and Frei, 2003, Ozdal et al., 2016).

Research in dogs has demonstrated proportional changes within bacterial makeup of the gut microbiome, and this study similarly indicated that medicinal effects of the introduction of green tea polyphenols can be directly correlated with microbiota induced changes, for example decreased expression of inflammatory cytokines (Li et al., 2020).

1.7.3.3. Cranberry

Cranberry has been shown to be beneficial in gut-related inflammatory diseases like IBD, where gut dysbiosis occurs(Wang et al., 2018). A study has shown that, by increasing the intake of dietary cranberry or the fruits, severity of IBD symptoms may be reduced. A significant decrease in severity of dextran sodium sulphate (DSS)-induced colitis was observed within a mouse model, decreasing disease activity and increasing colon length after dietary consumption of cranberry. This study also demonstrated reduced levels of pro-inflammatory cytokines and alterations in the faecal microbiota. Whilst there was a decrease of diversity with the diseased group compared to healthy control, cranberry treatment not only reduced this decline in diversity but also reversed the changes (Cai et al., 2017). This reversal of change is particularly interesting when considering soldiers as, not only is prevention or reduction of gut related diseases useful, but also the corrective reversal of changes in gut bacteria (increasing the abundance of beneficial bacteria such as *Lactobacillus and Bifidobacterium* whilst decreasing potentially harmful bacteria), is both useful and encouraging. What is interesting about this particular study is its use of dietary whole cranberry, which has large amounts of indigestible fibre and polyphenols, both of which can reach the colon. While it has not been confirmed that improvements were due to the polyphenols, it is important to consider that dietary polysaccharides found in the cranberry could increase SCFA production and alter bacterial composition in mice (Cai et al., 2017).

Other research into the beneficial effects of cranberry includes a study using cranberry extract in diabetic mice to evaluate whether modulations of the gut microbiota play a role in reducing type 2 diabetes. Again, there was a potential metabolic impact of cranberry interacting with the gut microbiome, whether this be polyphenolic or because of a prebiotic effect. Whilst perhaps not at first glance directly relevant, this study did show that treatment with cranberry extract not only had an anti-diabetic effect but also alleviated intestinal inflammation (Anhê et al., 2015).

The primary ‘active’ polyphenols in cranberries are proanthocyanidins (Blumberg et al., 2013). *In vitro* studies have shown that these polyphenols have an antimicrobial effect, reducing *E. coli* levels in the gut (Harmidy et al., 2011, Roque, 2015) and, *in vivo*, they have been known to help reduce leaky gut, or dysfunction (Blumberg et al., 2013). This is where the use of cranberry extract could be

particularly useful for the health and wellbeing of soldiers; not only is gut barrier dysfunction directly related to dysentery (König et al., 2016, Stewart et al., 2017), but it is also believed that stress exacerbates leaky gut (Vanuytsel et al., 2014, Wallon et al., 2008) which can then lead to inflammation and mental health conditions such as depression and PTSD. (Leclercq et al., 2016).

1.7.3.4. Cocoa

There has been a lot of research on the effects of cocoa polyphenols. Cocoa flavanols are able to cross the blood brain barrier and have been shown to improve cognition (Nehlig, 2013). Studies showed that cocoa flavonols were able to influence cognition in a number of ways although exact mechanisms have not yet been fully understood. Research has shown that, through both direct and indirect actions, cognitive decline was reduced and general cognition, such as working memory, improved (Mastroiacovo et al., 2015, Socci et al., 2017). The flavonoids in cocoa, primarily epicatechin, have also been found to initiate neurogenesis (Valente et al., 2009). In common with other polyphenols, blood flow can be improved and, in the case of cocoa polyphenols, cerebral blood flow may be stimulated (Sorond et al., 2008), which may help reduce neuronal death.

Neurodegenerative diseases are often related to neuroinflammation (Chen et al., 2016). Many studies have shown that polyphenols have anti-inflammatory effects throughout the body, and the same is likely to be true in the brain (García-Lafuente et al., 2009). Low grade inflammation associated with stroke (Zheng et al., 2003) and Alzheimer's is thought to be caused by an inflammatory cascade (McGeer & McGeer, 2003). Flavonols have been shown to reduce the effect of inflammation through cytokine release, amongst other processes (Leyva-López et al., 2016). Modulation of signalling pathways, like the MAPK signalling cascade, can affect neuronal function via inhibitory or stimulatory action that alters the target molecules, thereby altering gene expression (Spencer, 2007). One example of this comes from a study showing that oxidative damage could be prevented through anti-apoptotic action caused by direct action against the activation of caspase-3 (Schroeter et al., 2001).

Research has also been able to identify specific gene expression related to polyphenol intake where (when combined with ERK1/2) the polyphenol epicatechin regulated gene expression through

activation of CREB. This aids memory and neuroplasticity by promoting an expression of genes including those involved in angiogenesis (Schroeter et al., 2006, van Praag et al., 2007).

Although not directly related to polyphenol intake in military personnel under stress, studies have found that the intake of dark chocolate (i.e. cocoa) reduced urinary excretion of the stress hormone cortisol (Martin et al., 2009). However, research also concedes that chocolate feels comforting so some of the mood boosting effects may be psychosomatic (Parker et al., 2006). However, cocoa was seen to normalise the gut microbial activity in stressful situations, modifying the gut microbiome within two weeks (Martin et al., 2009).

Cocoa flavanols have also been seen to directly enhance pathways that increase brain-derived neurotrophic factor which, again, improves neuronal growth and health (Neshatdoust et al., 2016). Bacteria that have been identified as part of the gut microbial metabolism for cocoa polyphenols are *E. coli*, *Bifidobacterium* spp. (also found increased in faecal samples after the consumption of cocoa), *Lactobacillus* spp., *Bacteroides* spp., and *Eubacterium* spp. (Cardona et al., 2013). Research has shown that beneficial bacteria are increased and pathogenic species like *Clostridium* are decreased after the intake of polyphenols (Duda-Chodak et al., 2015).

It is, however, important to mention that cocoa is not the only food group that includes polyphenols that cross the blood brain barrier. In fact, in rats that had supplementation with blueberry polyphenols, specific polyphenols were subsequently found in the brain; anthocyanins were found in the cortex and hippocampus (Andres-Lacueva et al., 2005).

Whilst research has been carried out on individual polyphenols, most research focusses on the entire plant, that is a polyphenol mixture. There is likely much interaction, or crosstalk, between different compounds and phytochemicals within individual plants, and also within foods consumed alongside them.

It may be less useful to discuss research that focusses on individual polyphenols than those studies considering the effects of whole plant foods. The interaction of different foods and the gut microbiota should be studied, but also more research is needed into whether or not other foods consumed with polyphenols affect bioavailability and activity of these chemicals. Furthermore, given that research suggests certain food types improve the health of the gut microbiota, it may be worth considering

eliminating or reducing those foods that cause detriment to it, or rather cause dysbiosis (Brown et al., 2012). Otherwise, any benefits from introducing prebiotics and polyphenols into a diet could be negated.

Controversially, a recent study found that a 15 day consumption of a blend of flavonoids from cocoa, blueberry and green tea exerted none of the expected effects on aspects of the gut microbiome normally associated with good health, such as increased SCFA concentrations, gut inflammation, or diversity of the gut microbiome (Kung et al., 2020). This is interesting as studies that have looked at separate contents of these flavonoids have found them to have a considerable impact. It may be that more dramatic effects are seen when these foods are combined with prebiotic interventions. In this study, liquidised extracted samples were used in the intervention, so it may also be that whole food intake is more beneficial. Furthermore, in this study, only one faecal sample was taken between days 9–11 of the study, and a study in gnotobiotic mice showed that it might take 14 days to see differences in the gut microbiome after an increase of plants in the diet. Finally, it should also be taken into account that we may not see a massive change in the gut bacteria of people who already consume polyphenols, or rather those athletes who are likely to already have a good diet. These factors plus a fairly small sample size may be the reasons for this unexpected result. Notwithstanding, more research is needed to further investigate such findings.

1.8. Pharmacomicrobiomics

Not only is it important to explore nutrition as a treatment and preventative measure for PTSD, but understanding the impact of the gut microbiota on metabolism is also crucial given potential differences in metabolism of pharmacological treatments in individuals. Currently available medications, such as Selective Serotonin Reuptake Inhibitors (SSRIs), are limited in their benefit; they are not specifically designed for PTSD, and will often have less than a 30% patient full remit (Berger et al., 2009). Exploring the optimisation of drugs through pharmacomicrobiomics could be a way to improve patient remit and drug efficacy. It is also important to consider that drugs for PTSD have been shown to be effective prophylactically (Litz, 2008, Roque, 2015). Given that nutritional interventions have had the same effect in disorders such as depression, it is reasonable to posit that a

nutritional intervention could also be successful prophylactically in PTSD (Rechenberg & Humphries, 2013). Research has suggested that treating PTSD earlier or at the subclinical level is beneficial in terms of the reduction of symptoms and developmental trajectory of the disorder (Korte et al., 2016). Pharmacomicrobiomics considers the interplay of inter-individual microbiome variation and the response to drugs (Doestzada et al., 2018). Long term perturbations such as stress can disrupt the gut microbiome environment, causing detriment to the homeostatic environment surrounding the gut-brain axis (Carabotti et al., 2015). This disturbance is likely to worsen hypothalamic–pituitary–adrenal (HPA) axis function and immunity, given the ways in which the gut ecosystem interacts and triggers physiological changes in the brain.

Differences in an individual's drug response can not only cause detriment economically to society if it causes the treatment to fail, but can also seriously affect a patients wellbeing (Sultana et al., 2013). Improving efficacy in the kinetics of drugs is always desirable (Sharma et al., 2019). However, given that personalised medicine is expensive and time consuming, (Vogenberg et al., 2010) finding a nutritional intervention that could increase beneficial bacteria in the gut, reduce detrimental effects of certain disorders and potentially increase the efficacy of certain drugs could be of huge benefit.

1.9. Conclusion

By using the gut microbiota as a therapeutic target to exploit the bidirectional gut-brain axis, it may be possible to address neuropsychiatric conditions, such as PTSD. This is very exciting, as diet is one of the most modifiable factors of the gut microbiota, at all points in life, regardless of health status (Leeming et al., 2019). Effective remedies are urgently needed for the negative consequences of stress, dysentery and PTSD seen within military personnel. There appears to be no detriment to health from increasing authentic prebiotic and polyphenol intake in the diet, especially if it is through consumption of whole foods rather than supplementation of individual polyphenol extracts. The use of prebiotics and polyphenols to reduce symptoms of neuropsychiatric and physical conditions in military personnel looks very promising. That said, more research is needed to identify specific bacterial metabolites and specific bacterial gene expression for combinatorial polyphenol food groups before safety and efficacy can be fully confirmed.

Aims and objectives:

The aims of this thesis are as follows:

1) to explore whether a novel combination of prebiotics and polyphenols would have an effect on both bacterial composition and microbially derived neurotransmitters in vitro,

2) to explore how a novel combination of prebiotics and polyphenols could affect perceived stress levels and mood in healthy adults, as well as assess whether there are any key metabolite changes associated with mood states and

3) to investigate the effects of this novel combination on active military personnel under high altitude stress.

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Chapter 2 - *in vitro* effects of prebiotics and polyphenols on the human gut microbiota.

2.1 Introduction

In order to explore ways by which different nutraceuticals may affect the microbiota-gut-brain axis, in vitro batch culture fermentation may be used. Such in vitro methodologies allow for control and modification of the anaerobic environment in a way that in vivo work cannot. Batch cultures can be used to mimic the environment (temperature and pH) of the colon and pH modulated batch methods are able to replicate environments of the proximal, transverse, and distal colon. This section explores the use of these methods to assess: production of short chain fatty acids (SCFA), potential changes in bacterial composition, as well as production of neuroactive metabolites found in human faecal microbiota. Neuroactive compounds, such as serotonin and gamma-aminobutyric acid, may be microbially derived (Wall et al., 2014) . These compounds are thought to have an impact on cognition/cognitive function via the microbiota-gut-brain axis (Chen et al., 2021).

A growing body of research suggests that intake of certain foods/food supplements may affect microbially derived metabolites produced in the gut microbiota. as previously mentioned within this thesis (Ilyes et al., 2022; Cladis et al., 2021; Peredo-Lovillo et al., 2020). Some microbial metabolites, e.g. SCFAs, have been associated with GI function, immune function regulation, as well as having been associated with modifications to cognitive function (Silva et al., 2020). Recent evidence shows butyrate as a highly health promoting metabolite – including anti-inflammatory properties, as well as improving gut barrier function, it has also been associated with improvements to cognitive function (Amiri et al., 2020; Mcfarlane et al., 2012; Kelly et al., 2015). The primary source of SCFAs in foods is through colonic microbial fermentation of indigestible fibres, like inulin, pectin, xylan and resistant starch (Portincasa et al., 2022). Some research has suggested the use of prebiotic supplementation and high fibre diets in order to modulate the microbiota and attenuate stress responses and improve cognitive function through SCFA action (Portincasa et al., 2022; Davani-Davari et al., 2019; Dalile et al., 2020)

Beneficial microbes, such as *Lactobacillus* and *Bifidobacterium* spp. are associated with the prebiotic concept and concomitant health benefits (Gibson et al., 2017). Studies have demonstrated that these genera may favourably modulate the gastrointestinal composition, as well as enhancing immune function amongst other benefits, including potentially modulating the stress response (Schmidt et al., 2015; Messaoudi et al., 2005; Liu et al.,2022).

Prebiotics have been shown to ameliorate cognitive deficits – Inulin has been shown to increase *Bifidobacterium, Lactobacillus* and *Faecalibacterium prausnitzii*, as well as having favourable effects on mood in humans (Dewulf et al., 2013; Ramirez-Farias et al., 2009; Smith et al., 2015). A prebiotic GOS, has also been shown to increase *Bifidobacterium* and *Lactobacillus* and has been associated with a reduced stress response and anxiety in healthy adults (Monteagudo-Mera et al., 2016; Schmidt et al., 2015; Johnstone et al.,2021). Resistant starch may provide beneficial effects though the promotion of butyrate production, but also has been associated with increases in bifidobacteria and *F. prausnitzii* (DeMartino et al., 2020; Teichmann et al., 2020; Jung et al., 2023).

Polyphenols have low oral bioavailability (on average of 10% or less) and depend on the microbiota to produce bioavailable compounds (Di Lorenzo et al., 2019; Ming et al., 2007; Rajha et al., 2021). Whilst polyphenols may have a putative prebiotic function, (Rodríguez-Daza et al., 2022) their other beneficial properties include being anti-inflammatory, anti-tumourigenic and anti-microbial. They have also been associated with specific bacterial changes, as well as changes in cognitive function (Obrenovich et al., 2022, Wang et al., 2022. Cocoa has been associated with increased *Lactobacillus, Bifidobacterium* (Sorrenti et al., 2020), as well as improving working memory and reducing cognitive fatigue (Martin et al., 2021). Green tea has also been associated with increases in *Bifidobacterium* and has been shown in some studies to improve working memory (Jin et al., 2012; Schmidt et al., 2014). Blueberry intake again has associations with increased *Bifidobacterium* spp. and *Lactobacillus* spp., (Vendrame et al., 2011) as well as improved mood and emotion, executive function, and memory (Travica et al,. 2020; Wood et al.,2023). Cranberry, whilst less studied, has shown to increase certain beneficial genera of bacteria, as well as reducing intestinal inflammation (Ozcan et al., 2017; Anhe et al., 2015.

Whilst, as mentioned above, much research has looked into individual prebiotic and polyphenols on the gut microbiota and mood, combinations of prebiotics and polyphenols have not been investigated. The use of in vitro fermentation methods provides an opportunity by which to explore a novel combination within a controlled environment and compare to individual fractions.

The primary aims of this work were to assess changes in bacterial composition, SCFA and neurotransmitter content, under the influence of polyphenols and prebiotics.

Two forms of batch cultures were used, firstly, standard batch cultures. These were used to elucidate the effects of individual components, as well as a combination of all of these components. After confirming the potential benefit of a combination as opposed to individual prebiotics or polyphenols, a pH modulated model was employed to test a further breakdown of the combination – a combination of just prebioitic substrates, just polyphenol substrates, and then the final combination in order to potentially replicate the effects of the initial experiment. The pH modulated batch was used to better replicate the pHs of the gastrointestinal tract, and the transit times seen within these. It is a step towards more of a continuous model function.

2.2 Methods

2.2.1 Batch Culture

Simulated in vitro upper gut digestion

Simulated in vitro human digestion of food (from mouth to small intestine) was performed according to the protocol by Mills et al. (2008) with some adjustments. Different amounts of polyphenol sample were pre-digested dependent on the polyphenol concentration– i.e. in order to get 500mg blueberry 6.3g was required compared to 0.25g for green tea , 0.44g cranberry and 0.6g cocoa. These amounts were all corrected to achieve the final concentration of 500mg polyphenols per substrate. These data were provided by the substrate provider.

Samples were weighed and then added to distilled water and stomached for five minutes. To initiate the oral phase of digestion, the solution was mixed with amylase in $CaCl_2$(0.001 mol l-1, pH 7.0; 6.25 ml), incubated at 37°C on a shaker and then pH adjusted to 2.0 using 6M HCl.

To facilitate gastric breakdown of the sample, a pepsin solution (2.7 g in HCl (0.1 mol l-1; 25 ml)

was added and this was again incubated at 37ºC for 2h, followed by a small intestinal simulation, adding pancreatin (0.560g) and bile (3.5g) in $NaHCO_3$ (0.5 mol l-1; 125ml), and adjusting the pH to 7.0. This sample was then added to 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing and dialysed for 17 hours, with a change of dialysis fluid at 15h. These samples were then freeze dried (6 days) to use in the batch cultures. The samples were weighed and the loss of each in the upper gut was calculated, and a proportional amount was used for the batch cultures.

Faecal sample preparation

Faecal samples were obtained from three healthy volunteers who had not consumed antibiotics for at least 6 months before this, and who had not consumed probiotics or prebiotic supplements regularly at least within the last month. They had no history of gastrointestinal disease. Samples were prepared on the day of the experiment, in a 10% weight/volume phosphate buffer (0.1 M 0.1 mol l/1 anaerobically prepared PBS (pH 7.4), pH 7.4) solution. Samples were stomached for two minutes, (460 paddle beats/min) and the faecal slurry produced was inoculated into batch culture fermenters.

Batch culture fermentation

Three separate batch culture fermentations were ran, with three separate donors. The prepared vessels were autoclaved and then aseptically filled with a basal culture medium containing (L) 2g Peptone water, 2g yeast extract, 0.1g NaCl, 0.04g K_2HPO_4, 0.04g KH_2PO_4, 0.01g $MgSO_4.7H_2O$, 0.01g $CaCl_2.6H_20$, 2g $NaHCO_3$, 2ml Tween 80, 0.05g Hemin (dissolved in a few drops of 1M NaOH), 10µl Vitamin K, 0.5g L-Cysteine HCL, 0.5g Bile salts and 4ml resazurin solution in 1l deionised water. This basal medium was autoclaved after preparation. 135ml of sterile medium was placed into 300ml vessels and an anaerobic environment (oxygen free nitrogen) maintained through the introduction of nitrogen gas, left overnight. Before adding the faecal slurry, a circulating water bath was used to set the temperature of the basal medium at 37 °C, and a pH of between 6.7 and 6.9 was maintained using a pH meter (Electrolab pH controller, Tewksbury, UK) by the addition of 0.5 M HCl or 0.5 M NaOH. The medium was stirred using magnetic stirrers beneath the vessels. Nine vessels were used for the batch cultures, a positive prebiotic control (Orafti Synergy 1)(1.5g), a negative control (no substrate),

Hi-Maize resistant starch from Ingredion(6g), GOS from Bimuno (1.5g), and the pre-digested blueberry (1.89g), cranberry(0.132g), green tea (0.075g) and cocoa (0.18g), and one vessel containing a combination of all of the substrates listed above in the same quantities..

Substrate was added, and all vessels inoculated with 15ml of a 10% (w/v) faecal slurry from healthy adult volunteers (made with 0.1 mol l/1 anaerobically prepared PBS (pH 7.4)). 6ml of sample was taken from each vessel at times 0, 8, 24 and 48h. 48 hours samples were taken to align with research previously described, as well as to ensure reaching of a steady state. Sufficient sample was taken to enumerate bacteria by fluorescent in situ hybridisation (FISH), and analysed by Gas chromatography (GC) and Liquid chromatography–mass spectrometry (LC–MS). One set of samples was processed in order to carry out fluorescent in situ hybridisation flow cytometry (FLOW-fish), and one for gas chromatography, with others aliquoted into Eppendorf's then frozen at -20^{o}Cfor future use.

Preparation for FISH analysis

For each time point (baseline 0 hrs, 8hrs, 24hrs and 48hrs), 750 µl from each of the batch culture vessels was transferred to sterile 1.5ml microcentrifuge tubes, and this was then centrifuged at 1300xg or 5 minutes at room temperature at 13000xg. The supernatant was discarded, 375 µl of filtered sterile 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) added, and the pellet dispersed through aspiration. 1125 µl of Paraformaldehyde was then added and this was vortexed, then stored at 4 °C for 4-8 hours. After this period of time for fixation, samples were centrifuged at 13000g or 5 minutes at room temperature, and the supernatant discarded. The pellets were resuspended in 1ml cold 1x filtered 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) through aspirating. This was repeated twice. The washed cells were then suspended in 150 µl cold filtered 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) and then 150 µl ethanol was added. After vortexing this mixture, it was then stored at -20 °C. This process was completed twice per vessel per time point.

Preparation for GC/LCMS

1.5ml from each of the batch culture vessels was transferred to sterile 1.5ml microcentrifuge tubes and centrifuged at 13000xg for 5minutes at room temperature. The supernatant was transferred to a

new sterile microcentrifuge tube and then frozen at -20 °C. This process was completed in triplicate (three per vessel per time point.)

Preparation for Liquid Chromatography Gas Spectroscopy

Samples were defrosted (from storage at -20) and centrifuged for 5 minutes at 2000xg.
10 µL of supernatant was added to 0.99 mL of HPLC water to form a 1:100 dilution, which was then filtered using 0.22 µm syringe filters. 1 mL was added to a screwcap HPLC vial for analysis. Batch culture medium was also used as a control for comparison and prepared as above.
Individual stock solutions were prepared using analytical standards powders of dopamine hydrochloride (99%, Alfa Aesa), serotonin (Sigma-Aldrich), tryptophan (98%, Sigma-Aldrich), GABA (99%, Sigma-Aldrich), L(-)-epinephrine (99%, Acros Organics), L-noradrenaline (98%, Alfa Aesa) and kynurenic acid (98%, Sigma-Aldrich), each at 10000 ng/mL. A mixed standard solution was then prepared from the individual stock solutions and used to create a 7 level calibration series with the following dilutions: 10, 5, 1, 0.5, 0.25, 0.125, 0.0625 ng/mL.

FISH analysis

Labelled oligonucleotide probes were used to hybridise genus specific targets with fluorescent markers. Samples were screened using a BD Accuri™ C6 flow cytometer, measuring at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software.
Samples were prepared for flow-FISH analysis by thawing after -20 storage, and vortexing for 10s. 75 µL of the sample was added to 500 µL 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) in an Eppendorf tube (1.5 mL), vortexed and centrifuged at 13000 × g for 3 min. The supernatant was removed, and 100 µL Tris-EDTA buffer containing lysozyme added to the tube, mixed using a pipette and incubated in the dark for 10 minutes at room temperature. Samples were then vortexed and centrifuged for 3min at 13000xg. The supernatant was once again discarded, and the pellet washed with 500 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4). This was then vortexed gently and centrifuged for 3 minutes at 13000xg. The supernatant was discarded a final time and the pellet resuspended in 150 µl of hybridisation buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium

dodecyl sulphate, 30% formamide). Samples were vortexed and centrifuged at 13000xg for 3 minutes, and the supernatant discarded. The pellet was resuspended in 1ml hybridisation buffer.

Four µL (50 ng/µl^{-1}) of the selected oligonucleotide probe solutions (Table 1) was added to 50 µL of sample in Eppendorf tubes, vortexed and incubated at 36◦C overnight. After the incubation period, 125 µl of hybridisation buffer was added to each tube and they were subsequently vortexed, then centrifuged for 3 minutes at 13000 xg. Supernatants were removed and the each pellet resuspended in 175 µl washing buffer solution (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate). This was then incubated for 20 minutes, covered, at 38°C in a heating block. Following this, samples were centrifuged for 3 minutes at 13000 xg, and the supernatant discarded. 300 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) was added to each sample, and this was vortexed. Samples were held at 4 ◦C in the dark before flow cytometry was used. Bacteriology measurements were taken by a by a BD Accuri™ C6 flow cytometer, BD, Erembodegem, Brussels, and analysed used Accuri CFlow Sampler software.

Probes used were: Bif164 for *Bifidobacterium* spp, Lab158 for *Lactobacillus/Enterococcus*, Bac303 for *Bacteroides–Prevotella* group, Erec482 for *Eubacterium rectale–Clostridium coccoides* group, Rrec584 for *Roseburia–E. rectale* group, Ato291 for *Atopobium* cluster, Prop853 for clostridial cluster IX, Fprau 645 for *Faecalibacterium prausnitzii* spp, Dsv687 for *Desulfovibrio* genus and Chis 150 for most of the *Clostridium histolyticum* group (Clostridium cluster I and II). Total bacteria were enumerated by use of the Eub 338 probe mix (Eub338‡, Eub338II‡, Eub338III‡), and Non-Eub was used as a negative control. Table 1 shows individual probe sequences.

Probe name	Sequence (5' to 3')	Target groups
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338
Eub338‡	GCTGCCTCCCGTAGGAGT	Most Bacteria
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX
Fprau655	CGCCTACCTCTGCACTAC	*Feacalibacterium prausnitzii* and relatives
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)

Table 1: Oligonucleotide probe sequences.

Statistical analysis of FISH samples.

Statistical analyses was performed using SPSS software.

Analysis of variance analysis (ANOVA) was used to assess significance levels or any differences in bacterial counts between different time points and/or substrates. Differences were deemed significant at $p < 0.05$.

Gas Chromatography (GC) for Short Chain and Branched Chain Fatty Acid Analysis

GC/MS was performed as described by Richardson et al.,1989 using GC Agilent 7890B fitted with a flame ionisation detector and a HP-5ms column (30 × 0.25 mm, 0.25 μm film thickness (Agilent, Cheshire, UK)).). Elution times were recorded, and the data further analysed.

Preparation of samples

Individual standard solutions were prepared for acetate, iso-butyrate, butyrate, propionate, valerate, iso-valerate and lactate. These were prepared at 100mm and then diluted in serial dilutions to 50mM, 25mM, 12.5mM and 6.25mM.

The same compounds were then combined to form the external standard solution. (The external standard solution contained acetate (30 mM), iso-butyrate (5 mM), n-butyrate (20 mM), propionate (20 mM), n-valerate (5 mM), iso-valerate (5 mM) and lactate (10 mM))

The internal standard used was 2-ethylbutyric acid (100 mM) as described by Richardson et al. (1989).

The method, in brief, required that collected samples from fermentations be defrosted on ice. Each sample was vortexed and 1ml transferred into a prelabelled glass tube. This was combined with 50μL 2-ethylbutyric acid (100 mM; internal standard). 0.5 ml concentrated HCl and 3 ml diethyl ether were added, and these tubes were then vortexed in amulti-vortex for 1 minute at 1500 xg n. Samples were then centrifuged at 2000 xg for 10 minutes (SANYO MSE Mistral 3000i; Sanyo Gallenkap PLC, Middlesex, UK). The organic upper layer of each sample was then transferred to a clean separate tube, and, if needed, a further 1ml diethyl ether added. This was then vortexed and centrifuged in the same manner as the previous extraction. 400 μL of pooled diethyl ether layers were added to a screw

cap GC vial, with 50 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). This was then stored at room temperature for at least 48 hours (72 hours for lactic acid) prior to GC analysis.

Analysis of samples

Analysis was done using an HP GC 5690 ((Hewlett Packard, UK) fitted with a flame ionisation detector and a HP-5ms column (30 × 0.25 mm, 0.25 µm film thickness coating of crosslinked (5%-phenyl)- methylpolysiloxane (Hewlett Packard, UK).

Injector and detector temperatures were 275◦ C and the column temperature programmed from 63◦ C to 190◦ C by 5◦ C and held at 190◦ C for 30 min. Helium was used as the carrier gas at a flow rate of 1.7 mL/min (head pressure, 133 KPa).)). 1 µL of each sample was injected with a run time of 17.7 min.

The SCFA standard was run after each donor sample set to update the calibration as necessary. Response factor and peak area within samples, were calibrated and calculated using using Agilent Chemstation software (Agilent Technologies, Basingstoke, UK), and quantification of each SCFA (mM) was calculated using the following method:

Response factors were calculated using the following equation (Liu 2016):

$$Internal\ Response\ Factor = \frac{areaIS \times amountSC}{amountIS \times areaSC}$$

IS = Internal Standard; SC = Specific Compound of Interest

This equation was then used to calculate the amount of organic acids in the samples using the following secondary equation:

$$Amount\ of\ Specific\ Compound = \frac{amountIS \times areaSC \times IRFSC}{areaIS}$$

IS = Internal Standard; SC = Specific Compound of Interest; IRFSC = Internal Response Factor for Specific Compound of Interest

LC/MS analysis

Samples were analysed by liquid chromatography−mass spectrometry/mass spectrometry (LC–MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode. A gradient separation was carried using a 150 × 2.1 mm Discovery HS F5 – 3 column, with a 2 × 2.1 mm Discovery C18 Supelguard precolumn (both 3 µm particle size; Supelco, Dorset, UK). The column was maintained at 40 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The column flow rate was maintained at 0.4 mL/min. The timetable was as follows: 0–2 min, 100% A; 5 min, 75% A; 11 min, 65% A; 15–20 min 5% A; 20.1–30 min, 100% A. The injection volume was 10 µL. Eluent from the column was ran to waste from 0 to 1 min, and data collected from 1 to 18 min. Data were acquired in dynamic MRM mode. Transitions studied and voltages used are shown in (Table 2). Two transitions were acquired for each compound.

This method was based off previously published work adapted for our equipment and the dilution of samples were manipulated to ensure they fell within those bounds.

Compound name	Retention time (min)	Retention time window (min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision energy (V)	Cell (V)	Acc
GABA	1.90	3	104	87	50	4	7	
		3	104	45	50	20	7	

Norepinephrine[b]	2.50	3	152	107	116	16	7
		3	152	77	116	30	7
Epinephrine[b]	4.60	3	184	166	70	8	7
		3	184	107	70	24	7
Dopamine[b]	7.00	3	154	137	75	8	7
		3	154	91	75	28	7
Serotonin	9.70	5	177	160	45	4	7
		5	177	115	45	30	7
Kynurenic acid	9.77	5	190	144	100	16	7
		5	190	172	100	4	7
Tryptophan	10.20	5	205	188	78	4	7
		5	205	146	78	20	7

a

[b] Urinary Catecholamines, Metanephrines, and 3-Methoxytyramine in a Single LC/MS/MS Run Using Agilent Bond Elut Plexa SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS.

Statistical analysis.

Statistical analysis was performed using SPSS Statistics software.

Analysis of variance analysis (ANOVA) was used to assess significance levels or any differences in organic acid levels between different time points and/or substrates. Differences were deemed significant at $p < 0.05$.

pH modified batch Methods

Faecal sample preparation

Three faecal samples were obtained from three healthy volunteers who had not consumed antibiotics for at least 6 months before this, and who had not consumed probiotics or prebiotic supplements regularly at least within the last month. They had no history of gastrointestinal disease. Samples were prepared on the day of the experiment, in a 10% weight/volume phosphate buffer (0.1 M 0.1 mol l/1 anaerobically prepared PBS (pH 7.4), pH 7.4) solution. Samples were stomached for two minutes, (460 paddle beats/min) and the faecal slurry produced was inoculated into batch culture fermenters.

2.2.2 pH modified batch culture fermentation

Three separate batch culture fermentations were ran, with three separate donors. The prepared vessels were autoclaved and then aseptically filled with a basal culture medium containing (L) 2g Peptone water, 2g yeast extract, 0.1g NaCl, 0.04g K_2HPO_4, 0.04g KH_2PO_4, 0.01g $MgSO_4.7H_2O$, 0.01g $CaCl_2.6H_20$, 2g $NaHCO_3$, 2ml Tween 80, 0.05g Hemin (dissolved in a few drops of 1M NaOH), 10µl Vitamin K, 0.5g L-Cysteine HCL, 0.5g Bile salts and 4ml resazurin solution in 1l deionised water. This basal medium was autoclaved after preparation. 135ml of sterile medium was placed into 300ml vessels and an anaerobic environment maintained through the introduction of nitrogen gas, left overnight. Before adding the faecal slurry, a circulating water bath was used to set the temperature of the basal medium at 37 °C. An initial pH of between 5.4 and 5.6 (representing the proximal colon) was using a pH meter (Electrolab pH controller, Tewksbury, UK) by the addition of 0.5 M HCl or 0.5 M NaOH. This pH modified method differs in so far that the user manually adjusts the to mimic the pH of the intestinal tract. An initial pH of between 5.4 and 5.6 is used in order to better represent the proximal colon, followed by an adjustment at 14 hours a pH of between 6.2 and 6.4, representing the transverse colon, and at the 30-hour point, the distal colon is mimicked with a pH range of 6.7-6.9. The unmodified batch culture method is much more widely used, so has its benefits in being a trusted method, however being able to better mimic the environment of digestion and the intestinal tract can bring benefits. These time points are selected to better replicate transit times within the colon. The medium was stirred using magnetic stirrers beneath the vessels.

Four vessels were used for the batch cultures, a negative control (no substrate, a positive control containing a combination of prebiotics ((Orafti Synergy 1)(1.5g), resistant starch, (6g), GOS (1.5g),),

a vessel with polyphenols (blueberry (1.89g), cranberry(0.132g), green tea (0.075g) and cocoa (0.18g), and then a vessel containing a combination of all of the substrates listed above in the same quantities.

This was to further explore results from the previous batch cultures where individual ingredients were tested.

Substrate was added, and all vessels were inoculated with 15ml of a 10% (w/v) faecal slurry from healthy adult volunteers (made with 0.1 mol l/1 anaerobically prepared PBS (pH 7.4)). 6ml of sample was taken from each vessel at times 0, 14, 30 and 48. At the 14 hour time point, pH was adjusted to between 6.2 and 6.4, representing the transverse colon, and at the 30 hour point, the distal colon was mimicked with a pH range of 6.7-6.9.

Sufficient sample was taken to enumerate bacteria by fluorescent in situ hybridisation (FISH) and analysed by Gas chromatography (GC) and liquid chromatography–mass spectrometry (LC–MS), as well as for 16S rRNA partial gene sequencing. One set of samples was processed in order to carry out fluorescent in situ hybridisation flow cytometry, and one for gas chromatography, with others aliquoted into Eppendorf's then frozen at -20^{o}C for future use.

Preparation for 16S rRNA partial gene sequencing analysis

DNA isolation, library preparation and 16S rRNA gene sequencing

Both FLOW-fish and 16S rRNA partial gene sequencing were undertake to mitigate some of the limitations of each and ensure that a more comprehensive image of the data was achieved. FISH is able to provide high specificity, ensuring the identification of specific microbes, however it is limited by the number of species that can be simultaneously detected. Whilst the 16s rRNA sequencing can analyse a broader spectrum of diversity, it lacks the specificity and absolute quantification that FISH provides, rather giving an example more of diversity insights.

Extraction, lysis and DNA isolation was done according to manufacturer's recommendation (Fast DNA Stool Mini Kit Qiagen). Bead beating was run on a fastprep24 instrument (MPBiomedicals; 4 cycles of 45s at speed 4) in 2ml screwcap tubes containing 0.6g 0.1mm glass beads. 200µl of raw extract was prepared for DNA-isolation (DNeasy 96 Blood & Tissue Kit Qiagen). Concentration of

the isolated DNA was assessed with PicoGreen measurement (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Thermo Fisher) and integrity was checked for a random sample by agarose gel electrophoresis or capillary fragment analysis.

Subsequent PCR libraries were sequenced on an Illumina MiSeq platform using a v2 500 (2*250 bp read length). Pools were diluted to 9.2 pM and loaded together with 15% PhiX (Illumina, FC-110-3001) to increase the diversity of the run resulting in a raw cluster density of 631 and a cluster passed filter rate of 98%. Paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina's bcl2fastq software version v2.20.0.422. Quality of the reads was checked with the software FastQC version 0.11.8 and sequencing reads that fell below an average Q-score of 20 or had any uncalled bases (N) were removed from further analysis. The locus specific V4 primers were trimmed from the sequencing reads with the software cutadapt v3.2. Paired-end reads were discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to reform in silico the sequenced molecule considering a minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged sequences were again quality filtered allowing a maximum of one expected erroneous base per merged read. Reads that contain ambiguous bases or were outliers regarding the amplicon size distribution were also discarded. Samples that resulted in less than 5000 merged reads were discarded, to avoid distortion of the statistical analysis. Remaining reads were denoised using the UNOISE algorithm implemented in USEARCH to form Amplicon Sequencing Variants (ASVs) discarding singletons and chimeras in the process. The resulting ASV abundance table was then filtered for possible barcode bleed-in contaminations using the UNCROSS algorithm. ASV sequences were compared to the reference sequences of the RDP 16S database provided by https://www.drive5.com/usearch/manual/sintax_downloads.html and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. The resulting library was then corrected by including numbers of 16S copies and rarefying to an even sampling intensity to reduce bias in diversity metric calculations and quantified as described by Vandeputte et al., 2017. This was dobne by the sequencing provider, Microsynth.

2.3 Results

Enumeration of bacteria with flow-FISH – Batch Culture

Figures 1.1 and 1.2 illustrate the differences in bacterial groups at baseline, 8, 24 and 48 hours for batch culture and pH modulated batch culture, respectively. No significant difference in bacterial numbers was found between vessels at baseline.

For EUB I,II,III, a significant effect of substrate was found at time 48 between the negative control and combination vessel (P=0.034); inulin and blueberry (P=0.023), inulin and cocoa (0.030); and the combination vessel to blueberry(0.004), cranberry(0.028), green tea (0.013)and cocoa (0.006). A trend towards significance was seen between inulin and green tea (P=0.067), resistant starch and blueberry (P=0.053) and resistant starch and cocoa (0.068). Cranberry had a within substrate interaction that trended towards significance, with the bacterial numbers increasing between time 8 and time 48 (P=0.054). We saw a similar trend within the cocoa vessel – a trend (p=0.053) increasing from time 0 to time 8. When visually inspecting data for the combination vessel, it seems that for total bacteria, in contrast to other substrates, we saw a continual increase of bacterial counts.

For Bif164, bifidobacteria, we only see a significant decrease between time 24 and 48 in the cranberry vessel (P=0.035). A visual inspection, however, showed that the decrease of bifidobacteria was less apparent in the combination vessel when compared to inulin, and stayed higher for longer.

For EREC, at time 24 significant differences were seen between the cranberry vessel and inulin (0.032), GOS (0.049), resistant starch(0.030) , blueberry (0.043), green tea (0.049), and a trend was seen between cranberry against cocoa (0.083) and the combination vessel (0.054).

Significant differences were seen for ATO at time 24 between the cranberry vessel and the negative control (0.047) inulin (0.023), GOS (0.016), resistant starch(0.006) , blueberry (0.011), green tea

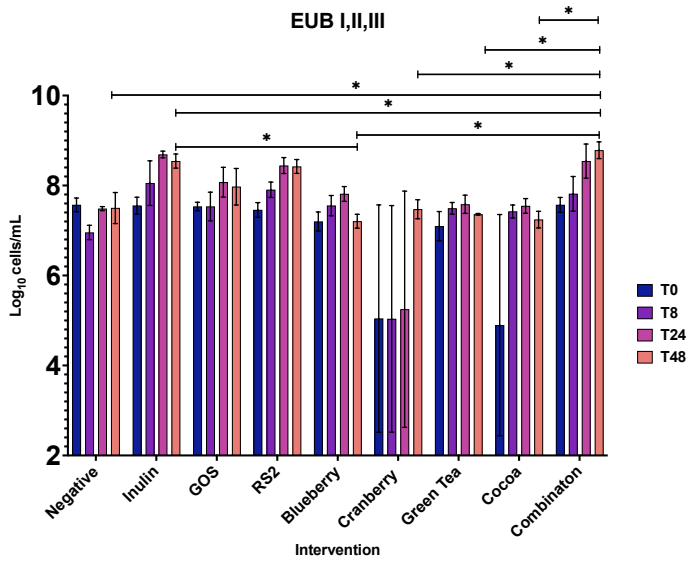
(0.047), and the combination vessel (0.022). a trend was seen between cranberry and cocoa(P=0.054) at the same time point.

Fprau also had significant differences between cranberry and other substrates at tiem 24. GOS (0.038, resistant starch (0.019), blueberry (0.022) and the combination vessel (0.044). Inulin had a trend of 0.077, as did green tea at p=0.091.

A significant difference was seen for CHIS within the resistant starch vessel, with an increase between time 0 and time 24. *Closer look at this data shows that this increase was skewed heavily by one donor.*

It is important to note that the substrate cranberry did not react particularly well within the flow-fish system so this may be the cause of the differences.

No significant differences were found for probes BAC, PROP, FPRAU or DSV so these are not represented in the figure below.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8
6
4
2
Negative
Inulin
GOS
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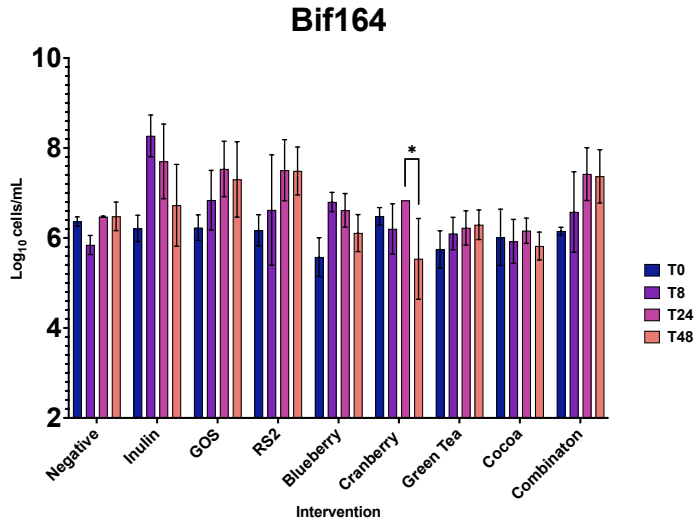
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention
T0
T8
T24
T48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4
2
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RS2
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Green Tea
Cocoa
Combinaton
Intervention
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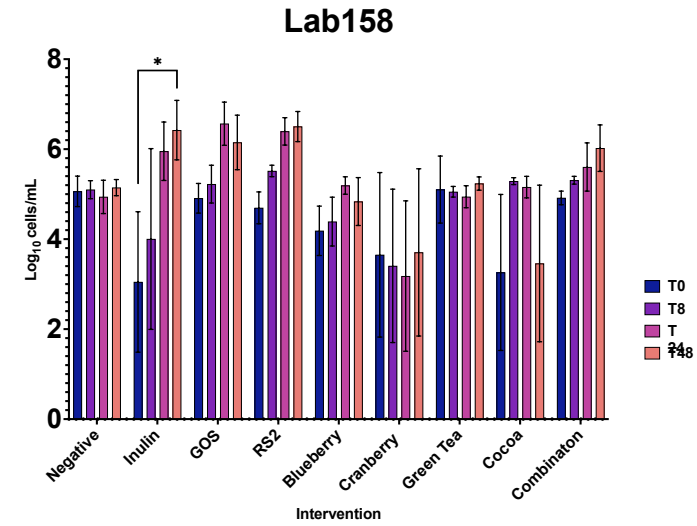
T24
T48


Lab158
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6
4
2
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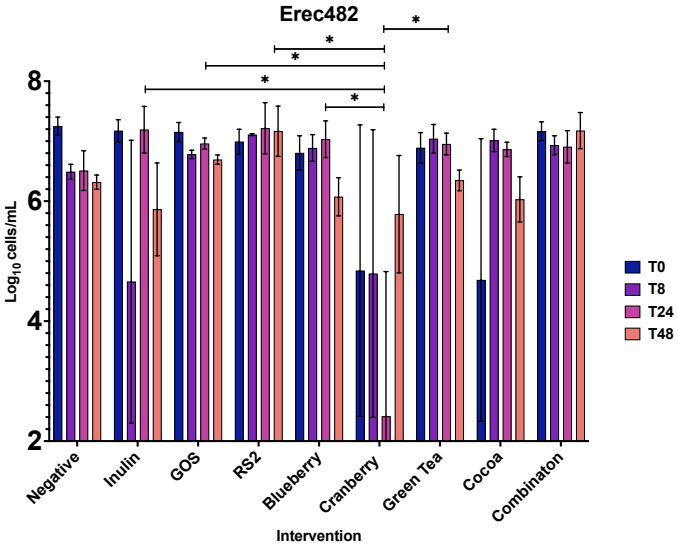
Combinaton
Intervention
T0
T8
T24
T48


Erec482
Log10 cells/mL
8
6
4
2
*
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*
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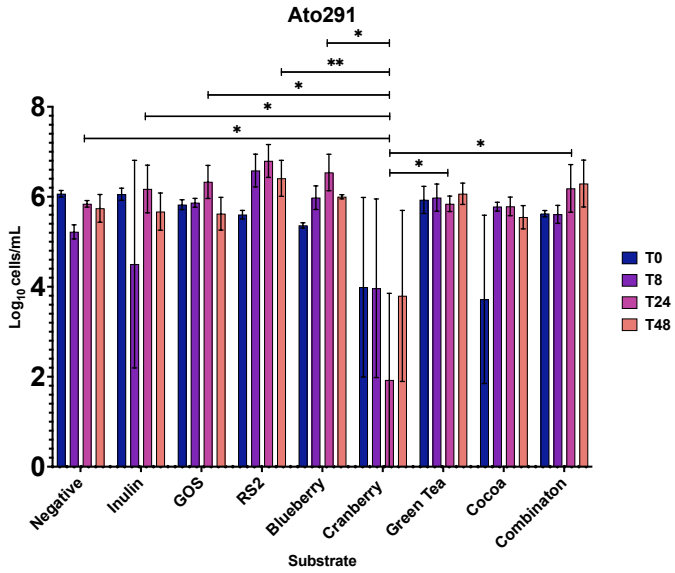
Cocoa
Combinaton
Intervention


Ato291
Log10 cells/mL
8
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4
2
0
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**
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*
*
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Cranberry
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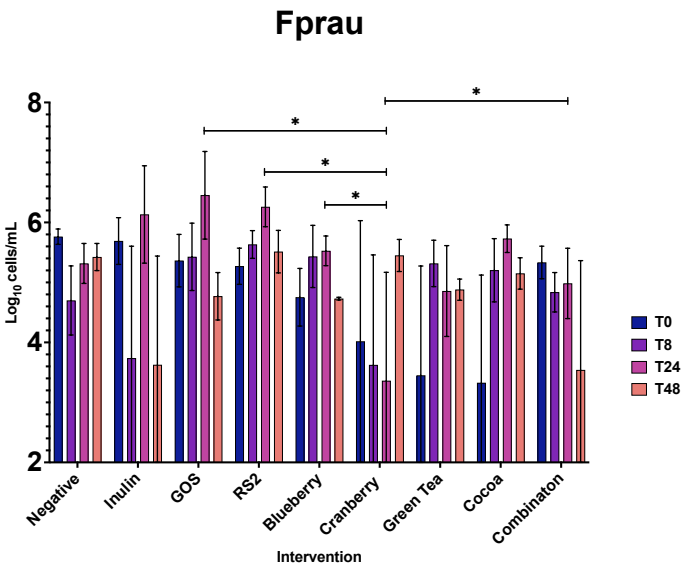
Cocoa
Combinaton
Substrate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Log10 cells/mL
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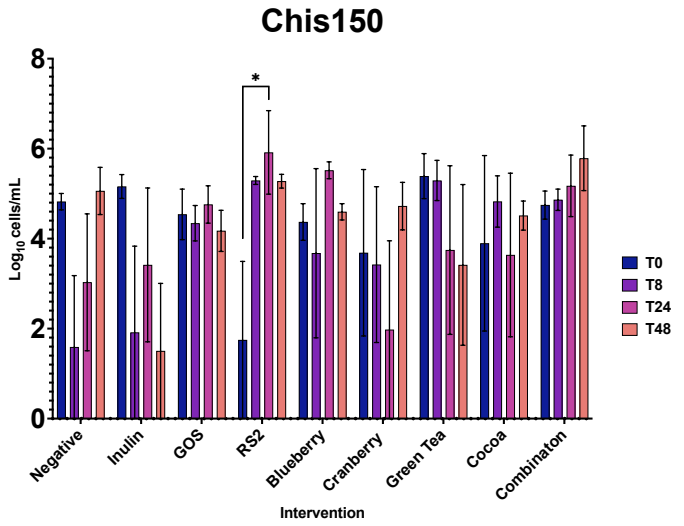
Combinaton
Intervention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Log10 cells/mL
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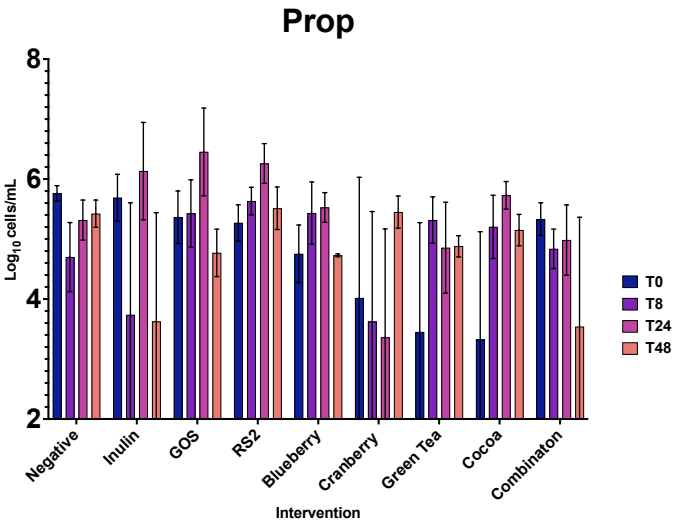
Combinaton
Intervention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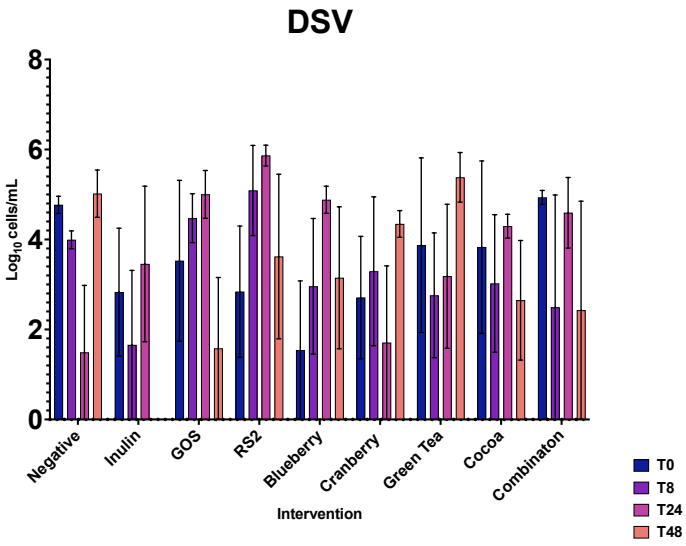
T48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Log10 cells/mL
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Cranberry
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Combinaton
Intervention
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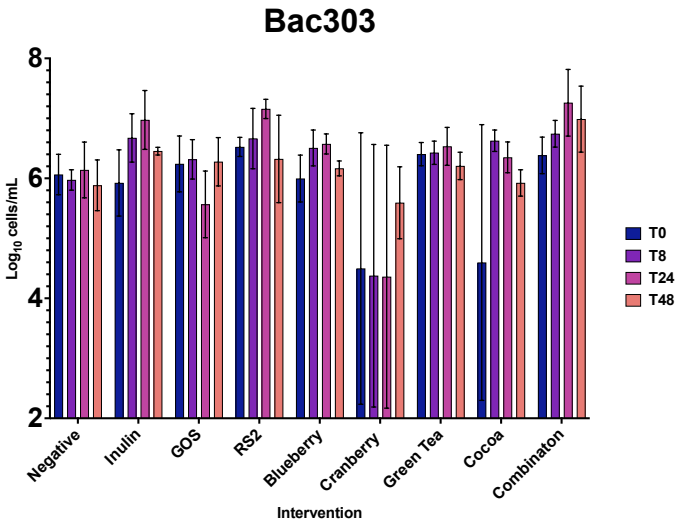
T8
T24
T48


Bac303
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6
4
2
Log10 cells/mL
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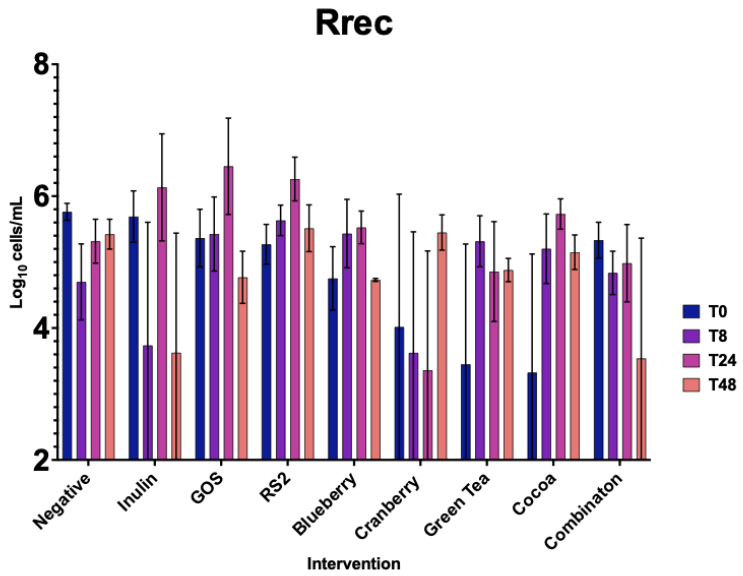
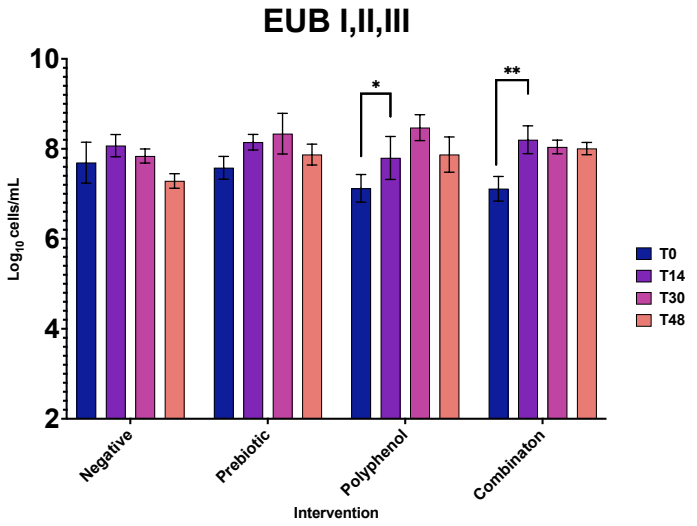


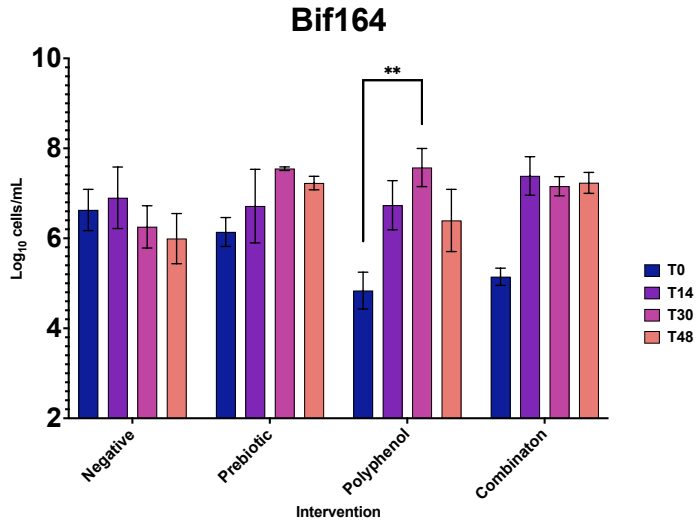
Fig. 1.1 Enumeration of bacteria by Flow-FISH at baseline (0) and following 8, 24 and 48 hours of fermentation. Bacterial groups shown were: A – total bacteria (EUB), *Bifidobacterium spp.*(BIF), *Lactobacillus* spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), *Clostridium coccoides–Eubacterium rectale* group (EREC), *Roseburia* subcluster (RREC), *Faecalibacterium prausnitzii* (FPRAU), *Atopobium- Coriobacterium* spp. (ATO), *Clostridium* cluster IX (PROP), *Desulfovibrio* (DSV) and *Clostridium histolyticum* (CHIS). Values are presented as mean ± standard error. * denotes significant difference, where $p < 0.05$. n=3.

Enumeration of bacteria with flow-FISH – pH modified batch

For total bacterial counts, a significant difference was seen between time 0 and time 8 in the polyphenol combination (0.046), and the polyphenol/prebiotic combination (0.003).

Bifidobacteria showed significant changes only in the polyphenol combination, between times 1 and 24. Ato showed significance within the polyphenol combination between time 0, 8, 24 and 48 with P values of 0.013, 0.008 and 0.033 respectively. In the overall combination vessel, significance was seen with an increase of ATO between times 0 and time 8 (P=0.021). Though trends did appear in the other vessels, no statistical significance was apparent.


EUB I,II,III
Log10 cells/mL
10
8
6
4
2
*
**
T0
T14
T30
T48
Negative
Prebiotic
Polyphenol
Combinaton
Intervention


Bif164
Log10 cells/mL
10
8
6
4
2
**
T0
T14
T30
T48
Negative
Prebiotic
Polyphenol
Combinaton
Intervention

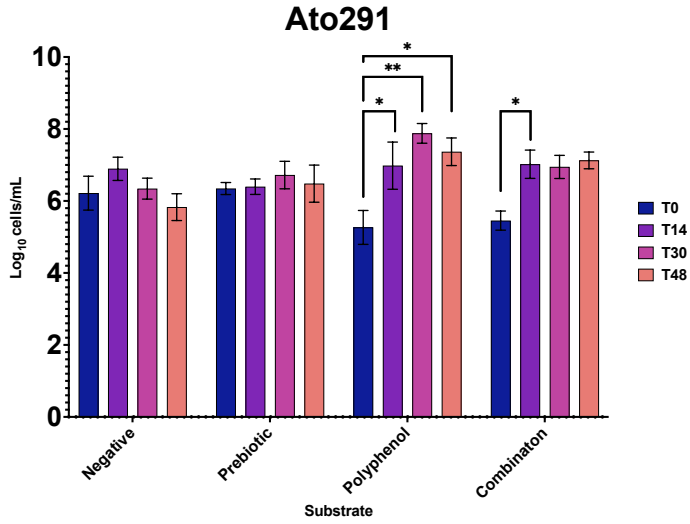

Ato291
Log10 cells/mL
10
8
6
4
2
0
*
**
*
*
*
T0
T14
T30
T48
Negative
Prebiotic
Polyphenol
Combinaton
Substrate

Fig. 1.2 Enumeration of bacteria by Flow-FISH at baseline (0) (pH representing the proximal colon), 14 (transverse colon), 30 (distal colon) and 48.. Bacterial groups shown were: A – total bacteria (EUB), *Bifidobacterium spp.*(BIF), *Lactobacillus* spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), *Clostridium coccoides–Eubacterium rectale* group (EREC), *Roseburia* subcluster (RREC), *Faecalibacterium prausnitzii* (FPRAU), *Atopobium- Coriobacterium* spp. (ATO), *Clostridium* cluster IX (PROP), *Desulfovibrio* (DSV) and *Clostridium histolyticum* (CHIS). Not all are pictured in main text. Values are presented as mean ± standard error. * denotes significant difference, where p <0.05. n=3.

Short-chain fatty acids – Batch cultures

Figure 2.1 shows change in SCFA concentration throughout the fermentation process. No significant difference between vessels at time 0 was found.

Within the inulin vessel, there was a significant increase in acetate from time 0 to 24 hours (P=0.043) and again, a significant increase from time 0 to 48 hours (P=0.004). No significant data was seen for the negative control, GOS, blueberry, cranberry, green tea or cocoa. Again, for resistant starch, a significant difference was seen between baseline and 24 and baseline and 48 hours (P=0.033 and P =0.005 respecitvely). The combination vessel had a significant increase in acetate between baseline and 24 and 48 hours (P<0.001 for all). The same was also seen for 8 hours and 24 and 48 hours (P<0.001 for all). The combination vessel seemed to be outperforming all other vessels in terms of acetate production, although no significant differences between substrates were seen.

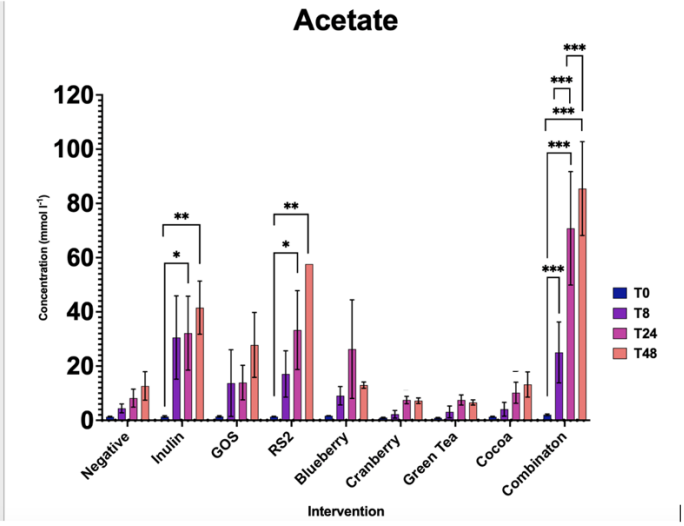
Propionate had significance within the resistant starch vessel, between times 0 and 8 (P=0.020) and in the combination vessel between times 0 and 8 (P=0.003), 0 and 24 (P=0.001) and 0 and 48 (P<0.001).

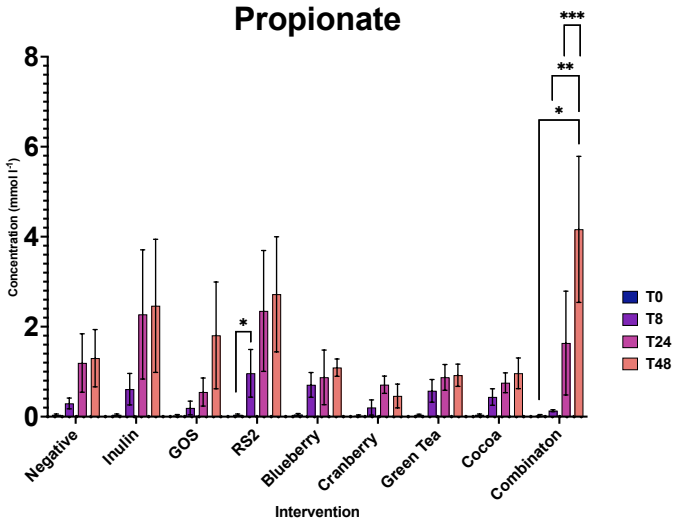
Lactate showed a significant increase in the combination vessel, between times 0 and 8 (0.036), times 0 and 24 (P=0.007)and 0 and 48(P=0.002), continuing the trend of the SCFAs still increasing at 48 hours.

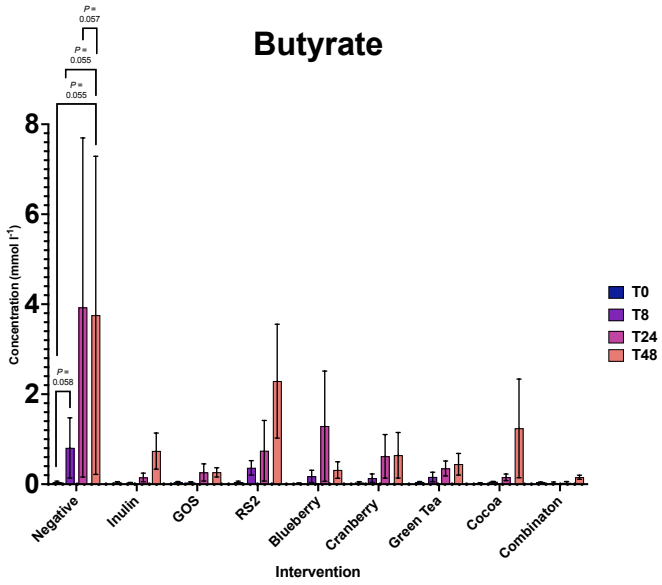
Within the negative control vessel for isobutyrate, there were some significant changes - between time 0 and 8, 24, 48, as well as between time 8 and 24, and 8 and 48, and time 24 and 58. All these

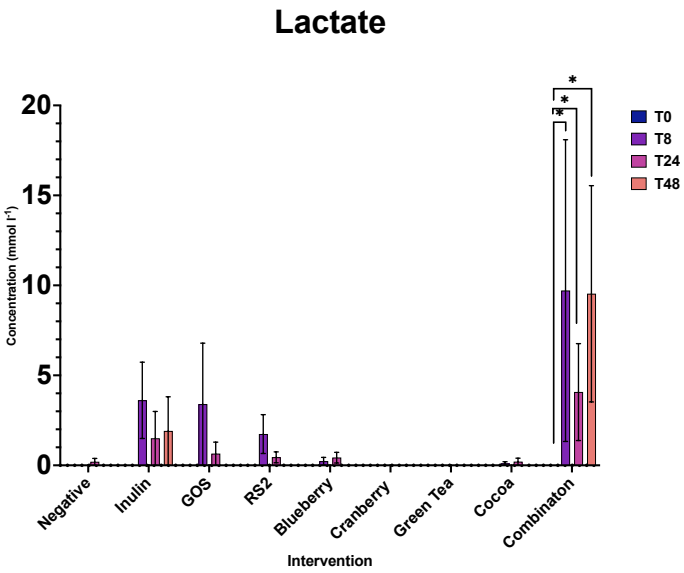
had a P value of 0.045. For isovalerate, green tea had a significant increase between time 0 and time 8 (P=0.046).

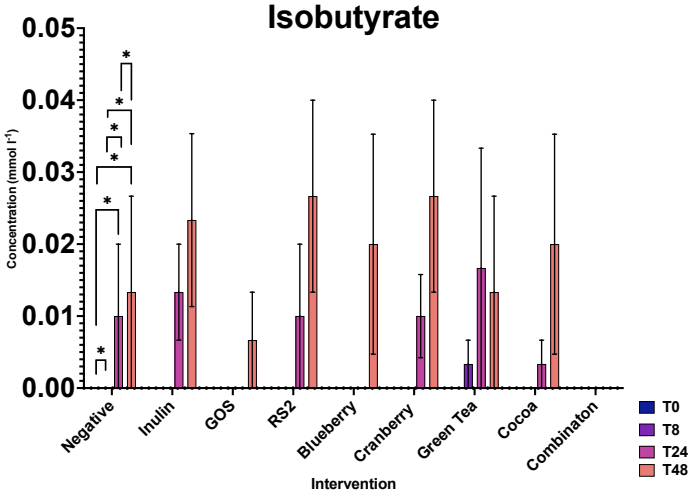
No significant changes occurred for butyrate, but there is a general visual increase within the resistant starch vessel. No significant data were seen for valerate.






Butyrate
P = 0.057
P = 0.055
P = 0.055
P = 0.058
Concentration (mmol l-1)
0
2
4
6
8
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention
T0
T8
T24
T48


Lactate
Concentration (mmol l-1)
0
5
10
15
20
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention
T0
T8
T24
T48


Isobutyrate
Concentration (mmol l-1)
0.00
0.01
0.02
0.03
0.04
0.05
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention
T0
T8
T24
T48

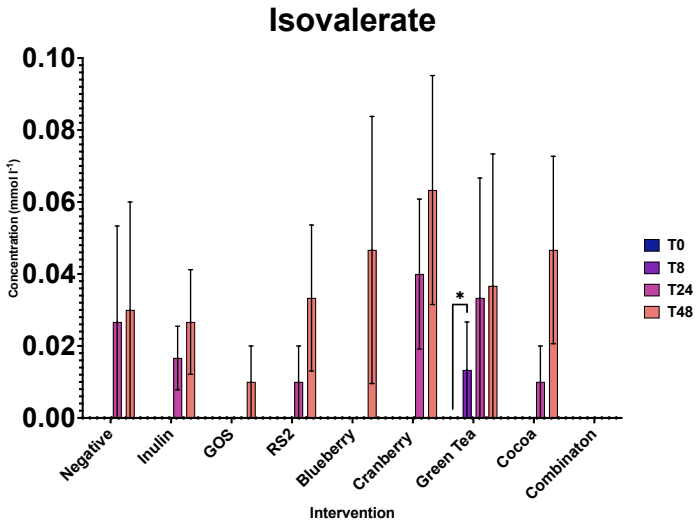


Fig 2.1. SCFA concentrations of acetate, propionate, butyrate, and lactate, Isovalerate andisobutyrate (mM) per vessel at baseline (0 hours) and following 8, 24 and 48 hours of fermentation. Values are mean ± standard error. Significant change within vessels is indicated as * $p < 0.05$. n=3

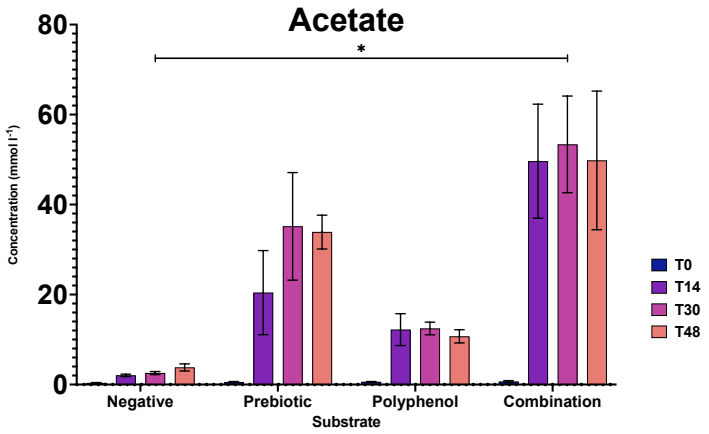
Short-chain fatty acids – pH modified batch

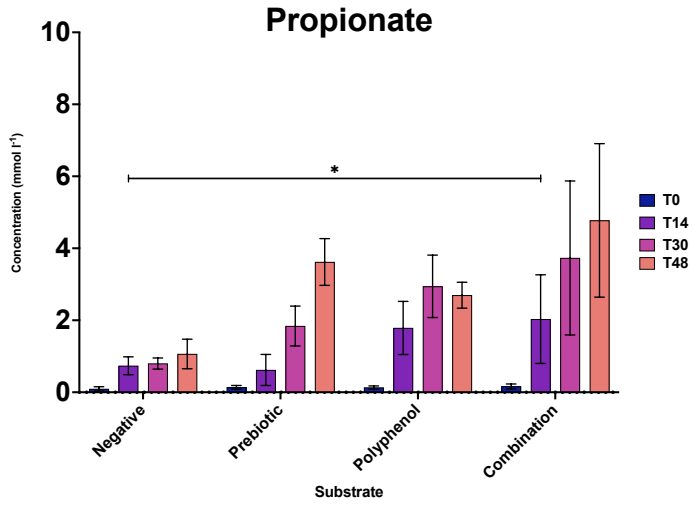
Acetate showed a significance increase between the negative control and the combination of prebiotics and polyphenols at 24hours (P=0.045). At time 8, there was a similar trend between these vessels (P=0.063). Visually, production of acetate was higher in the combination vessel than for any other substrate.

Propionate showed a significant difference within the combination vessel – between times 0and 48hr (P=0.038).We saw that within the prebiotic substrate and the combination substrate, production of propionate continued to increase at 48 hours. This differs from the individual inulin//GOS substrates reported in the batch cultures.

Butyrate significantly increased in the polyphenol vessel, between times 0 and 24hrs(P=0.026), ad 8 and 24hrs(P=0.043).

Lactate was significantly higher in the combination vessel at time 8, when compared to the negative control (P=0.015)and the polyphenol (0.025). A similar occurrence happened at 24hours, where there was a significantly higher level of lactate in the combination vessel than in the negative control (0.007) or the polyphenol vessel (0.004). It was also higher than the prebiotic vessel (p=0.053). Within the prebiotic vessel, the level of lactate was significantly higher at 24hours than at baseline (P=0.007). In the combination vessel, this also occurred, between baseline and time 8 (p=0.001), and baseline and time 24(P<0.001). Interestingly, there was a decline throughout the time points of lactate, across all substrates.


Acetate
80
60
40
20
0
*
Concentration (mmol l-1)
Negative
Prebiotic
Polyphenol
Combination
Substrate
T0
T14
T30
T48


Propionate
10
8
6
4
2
0
*
Concentration (mmol l-1)
Negative
Prebiotic
Polyphenol
Combination
Substrate
T0
T14
T30
T48

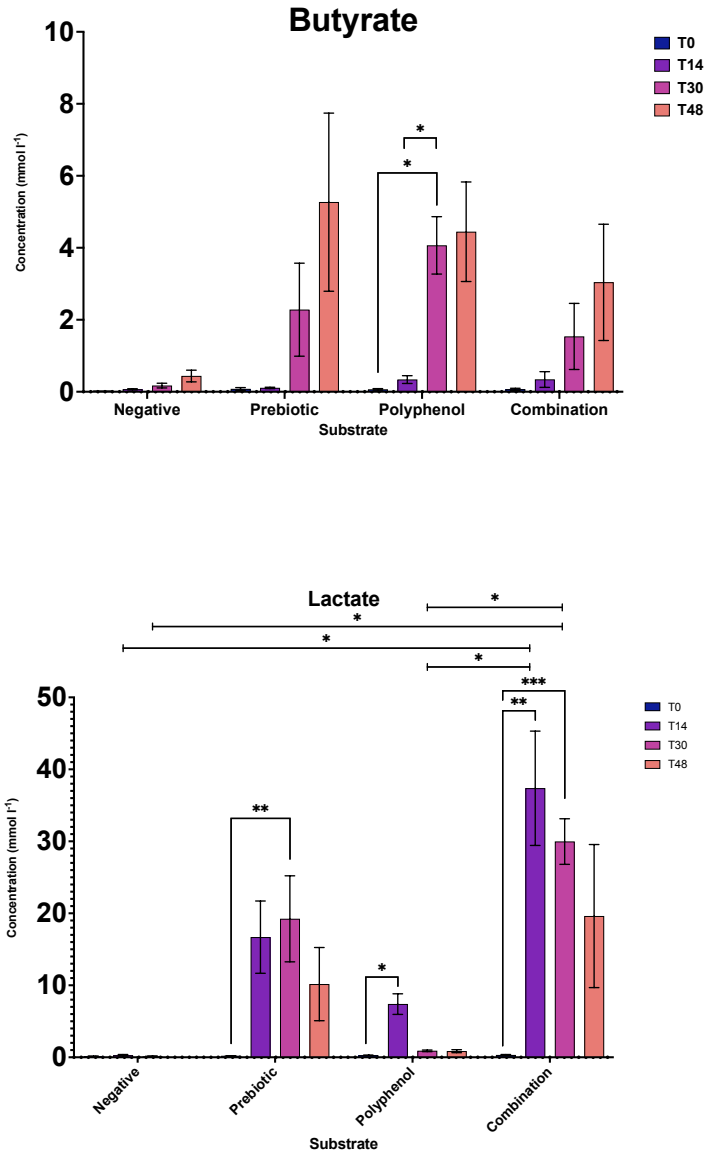


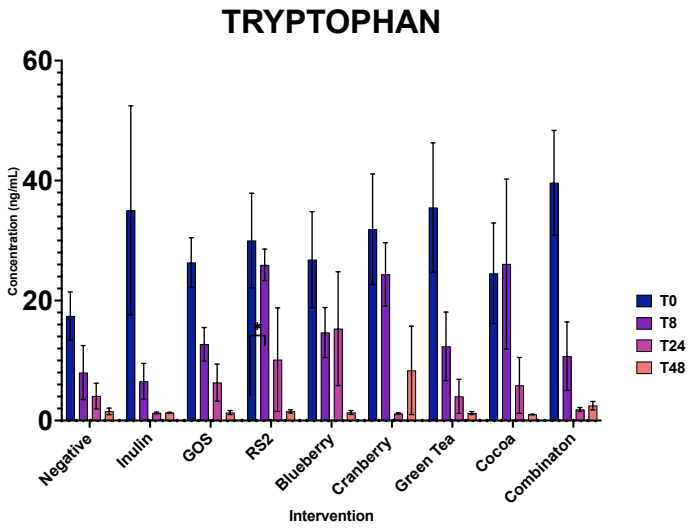
Fig 2.2 SCFA concentrations of acetate, propionate, butyrate, and lactate (mM) per vessel at baseline (0 hours) and following 14, 30 and 48 hours of fermentation. Values are mean ± standard error. Significant change within vessels is indicated as * $p < 0.05$. n=3

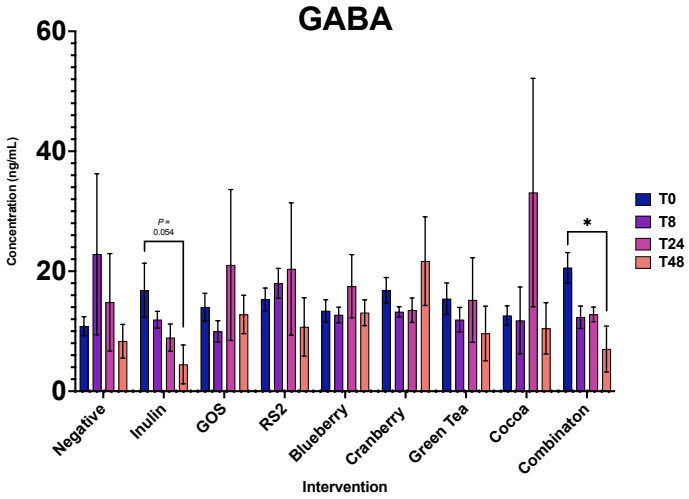
LC-MS – Batch Cultures

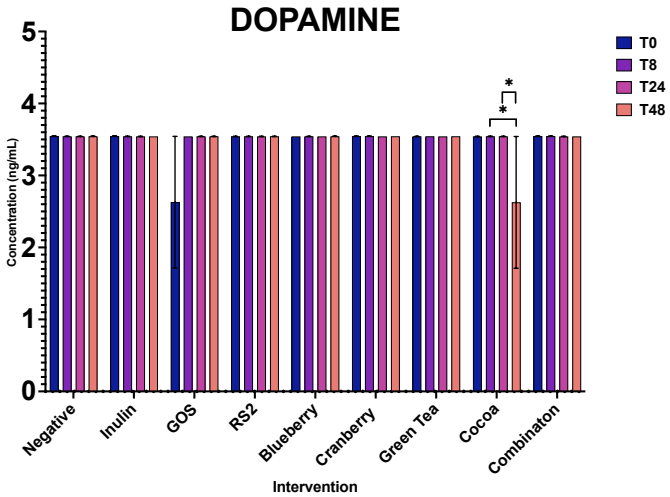
As shown in figure 3.1, the neurotransmitter GABA showed a significant decrease in the combination vessel between time 0 and 48 (P=0.029). There was also a trend in the inulin vessel, again, a decrease from time 0 to 48 (P=0.054)

Kynurenic acid has a significant increase from time 8 to 48hours in the GOS vessel (P=0.049). Norepinephrine showed significant changes within the Inulin, resistant starch, cranberry, green tea, cocoa and combination vessels. For Inulin, we saw a significant change between baseline and 24 hours. (P=0.027), as well as baseline and 28 hours (P=0.011). There was also a trend towards significance for between baseline and time 8 (P=0.051). In the resistant starch vessel there was a significant change between baseline and time 48(P=0.038), as well as between time 8 and 48hours (P=0.005). Cranberry showed a significant change between baseline and 24 hours, with a P value of 0.033. Green tea has significant changes from baseline to 24 and 48 hours – P=0.033 and P=0.009 respectively. Cocoa was significantly different between 8 hours and 48 hours, P=0.004, and the combination vessel shows significant changes from baseline to 8 hours,(p=0.047) 24 hours (P=0.011), and 48 hours (P=0.005).

Dopamine showed a significant decrease in the cocoa vessel from time 8 and time 24 to time 48, both P=0.046. There was a significant increase in the GOS vessel from baseline to 8hours and 24 hours, again with a P value of 0.046. Tryptophan demonstrated no significance, and the levels of epinephrine and serotonin were below detection.


TRYPTOPHAN
Concentration (ng/mL)
60
40
20
0
T0
T8
T24
T48
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention


GABA
Concentration (ng/mL)
60
40
20
0
P=
0.054
*
T0
T8
T24
T48
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention


DOPAMINE
Concentration (ng/mL)
5
4
3
2
1
0
*
*
T0
T8
T24
T48
Negative
Inulin
GOS
RS2
Blueberry
Cranberry
Green Tea
Cocoa
Combinaton
Intervention

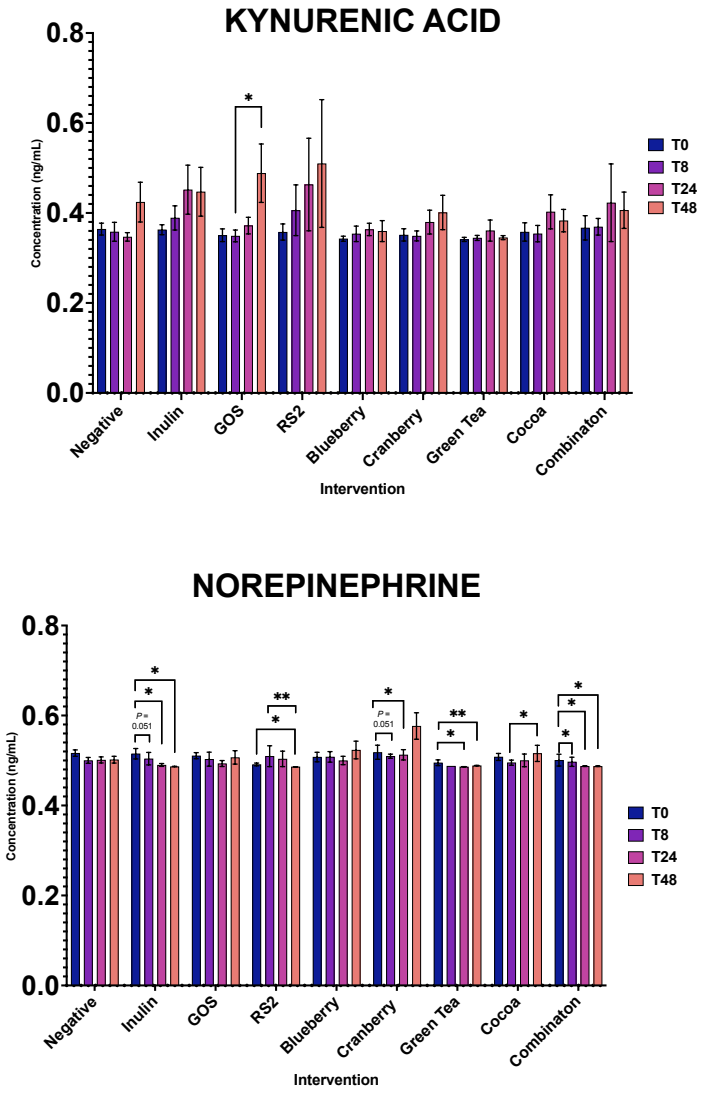


Fig 3.1 Concentrations of GABA tryptophan, norepinephrine, kynurenic acid and dopamine (ng/mL) per vessel at baseline and following 8, 24 and 48 hours of fermentation. Values are mean ± standard error. Significant change is indicated as as * ($p < 0.05$). Dopamine is displayed however it was affected by the limit of detection and is likely not representative data. n=3

LCMS. – pH modified batch

LCMS data are displayed below in Figure 3.2.

Tryptophan showed a decrease after baseline for all substrates. For the negative control vessel, differences were significant between 8 hours and 24 (0.024), 8hours and 48 (P=0.034), and trended towards significance between baseline and 24 hours (P=0.062) and 48 hours (P=0.050). The prebiotic

vessel was significantly different between baseline and time 24(P=0.007) and baseline and time 48 (P=0.006).

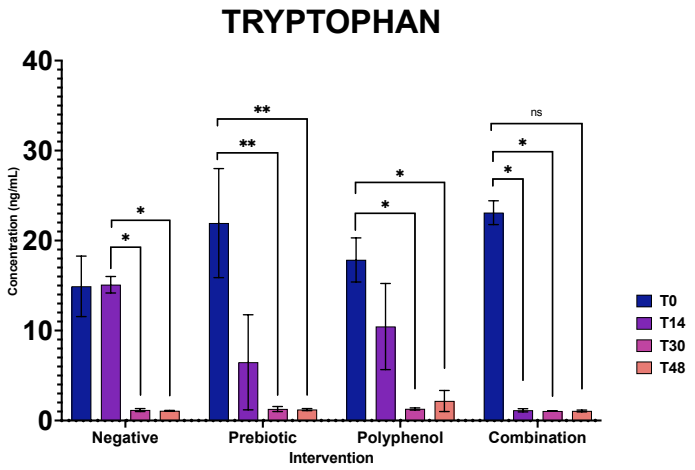
The polyphenol vessel was again significantly lower in tryptophan at time 24 when compared to baseline (P=0.025) as well as at time 48 when compared to baseline (0.027). The combination of prebiotics and polyphenols was significantly decreased at 8 hours (0.049, 24 hours (P=0.016)and 48 hours (P=0.013), all when compared to baseline.

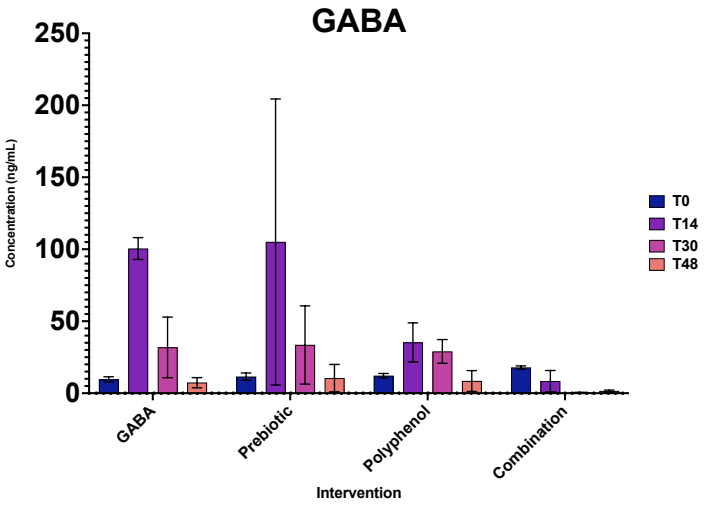
Serotonin was significantly different at baseline between the negative control and the combination vessel (P=0.003), the prebiotic vessel and the combination vessel(P=0.003), and the polyphenol and combination vessel(P=0.007). Visually, serotonin peaked at time 8 for both the polyphenol and combination vessel.

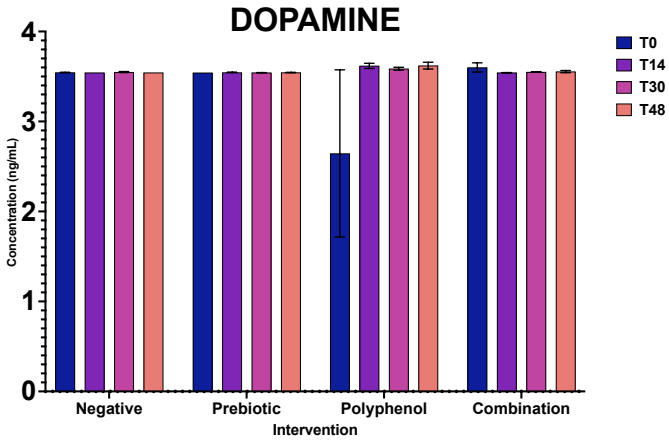
Kynurenic acid was significantly different at 8 hours between the negative control vessel and the polyphenol vessel (P=0.009) and the negative control and the combination vessel (P=0.041). Both e were higher than the negative control. The prebiotic vessel was also significantly lower than the polyphenol and the combination vessel, with P values of 0.004 and 0.016 respectively.

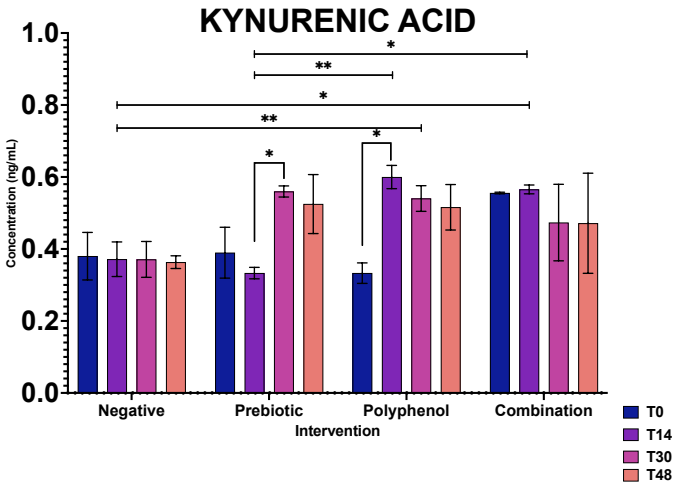
Within the prebiotic vessel, there was a significant increase from 8 hours to 24 hours (P=0.025). There was also a significant increase in the polyphenol vessel from baseline to 8 hours (P<0.001).

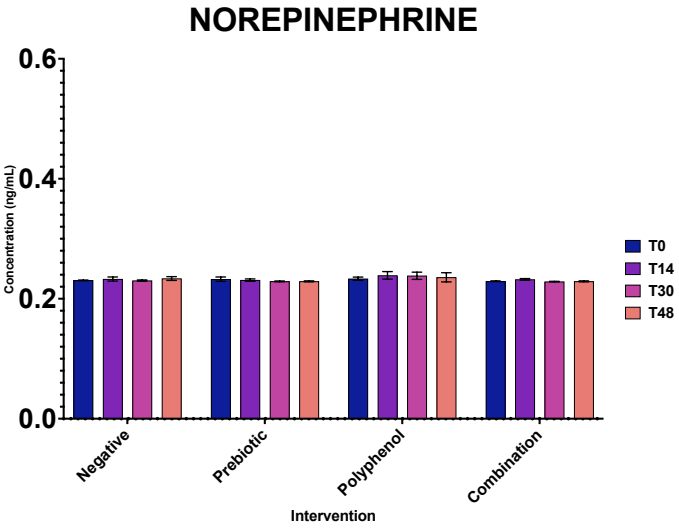
No significant differences were found for dopamine, GABA, or norepinephrine, and the levels of epinephrine were below detection.


TRYPTOPHAN
Concentration (ng/mL)
40
30
20
10
0
*
*
**
**
*
*
*
ns
Negative
Prebiotic
Polyphenol
Combination
Intervention
T0
T14
T30
T48


GABA
Concentration (ng/mL)
250
200
150
100
50
0
GABA
Prebiotic
Polyphenol
Combination
Intervention
T0
T14
T30
T48


DOPAMINE
Concentration (ng/mL)
4
3
2
1
0
Negative
Prebiotic
Polyphenol
Combination
Intervention
T0
T14
T30
T48


KYNURENIC ACID
Concentration (ng/mL)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
*
**
*
*
Negative
Prebiotic
Polyphenol
Combination
Intervention
T0
T14
T30
T48


NOREPINEPHRINE
Concentration (ng/mL)
0.6
0.4
0.2
0.0
Negative
Prebiotic
Polyphenol
Combination
Intervention
T0
T14
T30
T48

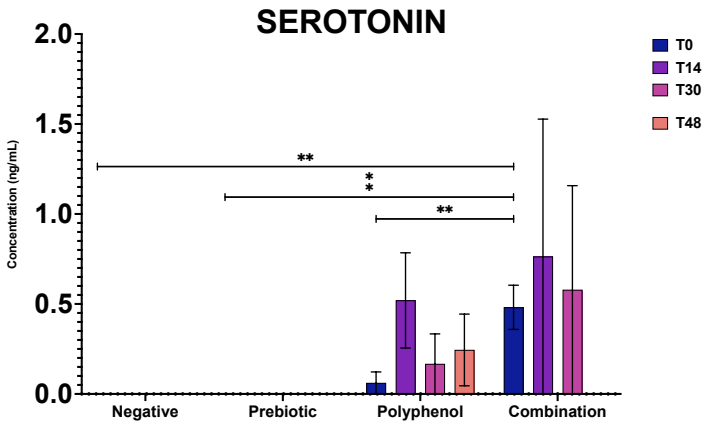
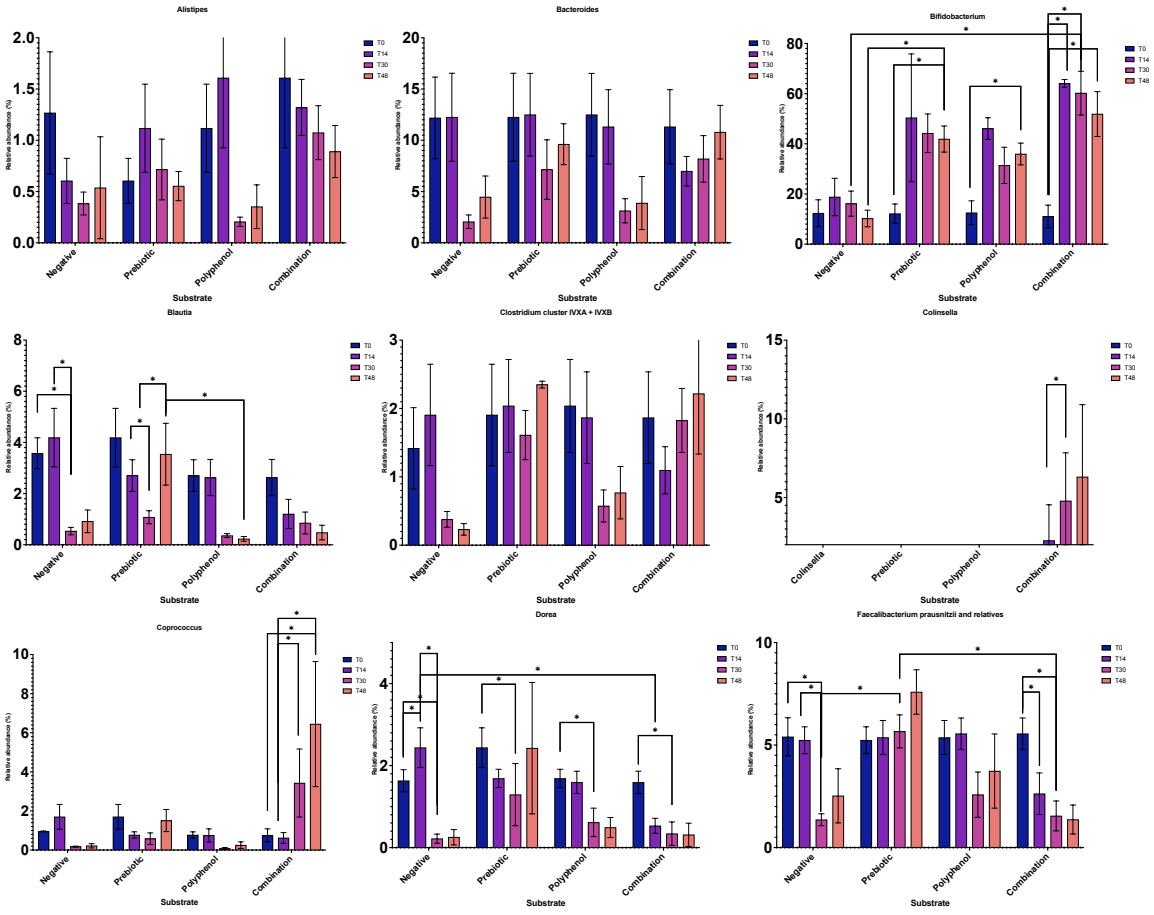

SEROTONIN
Concentration (ng/mL)
2.0
1.5
1.0
0.5
0.0
**
**
**
Negative
Prebiotic
Polyphenol
Combination
T0
T14
T30
T48

Fig 3.2 Concentrations of GABA tryptophan, norepinephrine, kynurenic acid, serotonin and dopamine (ng/mL) per vessel at baseline and following 14, 30, and 48 of fermentation. Values are mean ± standard error. Significant change is indicated as as * ($p < 0.05$). n=3

16S rRNA partial gene sequencing – pH modified batch.

Relative abundance data was calculated using the raw ASVs. These results are displayed below in fig 4.1. Of note, bifidobacteria significantly increased between the control vessel and the combination. Bifidobacteria was highest in relative abundance throughout the fermentations. The polyphenol vessel began to increase again at the 48 hour time point, mimicking what we saw in the combination vessel for many bacterial groups in the flow fish. This is interesting and suggests that the polyphenols may be the reason we see an increase in bacterial growth, or rather a slowed decrease, at this time point.

For clarity, the significant data following two-way Anova is presented below the figures in a table in a table (table 1.), indicating where, and what changes occurred.



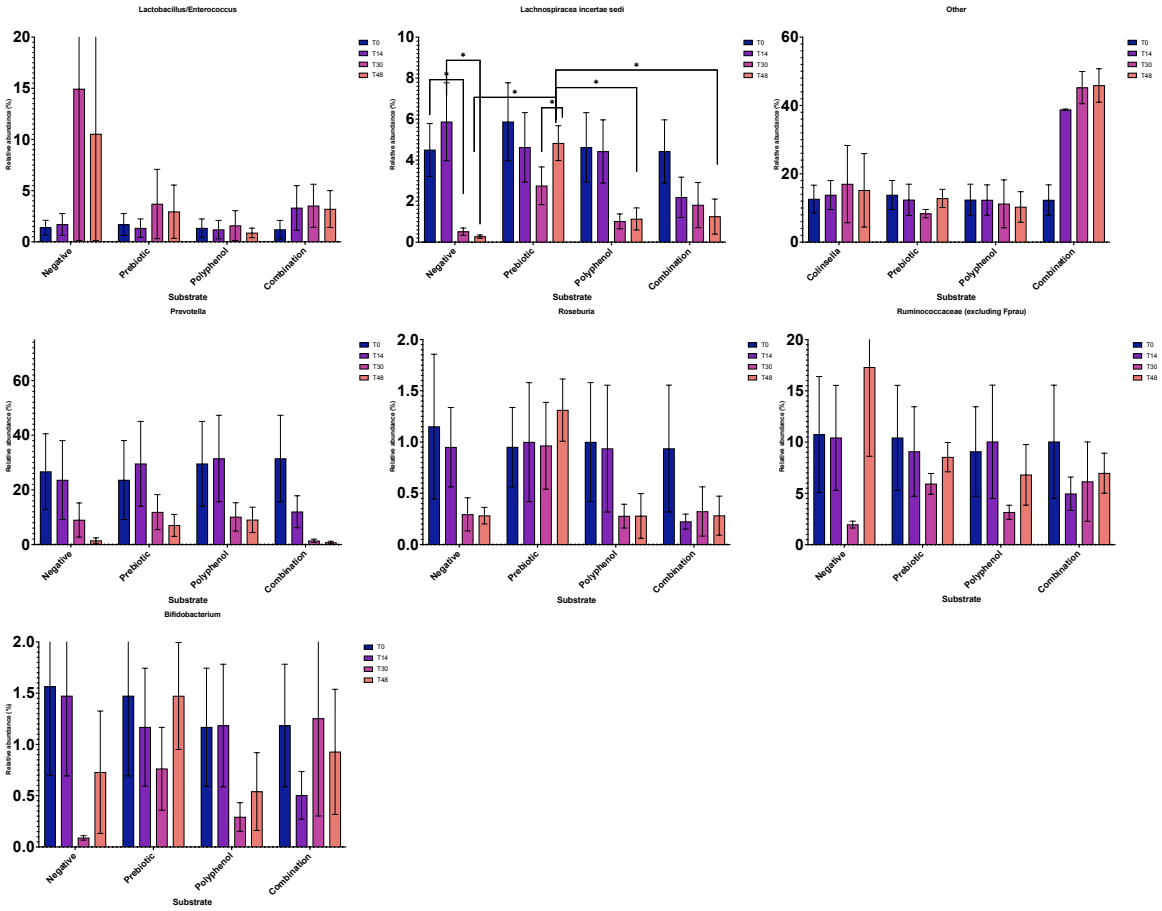


Fig. 4.1 Relative abundance (%) of bacterial groups are represented, per vessel at baseline and following 14, 30, and 48 of fermentation. Values are mean ± standard error. Significant change is indicated as as * ($p < 0.05$). n=3.

Bacterial Group	Time/Vessel interaction	Change	Significance Level (P<0.05)
Lachnospiracea incertae sedi	48 hours; negative control and prebiotic	Higher in prebiotic	0.007
	48 hours; prebiotic and polyphenol	Higher in prebiotic	0.025
	48 hours; prebiotic and combination	Higher in prebiotic	0.03
	Negative control; 0 hours to 30 hours	Lower at 30 hours	0.042
	Negative control; 14 hours to 30 hours	Lower at 30 hours	0.009
	Negative control; 14 hours to 48 hours	Lower at 48 hours	0.024
	Prebiotic; 30 hours to 48 hours	Higher at 48 hours	0.02
Faecalibacterium prausnitzii and relatives	30 hours; negative control and prebiotic	Higher in prebiotic	0.029
	30 hours; prebiotic and combination	Higher in prebiotic	0.036

	Negative control; 0 hours to 30 hours	Lower at 30 hours	0.024
	Negative control; 14 hours to 30 hours	Lower at 30 hours	0.013
	Combination; 0 hours to 14 hours	Lower at 14 hours	<0.001
	Combination; 0 hours to 30 hours	Lower at 30 hours	0.025
Dorea	14 hours; negative control and combination	Lower in combination	0.016
	Negative control; 0 hours to 14 hours	Higher at 14 hours	0.038
	Negative control; 0 hours to 30 hours	Lower at 30 hours	0.008
	Negative control; 14 hours to 30 hours	Lower at 30 hours	0.004
	Prebiotics; 0 hours to 30 hours	Lower at 30 hours	0.028
	Polyphenols; 0 hours to 30 hours	Lower at 30 hours	0.04
	Combination; 0 hours to 14 hours	Lower at 14 hours	0.008
	Combination; 0 hours to 30 hours	Lower at 30 hours	0.017
Coprococcus	Combination; 0 hours to 48 hours	Higher at 48 hours	0.038
	Combination; 14 hours to 48 hours	Higher at 48 hours	0.037
	Combination; 30 hours to 48 hours	Higher at 48 hours	0.041
Colinsella	Combination; 14 hours to 30 hours	Higher at 30 hours	0.024
Blautia	48 hours; prebiotic and polyphenol	Lower in polyphenol	0.046
	Negative control; 0 hours to 30 hours	Lower at 30 hours	0.02
	Negative control; 14 hours to 30 hours	Lower at 30 hours	0.01
	Prebiotics; 0 hours to 30 hours	Lower at 30 hours	0.025
	Prebiotics; 30 hours to 48 hours	Lower at 30 hours	0.039
Bifidobacteria	30 hours; negative control and combination	Higher in combination	0.016
	48 hours; negative control and prebiotics	Higher in prebiotics	0.03
	48 hours; negative control and combination	Higher in combination	0.006
	Prebiotics; 0 hours to 30 hours	Higher at 30 hours	0.007
	Prebiotics; 0 hours to 48 hours	Higher at 48 hours	0.002
	Polyphenols; 0 hours to 48 hours	Higher at 48 hours	0.011

	Combination; 0 hours to 14 hours	Higher at 14 hours	0.02
	Combination; 0 hours to 30 hours	Higher at 30 hours	<0.001
	Combination; 0 hours to 48 hours	Higher at 48 hours	<0.001

Table 1. Representing and quantifying any significant changes within the relative abundance level data from 16S rRNA partial gene sequencing.

2.4 Discussion

This study aimed to evaluate the potential effects of a novel combination of prebiotics and polyphenols on bacterial proliferation, SCFA and neurotransmitter levels in vitro using gut models.

Through the use of pH controlled, anaerobic *in vitro* batch culture models, the impact of varying substrates on faecal samples was explored. For the first exploratory batch cultures, Inulin was considered to be a positive prebiotic control. The second, pH modulated batch cultures, used a prebiotic combination that also included inulin as a positive control.

In these inulin containing vessels, there was an increase in bifidobacteria, and modifications to the short chain fatty acid content– as expected in a prebiotic effect (the selective stimulation of beneficial bacteria within the gut). Previous research has demonstrated that the inclusion of oligofructose or inulin in a diet led to *Bifidobacterium* becoming predominant within fecal samples (Gibson et al., 1995).

Initially, static batch cultures were used to assess separate substrates that could be included within a novel combination had effects. As shown in the results, there did seem to be better longevity of action from this vessel in terms of bifidobacterial production, however what was not known was whether this was due to the combination of other prebiotics or addition of polyphenols. This therefore provided an opportunity to further explore this combination through the use of a pH modified batch system, wherein the pH was manually controlled to better reflect the colonic ecosystem. In this model, the prebiotic combination was separated out from the polyphenol combination, by only including the

prebiotics in one vessel, the polpyhenols in another, and the combination of both in a final exploratory vessel. As a note, we will not be able to separate out the prebioitic effect completely from the polpyhenol action – these are still whole food powders and may provide a prebiotic effect themselves. The results amy also be affected by the modified pHs.

Through the addition of these substrates, the models provided an opportunity to explore effects of prebiotics and polyphenols on the human microbiota and associated metabolite production.

The most interesting, perhaps, of these results, as briefly mentioned above, was longevity of bacterial abundance. Both type of batch culture models have a limited nutrient supply from the basal medium, and in the 48 hour sampling period a decline in these bacteria was expected. We can, through the use of FLOW fish, see that this was the case for most bacterial groups and most substrates in the batch culture models – after 24 hours there was a decline.

However, further analysis showed that the decline was less pronounced in some groups – for total bacteria, the novel combination vessel had an increasing trajectory even at 48 hours, which was unusual, as in previous research we would expect a steady state, or decline of bacteria as nutrients are used up within the batch system. Whilst not reflected exactly with the bifidobacterial probe, the decline was much less sharp than when compared to the positive control. Resistant starch had a similar effect, so it may be expected that the combination vessel showed this too. *Lactobacillus* spp. seemed to continually grow in most of the vessels, with only the inulin vessel showing significantly increased results. Research has shown that *Lactobacillus* is associated with the intake of both prebiotics and polyphenols, so this is expected within the current conditions (Piekarska-Radzik et al., 2021; Śliżewska et al., 2020).

The EREC group was maintained at a high level within this combination vessel, when compared to all other substrates, continuing to increase at a 48 hour time point sample. Though the RS2 vessel did not continue this increase, it did maintain a high level of EREC, so again, this substrate could be partially responsible for this action within the combination vessel. This would make sense when compared to

the literature - through surprising as RS2 is more highly associated with F. prau. In vivo, *E. rectale* increases when a diet high in resistant starch is consumed – so the results here are as expected.

Ato was another group that increased at 48 hours within the combination vessel. Though a commonly occurring genus in human faeces, thought to be increased by prebiotic supplementation, there is no currently identified beneficial or detrimental effect to human health associated with modulation of this bacterial group (Kwok et al., 2014; .Wang et al., 2020)

It is important to note that whilst the highest $\log_{10}$ numbers may not have been seen in the combination vessel, many positive bacterial groups continued to increase at the 48 hour mark within this novel combination, as well as the decline being less sharp where there was a decline. This may be useful in terms of supplementation if the action of the novel combination provides a prolonged effect.

Transient changes were seen within CHIS (clostridia), however there was one significant increase. This was potentially skewed by one donor, as this was not echoed in follow up pH modulated batch experiments. Ideally the results would have shown a decrease in this negative bacteria – this would better align with other research (Liu et al., 2016), where, although similarly to this research, transient changes were seen, an overall decrease was seen in CHIS when inulin and galactooligosaccharides were utilisied within the batch culture vessels. The increase in this group is associated with exposure to stress (Lee et al., 2011), as well as with ulcerative colitis (Kleessen et al., 2002)

Cranberry, as a substrate, showed continually lower bacterial numbers, however it was quite insoluble and this may have not been optimal for the flow cytometry. Another explanation is that the cranberry selectively stimulates other bacteria – not much is known about the specific mechanisms for cranberry use within the gut microbiome – though ordinarily the expectation would be that it would increase bifidobacteria, as has been shown previously (Özcan et al,. 2017; Karboune et al., 2022)

The second set of fermentations- the pH modulated batch cultures – had fairly transient data for total bacteria. As expected, across all substrates the total bacteria count did decline towards 48 hours. For bifidobacteria, we saw that, whilst not reaching as high a peak log10 value as the prebiotic or the polyphenol combination, values did seem to begin to increase again towards the 48 hour mark, suggesting that it was not just the combination of the prebiotics that provide the combination with its

potential extra longevity of action. The same trend of the combination vessel increasing towards 48 hours was seen with Ato.
These two fermentation types seemed to present fairly similar results/trends, reinforcing the thought that the novel combination may be a more effective and efficient way of supplementing.

Within the batch culture fermentation period, concentrations of acetate remained steady for most substrates, however within the combination vessel, the inulin and the resistant starch they significantly increased through to the 48 hour sample, with the most acetate being produced in the combination vessel.. Propionate increased throughout all substrates, except cranberry, but the concentration was highest within the combination vessel. Conversely, production of butyrate was lowest within the combination vessel, but the concentration of this was lower across all substrates when compared to the negative control. Though present only in low concentrations, isovalerate and isobutyrate seemed to increase across the fermentation periods with the exception again of the combination vessel, where no production was seen. Lactate was higher level in the combination vessel than any other substrate. It seemed to dip at 24 hours and then began to increase again – explained by the fact that it is an electron sink product in anaerobic ecosystems (Rowland et al., 2018). It is very interesting that the polyphenol/prebiotic substrate combination produced more lactate than any of the individual substrates, indicting optimal energy generation for the microbiome.

The pH modulated batch fermentations also showed that the combination vessel outperformed production of SCFAs for all but the butyrate, where prebiotics alone seemed to have the largest peak, followed by polyphenols. This being said, there was a continuing increase of butyrate across all three substrate groups when compared to the negative control. Again, combining prebiotics and polyphenols seemed to provide better longevity of action, including microbiota outputs like SCFAs.

General increases of SCFAs were likely to be at least slightly affected by fermentation of the basal medium, however, the dramatic difference especially within lactic acid of the combination vessel cannot be explained by this. As mentioned, lactate is an important gut bacteria derived metabolite and

is thought to make valuable contributions to host health. For example, research suggests that intraluminal levels of lactate (produced by lactobacilli or bifidobacteria) can have modulatory effects on inflammation of the intestinal lining (Zhou et al.,2022; Iraporda et al., 2015; Wang et al., 2020). This is beneficial in terms of modulation of gut symptoms, but also in mood state and neurological health - chronic psychological stress has been shown to increase intestinal mucosal mast cells (Yang et al., 2006)– which are key effectors of the gut-brain axis. These are key players within the HPA axis and respond to stressors by way of altering permeability of the gastrointestinal tract (Carabotti et al., 2015; Appleton. 2018). Some research has shown that by suppressing pro-inflammatory IL6, lactate can minimise inflammation (Manosalva et al., 2021). Lactate can also be converted into other health promoting SCFAs, for example, to acetate or butyrate (Oh et al., 2021). The continued production of butyrate within the vessel is also of potential benefit – with butyrate being related to improved mood, cognition and improved GI tract function (Bourassa et al., 2016).

In both the batch, and the pH modulated batch fermentation models, some levels of neurotransmitters were detected. Whilst GABA, Norepinephrine, Epinephrine, Dopamine Serotonin, Kynurenic acid and Tryptophan were all outlined as possible targets for LCMS in the methodology, some were below levels of detection. This is not surprising, as the batch cultures were not optimised for detection of neurotransmitters, rather, for the detection of faecal microbial output. The presence, however, of some neurotransmitters throughout these fermentations is of relevance.

Whilst significant changes were only seen in the combination vessel, it was apparent that the GABA increased throughout the time period for some substrates, including some of the polyphenols.. GABA has been associated in research with various mood states, and is known to be a primary inhibitory neurotransmitter, so it is an excellent target for nutritional interventions (Hepsomali et al., 2020) Research has suggested that the production of GABA in the brain can be modified by intake of probiotics (Sarkar et al., 2019; Monteagudo-Mera et al., 2023) . As such, GABA produced from the gut microbiome may also indirectly modify brain function. This may be through GABAergic signalling (Tette et al., 2022).

Kynurenic acid shows a significant increase in the GOS and resistant starch vessels. This may suggest a conversion of tryptophan through the kynurenic pathway- in silico analyses have demonstrated that GI bacteria have the capability to metabolise tryptophan into metabolites like kynurenine –through this pathway (Kaur et al., 2019). This would also explain low tryptophan levels. Dopamine and norepinephrine were present at very minimal levels and varied very little across substrate and time. Levels of epinephrine and serotonin were below detection.

In pH modulated batch cultures, we again detected the presence of GABA, tryptophan, norepinephrine and dopamine, but what was perhaps the most interesting is that within the pH modulated batch we begin to see (albeit low) levels of serotonin in the polyphenol and the combination vessel. This is an important observation, that higher polyphenol intake (i.e. the combination) may begin to influence the production of gut derived serotonin. This is a fairly novel observation, though similar data has been reported in very studies such as Eastwood et al., 2023, it is still exciting, as it suggests gut/microbial derivation of serotonin, in the absence of colonic cells, whilst under physiologically relevant conditions - and that this is enhanced within a combination of polyphenols and prebiotics.

Briefly returning to the GABA production, interestingly, we would expected to have seen more GABA within the beginning of the fermentation in the pH modified batch cultures, as GABA production is higher in the proximal region of the colon, with a more acidic environment tending to favour production (Xie et al., 2017). This is due to bacteria such as *Lactobacillus* and *Bifidobacterium* utilising it as a mechanism to survive the pH of the intestinal environment, and produce it through the decarboxylation of glutamate (Monteagudo-Mera et al., 2023). This lower pH of the proximal colon seen within the pH modified batch culture should encourage the enzymatic activity responsible for GABA synthesis. In this study, we saw higher levels of GABA production at time points 14, which was at a pH of 6.2 and 6.4, which was not expected.

As such, the novel combination of prebiotics and polyphenols may be a useful tool in the modulation of human health. Tryptophan, the sole precursor to serotonin (Colle et al., 2020), was seen to decrease over time throughout these fermentations – this may be explained by the gut mediated breakdown of tryptophan into other compounds. The gut microbiome is through to play a role in the mediation of pathways for tryptophan use. For example, *Lactobacillus* and *Bifidobacterium* spp. are thought to play a role in the upregulation of serotonin transporter expression within the GI tract (Cao et al., 2018). Though this does not necessarily correlate with the data presented above, – we can see that there is an increase *Lactobacillus* which supports this potentially occurring.

In terms of the overall potential superior effect of the combination vessel, it is also likely that the effects seen in the combination vessel may be as a result of the increase in nutritional intake - dose dependent studies (Bothe et al., 2017) demonstrate that increased doses of prebiotics do indeed provide a superior prebiotic effect. However, a 2023 study by Kaewarsar et al suggested that there were optimum ratios of combinatorial prebiotics that improve the prebiotic effect in vitro, so it may not just be the effect of increased dose. It is, however, important to note that by the definition of a prebiotic effect, there needs to be a positive effect on host health (Gibson., 2017), which of course cannot be seen *in vitro.*

In conclusion, the combination mixture of polyphenols and prebiotics showed most promise for beneficial effects following microbial fermentation. It is worth exploring this further through more expansive study, including in vivo, in part to confirm a prebiotic effect.

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Chapter 3 – A human intervention study on prebiotics and polyphenols

3.1 Introduction to gut, brain and stress.

Stress is a ubiquitous part of everyday life. The unavoidable effects of a modern world, where many persons may experience physiological or psychological stressors on a daily basis, can have an effect on health and wellbeing. Whilst most people recognise the feeling of being 'stressed out', the word 'stress' is used to describe many different life events – with the colloquial definition varying from person to person.

Whilst a broadly accepted definition may be – any type of change that causes physical, emotional or psychological strain that the body can not cope with (Lazarus et al., 1985). We often refer to fight or flight, or the production of cortisol when discussing stress, but it is suggested that a focus on specific hormones is too narrow and ignores other effects (Kagias et al., 2012; Yaribeygi et al.,2017;. We can also describe stress as an external or internal condition that challenges homeostatic state of a cell or organism (Kagias et al., 2012).

For the purpose of this paper, it is important to utilise a broader definition, so we will define stress in the broad definition of any condition that causes homeostatic challenge, and therefore the stress response as however the body responds to this, regardless of mechanism (Lu et al., 2021;Chu et al., 2021). Moreover, within this definition, for modern life, stress can be 'perceived'. (Epel et al., 2018)

Whilst often at least partly managed, stress can have debilitating effects on human health, such as oxidative stress, inflammation, hypertension etc (Mariotti et al., 2015). Whilst sometimes managed so successfully that people do not notice they are under stress, the toll it can take on our bodies in the long term is intense. There has been research suggesting that chronic stress actually ages us – potentially through the damage of telomeres by reactive oxygen species (Yegorov et al., 2020).

Stress can affect cognition, memory, learning, as well as damage the body though oxidative stress (Juszczyk et l., 2021; Salim et al., 2017. There have been associations with cellular changes in the brain (primarily animal studies) (Khan et al., 2020; Rinaldi et al., 2010; Zurawek et al., 2016),

decreased neuron presence (McEwan et al., 2016; Woo et al.,2021) and physical consequences such as hypertension (Kulkarni et al., 1998), nutrient insufficiencies (Tomasello et al.,2016; Kaplan et al., 2015), hormone disruption (Ranabir et al., 2011), injury or impairments (musculoskeletal and cognitive) (Nippert et al., 2008), inflammation and immune suppression (Slavich et al., 2014; Glaser et al., 1987)), as well as general illness and infection (Salleh et al., 2008). It can also potentially lead to an elevated risk of cardiovascular disease mortality and morbidity (Wentworth et al., 2013). It is also important to consider oxidative stress – whereby an imbalance occurs of the production and accumulation of oxygen reactive species in cells and tissues, where the body's natural anti-oxidant defence becomes overwhelmed and unable to remove these products, causing the cells to be damaged (Pizzino et al., 2017).

Oxidative stress is seen in various pathologies, such as neurological conditions (Alzheimer's, depression, Post Traumatic Stress Disorder (PTSD)) (Pizzino et al., 2017) or physiological disorder like cardiovascular disease (Wentworth et al., 2013). Some evidence has also shown support for telomere attrition caused by oxidative stress (Kawanishi et al., 2004). Perceived stress has been linked to this phenomenon, as well as to increased inflammation and immunosenescence (Martinez de Toda et al., 2019). Stress has also had strong links to Irritable Bowel Syndrome (IBS) (Qin et al., 2014), including an association between PTSD and IBS (Ng et al., 2019). This association has led to an exploration into communication between stress, mood and the gut microbiome. If we can suggest that diet is one of the most modifiable contributors to human health, and certainly one of the most identifiable ways we can modify composition of the gut microbiota (Leeming et al., 2019), exploring avenues involving dietary change and/or the inclusion of nutraceuticals seems to be a next step in healthcare. Inclusion of dietary changes into such medical care would potentially be an accessible, abundant form of treatment that may potentially counter oxidative damage, and gut dysbiosis (amongst other things) associated with a non-optimal mood state.

3.1.1 Stress and the gut

One of the reasons it may be sensible to try counter stress through dietary changes, aside from the low risk of side effects when compared to medication (Downer et al., 2020), is that the gut microbiota is

influenced by stress (Madison et al., 2019). Stress, and other psychological conditions like depression, can modify composition of the gut bacteria through various mechanisms, i.e. via the endocrine system or through inflammation (Madison et al., 2019). As the gut microbiome is reshaped, metabolic products of this environment may also change – releasing, again, hormones or metabolites that may affect mood states (Silva et al., 2020).

Research into gastrointestinal disorders like IBS and cognitive disorders have shown correlations between the microbiota and gut brain axis (GBA) (Appleton et al., 2018; Carabotti et al., 2015). Moreover, there is more specific research that directly links the hyper arousal of PTSD to IBS, as a result of GBA bidirectional signalling (Ng et al., 2019; Oroian et al., 2021). Although much research within this emerging field has been in animals, such as mice and rats, human trials that have featured pro/prebiotic supplementation have linked the gut microbiota to the stress response (Nishida et al., 2017)

Recently, there has been interest into nutrition and mental health, though the concept is of course not novel – you are what you eat is a term often used. Early Greek philosophers suggested that food was medicine, those who eat a Mediterranean diet have long been associated with a higher quality of life (Guasch-Ferré et al., 2021), indeed- studies have shown that those consuming this highly recommended diet can have a lower risk of depression (Oddo et al., 2022). Most people can recognise that in times of low mood, stress or excitement even, one's appetite can be altered (Hepworth et al., 2010).

It is, however, difficult to establish causal links, considering potential interplays between diet, lifestyle and habit. Studies have demonstrated that diet may play a significant row in prevention and treatment of some mood disorders (Chopra et al., 2021; Perez, 2018). As mentioned above, there has been preclinical evidence in animal studies demonstrating links between mood disorders and the gut, but some human evidence has also shown links between dietary supplementation, and reduced stress levels in students (Slykerman et al., 2022), consolidated by animal research suggesting that an exaggerated stress response in could be reversed by specific bacterial changes- e.g. probiotic use of a bifidobacterial species (Sakar et al., 2016).

The Gut-Brain Axis

Though perhaps intrinsically accepted in modern language, the concept of a gut brain axis (GBA) is still an emerging field. Briefly, the GBA can be explained by research showing that the brain can affect structure and function of the gut microbiota, and vice versa. This is a bidirectional mechanism and can act through modulation of gut motility and permeability, as well as hormone secretion (Carabotti et al., 2015).

Though there is much current exploratory research, potential mechanisms by which GBA may act include the vagus nerve, microbially derived metabolites, and hormone signalling, as well as being modified by the immune system (Navidinia et al., 2023).

Recent research shows that there is potential for gut mediated therapies to reduce or at least control symptoms of psychological disorders, such as PTSD (Bersani et al., 2020, Leclercq et al., 2016). The ability of the gut microbiota to influence biological states of an individual has led to an acknowledgement that research into the microbiome is an essential part of current and future healthcare strategies (Hadrich, 2018). Research has shown that the brain can affect structure and function of the gut microbiota through modulation of gut motility and permeability. It has also been shown that through this bidirectional mechanism, secretion of hormones may directly affect microbial gene expression (Martin et al., 2018). These interactions are thought to be a circular communications loop and any disturbance can result in dysregulation. One example of this is where secretion of hormones such as 5-HT from enterochromaffin cells may travel towards the gut lumen, potentially resulting in microbial alterations (Lund et al., 2018). This is likely a bidirectional relationship, whereby secondary bile acids and short chain fatty acids derived from the gut bacteria are responsible for regulation of enterochromaffin cell derived 5-HT synthesis (Mandić et al., 2019).

Hormones can affect microbial gene expression in other ways, such as in the case of increased virulence of *Pseudomonas aeruginosa*, by norepinephrine (Hegde et al., 2009). Though mechanisms for this are not fully understood, it is thought that direct affectation of norepinephrine on the virulence of bacteria is through enhancement of bacterial attachment to host tissue (Freestone, 2013).

Not only is IBS associated with general detriments to cognitive function, but research has directly linked the hyper-arousal and hyper-vigilant state of PTSD to IBS as a result of bidirectional signalling of the GBA (gut-brain axis) (Ng et al., 2019). Traditional diagnoses of PTSD rely on examination of behavioural symptoms (Spoont et al., 2010) but more recent evidence (as mentioned) has shown PTSD to be linked with immune system and inflammatory changes. IBS has been independently associated with PTSD (Iorio et al., 2014). One study showed that 36% of patients with IBS met behavioural and psychological criteria for PTSD diagnosis (Irwin et al., 1996). It has also been reported that, specifically in female veterans, there was an increase of IBS in those diagnosed with PTSD (Savas et al., 2009).

Psychiatric illness is highly debilitating to some and often one of the most dangerous aspects is risk of relapse (Moges et al., 2021). By taking a more holistic approach to the treatment of cognitive perturbations, such as exploring the potential for modulation of the gut microbiota to reduce symptoms or lessen these, recovery may be improved.

Through targeting the gut microbiota, it may be able to co-opt host-specific responses to foods, in terms of how quickly and effectively responses to functional foods or supplements can occur. This section reports a human intervention study carried out at University of Reading.

3.1.3 Microbially derived metabolites:

Short Chain Fatty Acids

One of the most abundant microbially derived metabolites are short chain fatty acids (SCFA). These are produced by the bacterial fermentation of dietary substrates. SCFAs are thought to affect the brain and body in several ways – as well as influence the gut-brain axis (Silva et al., 2020).

Primary SCFAs produced in the colon are acetate, propionate and butyrate – though others, like valerate, are also present (Den Besten et al., 2013). Lactate is often included in the same category as the previously mentioned SCFAs, however it is not a SCFA, rather, another microbially derived metabolite that potentially exerts benefit to the host through electron transfer (Sheridan et al., 2022).

There is a growing body of research suggesting and supporting the idea that manipulation of the gut

microbiota, and thereby its metabolites, can be a treatment target for neurological diseases - especially those that are highly associated with microbiota dysbiosis like depression, Parkinson's, etc (Zhu et al;., 2022; Kandpal et al., 2022).

Whilst SCFAs may improve the gut health locally, including maintaining the gut barrier and reducing inflammation, SCFAs also potentially are key players in the GBA – affecting it both directly and indirectly. SCFAs can regulate the secretion of interleukins, and thereby influence systemic inflammation (Silva et al., 2020; O'Riordan et al.,2022).

Research has shown that some SCFA can cross the blood-brain barrier (BBB), potentially triggering peripheral nervous system signalling, a very interesting concept considering that many mood associated disorders are associated with the dysregulation of the peripheral nervous system (Silva et al., 2020).

Another method by which SCFAs may affect the gut brain axis is through binding to G protein-coupled receptors – specifically free fatty acid receptor 2 and 3 (FFAR2 and FFAR3). It has been suggested that these receptors may be the predominant receptor type mediating protective benefits that SCFAs can provide (Mishra et al., 2020). Research has shown that both propionate and butyrate were able to prevent a decline in BBB function caused by endotoxin (LPS) exposure (Hoyles et al., 2018).

SCFAs also interact with enteroendocrine cells, through indirect production of gut hormones like GABA and serotonin. This can promote signalling to the brain, through vagal or systemic pathways (Silva et al., 2020).

Polyphenols

Polyphenols may have a variety of mechanisms in their effect on stress, including but not limited to the potential antioxidant MOA. Though previously thought to be solely due to direct antioxidant effects, beneficial modulation of both physical and cognitive health by polyphenols is now widely accepted to be due to interactions with the gut microbiota, with metabolites of these interactions providing beneficial effects throughout the host (Kennedy, 2014).

Polyphenols are found in all plants and are thought to be a crucial modulator of host health. Polyphenols provide benefits throughout the host system largely due to metabolites produced through gut microbial interactions. Some foods that have high polyphenol content include green tea, cocoa, blueberry and cranberry, coffee, cereals and some vegetables (Wang et al., 2022; Pandey et al.,2009). Blueberries, high in polyphenols, may reduce oxidative stress in athletes (Bowtell et al., 2019), as well as having beneficial preventative effects on metabolic disease, due to interactions with the gut microbiome (Rodríguez-Daza et al., 2020). Studies have demonstrated the impact of green tea extract on athletic performance, with beneficial impacts being linked to lessening of cumulative fatigue damage (Machado et al., 2018). Cranberry has been linked to both improving IBD, as well as modulating diversity of the gut microbiota (Cai et al., 2019). Finally, cocoa has been shown to influence cognition, and reduce cognitive decline (Mastroiacovo et al., 2015).

Prebiotics

A prebiotic is defined as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson et al., 2017). They stimulate bacterial growth, and thereby may have an effect on host health. There are many potential health benefits of taking prebiotics, some of which are improved metabolic, allergic and gastrointestinal health (Davani-Davari et al., 2019). There is also research linking immune activity with prebiotic effects (Pujari et al., 2021). Prebiotics can be found 'naturally' in food sources, but can also used as supplements.

3.1.4 Aim

A human volunteer study was undertaken to assess the effects of prebiotic and polyphenol intervention on gut health related to stress. The study was carried out in a double-blind, cross-over manner with healthy volunteers

3.2 Methods

Participant recruitment: Healthy adults between 18 and 65 years old were recruited from local area through poster advertising, through the University of reading internal email, and the Hugh Sinclair Volunteer Panel.

Screening of participants: All potential participants were emailed a study information document outlining the study protocol and inclusion/exclusion criteria to be read prior to study intake. Inclusion and exclusion criteria were as follows: Written consent was obtained from each person and selection will take place after determination of health status through a medical interview and adherence to the inclusion/exclusion criteria.

Inclusion criteria-

1. Volunteer is healthy at the time of pre-examination
2. Volunteer has high perceived stress levels (own self-assessment)
3. Volunteer is aged ≥ 18 to ≤ 65 years at the time of pre-examination
4. Volunteer is able and willing to comply with the study instructions
5. Volunteer is suitable for participation in the study according to investigator/study personnel
6. Written informed consent is given by volunteer

Exclusion criteria-

1. No command of any local language
2. Gastrointestinal disorders including IBS, IBD or other conditions that might affect the gut environment
3. Food allergies or intolerances
4. Using drugs (e.g. antibiotics) influencing gastrointestinal function (at least 8 weeks before intervention)

5. Use of laxatives
6. Participants with any form of diagnosed diabetes (types I and II)
7. Volunteers currently involved or will be involved in another clinical or food study
8. History of drug (pharmaceutical or recreational) or alcohol abuse.
9. If participants are pregnant or are lactating
10. Regular intake of probiotic or prebiotic supplements
11. Smoking

This study was given ethical approval by the The University of Reading Research Ethics Committee (UREC 21/19).

Study design: The trial was a two week, randomised, placebo controlled study, with measures being taken at day 0 and day 14/15. Supplementation (specifics below) was taken every day for 2 weeks.

Intervention: The intervention used was a mixture of prebiotics and polyphenols, as follows: 5g inulin, 5g galactooligosaccharides, 20g resistant starch. These dosages were chosen to align with the pre-decided and provided dosages from the corresponding study at Natik. Cocoa was at 200mg, 125mg wild blueberry, 323mg green tea, and 150mg cranberry. The polyphenols given were all extracts from wholefoods and pre-chosen in the concurrent study. The placebo was a maltodextrin placebo. As participants were only assigned to one group, it did not matter that the interventions differed visually from one another. Each participant was randomly assigned to a group, ensuring that even numbers in each group as far as possible. Wellbeing (PSS, GAD-7 and PHQ-9) questionnaires (Appendix A) were also used. A food diary was taken to ensure that probiotic or prebiotic containing products were not consumed on a regular basis throughout the study.

Procedure: This human trial was a randomised placebo controlled pilot study, enrolling 20 participants. Participants consumed either: a combination of prebiotics (inulin, RS2, GOS);

polyphenols (Cocoa, wild blueberry, cranberry, green tea); a combination of both prebiotics and polyphenols, or a maltodextrin control. Volunteers consumed these for two weeks and donated a urine and faecal sample at baseline and at 14/15 days. Stress was evaluated at baseline and at final sample collection using a the perceived stress questionnaire (appendix A)Various mood state questionnaires were taken, also at baseline and study completion (Appendix A). Blood pressure measurements were taken throughout by the participants themselves, in triplicate, and asked to be taken on day 0 and day 14 and at least 3 times per week.

Questionnaires:

The questionnaires were all self administered by participants, though they can be found in Appendix A, a brief description of these follows;

The Perceived Stress Scale (PSS-10) is a 10-item questionnaire originally developed by Cohen et al. (1983). It is a widely used questionnaire and is used to assess stress levels in adults, as well as in young people aged 12 and above. It is intended to evaluate the degree to which a user has perceived life as unprefictable, uncontrolloable and overloading over the previous month. The scoring is from 0-4 with reverse scoring for items that are positive. These are then totalled to give a total score, with scores between 0-13 indicating low perceived stress, 14-26 moderate perceived stress, and 27-40 indicating high perceived stress (40 being the maximum score).

Generalized Anxiety Disorder 7-item (GAD-7) is a seven-item instrument that is used to assess feelings of ancxiety). Each item asks the individual to rate the severity of his or her symptoms over the past two weeks. Response options include “not at all”, “several days”, “more than half the days” and “nearly every day”. Scores of 5, 10, and 15 are taken as the cut-off points for mild, moderate and severe anxiety, respectively.

Patient Health Questionnaire-9 (PHQ-9) is a self-administered instrument for common mental disorders. It scores each of the nine questions/criteria as "0" (not at all) to "3" (nearly every day).

PHQ-9 scores of 5, 10, 15, and 20 represented mild, moderate, moderately severe, and severe depression, respectively (Kroenke et al., 2001).

Baseline Characteristics

Gender	Age	Height	Weight	BMI
Female	26	163	52	20
Female	54	157	60	24
Female	64	153	70	30
Male	27	178	90	28
Female	19	174	70	23
Female	55	170	70	24
Female	63	165	61	22
Female	24	165	64	24
Female	28	162	62	24
Female	60	175	76	25
Male	28	181	79	24
Female	27	164	53	20
Male	33	180	93	29
Male	26	180	93	29
Female	24	156	49	20
Female	41	157	65	26
Female	51	167	59	21
Male	24	186	97	28
Female	27	160	60	23

Table illustrating the general baseline characteristics of the study population,

Samples: Faeces was taken for flow-FISH analysis of microbiota changes by thawing after -80 storage, and diluted to a 1:10 ratio. Labelled oligonucleotide probes were used to hybridise genus specific targets with fluorescent markers. Samples were screened using a BD Accuri™ C6 flow cytometer, measuring at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software.

75 µL of the sample was added to 500 µL 0. .1 mol l/1 anaerobically prepared PBS (pH 7.4) in an Eppendorf tube (1.5 mL), vortexed and centrifuged at 13000 × g for 3 min. The supernatant was

removed, and 100 µL Tris-EDTA buffer containing lysozyme added to the tube, mixed using a pipette and incubated in the dark for 10 minutes at room temperature. Samples were then vortexed and centrifuged for 3min at 13000xg. The supernatant was once again discarded and the pellet washed with 500 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4). This was then vortexed gently and centrifuged for 3 minutes at 13000xg. The supernatant was discarded a final time and the pellet resuspended in 150 µl of hybridisation buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium dodecyl sulphate, 30% formamide). Samples were vortexed and centrifuged at 13000xg for 3 minutes, and the supernatant discarded. The pellet was resuspended in 1ml hybridisation buffer.

Four µL (50 ng/μl^{-1}) of the selected oligonucleotide probe solutions (Table 1) was added to 50 µL of sample in Eppendorf tubes, vortexed and incubated at 36◦C overnight. After the incubation period, 125 µl of hybridisation buffer was added to each tube and they were subsequently vortexed, then centrifuged for 3 minutes at 13000rxg. Supernatants were removed and each pellet resuspended in 175 µl washing buffer solution (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate). This was then incubated for 20 minutes, covered, at 38°C in a heating block. Following this, samples were centrifuged for 3 minutes at 13000xg, and the supernatant discarded. 300 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) was added to each sample, and this was vortexed. Samples were held at 4 ◦C in the dark before flow cytometry was used. Bacteriology measurements were taken by a by a BD Accuri™ C6 flow cytometer, BD, Erembodegem, Brussels, and analysed used Accuri CFlow Sampler software.

Probes used were: Bif164 for *Bifidobacterium* spp, Lab158 for *Lactobacillus/Enterococcus*, Bac303 for *Bacteroides–Prevotella* group, Erec482 for *Eubacterium rectale–Clostridium coccoides* group, Rrec584 for *Roseburia–E. rectale* group, Ato291 for *Atopobium* cluster, Prop853 for clostridial cluster IX, Fprau 645 for *Faecalibacterium prausnitzii* spp, Dsv687 for *Desulfovibrio* genus and Chis 150 for most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II). Total bacteria were enumerated by use of the Eub 338 probe mix (Eub338‡, Eub338II‡, Eub338III‡), and Non-Eub was used as a negative control. Table 1 shows individual probe sequences.

Probe name	Sequence (5' to 3')	Target groups
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338
Eub338‡	GCTGCCTCCCGTAGGAGT	Most Bacteria
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and relatives
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)

Table 1: Oligonucleotide probe sequences.

16S rRNA partial gene sequencing:

DNA was extracted by using Magnetic Soil and Stool DNA Kit (TianGen, China, Catalog #: DP712). 16S rRNA/18SrRNA/ITS genes of distinct regions were amplified used specific primers. All PCR reactions were carried out with 15 μL of Phusion® High -Fidelity PCR Master Mix (New England Biolabs); 2 μM of forward and reverse primers, and 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98℃ for 1min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. PCR products were purified and sequencing libraries generated using NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs, USA, Catalog #: E7430L). The library was checked with Qubit and real-time PCR for quantification and bioanalyser for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required. For bioinformatics, paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Sequence Assembly:Paired-end reads were merged using FLASH (V1.2.11, http://ccb.jhu.edu/software/FLASH/) (Magoc T et al.,2011). Quality filtering on the raw tags was performed using the fastp (Version 0.23.1) software to obtain high-quality Clean Tags (Bokulich NA et al.,2012). Tags were compared with the reference database (Silva database (16S/18S),https://www.arb-silva.de/; Unite Database (ITS), https://unite.ut.ee/) using UCHIME Algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed (Edgar RC et al.,2011). Then, effective tags were finally obtained.

3.3 Results

Enumeration of bacteria with flow-FISH

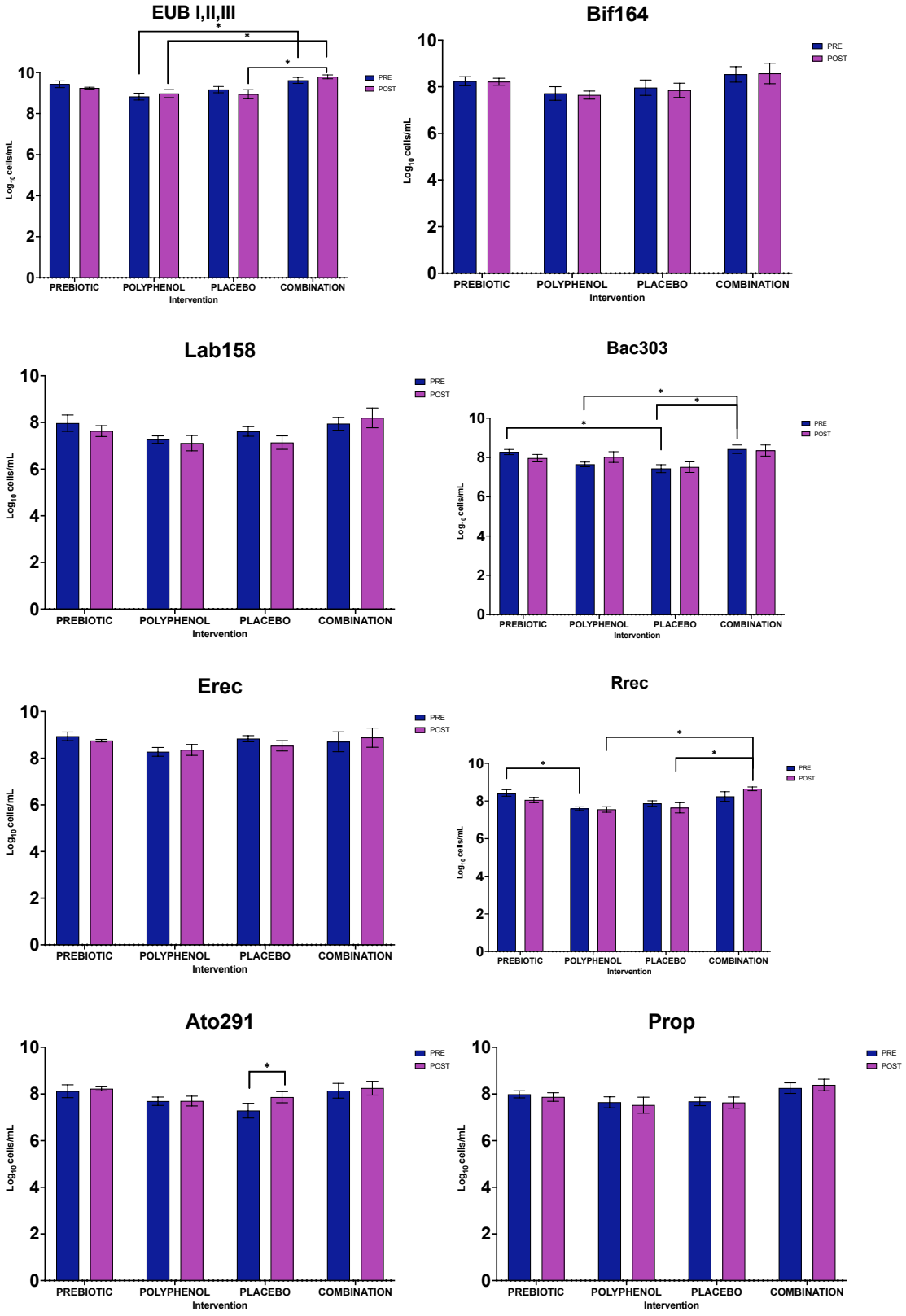
Figure 1. displays the differences in bacterial groups, pre and post intervention for each trial group, prebiotic, polyphenol, placebo and combination.

Significant differences were seen in the total bacteria (EUB I,II,III) – a significant increase was seen post-supplementation in the combination group when compared to the polyphenol ($p=0.011$), and when the placebo was compared to the combination – ($p=0.008$). a significant difference was also seen at baseline between the polyphenol group and the combination group($p=0.017$).

A significant difference at baseline between prebiotic and polyphenol group was seen for RREC ($p=0.26$), as well as a significant increase at the post supplementation time point from both the polyphenol group ($p=0.003$) and the placebo control ($p=0.006$)to the novel combination group. Ato showed significant changes within the placebo group ($p=0.38$)

Baseline significant differences between groups were seen in CHIS, and Bac, not as a result of any substrate intake. DSV decreased in all groups except the placebo.

Bifidobacteria was also the highest within the combination group, though not statistically significant – it was also increased post supplementation.


EUB I,II,III
Bif164
Lab158
Bac303
Erec
Rrec
Ato291
Prop
PRE
POST
Log10 cells/mL
PREBIOTIC
POLYPHENOL
PLACEBO
COMBINATION
Intervention

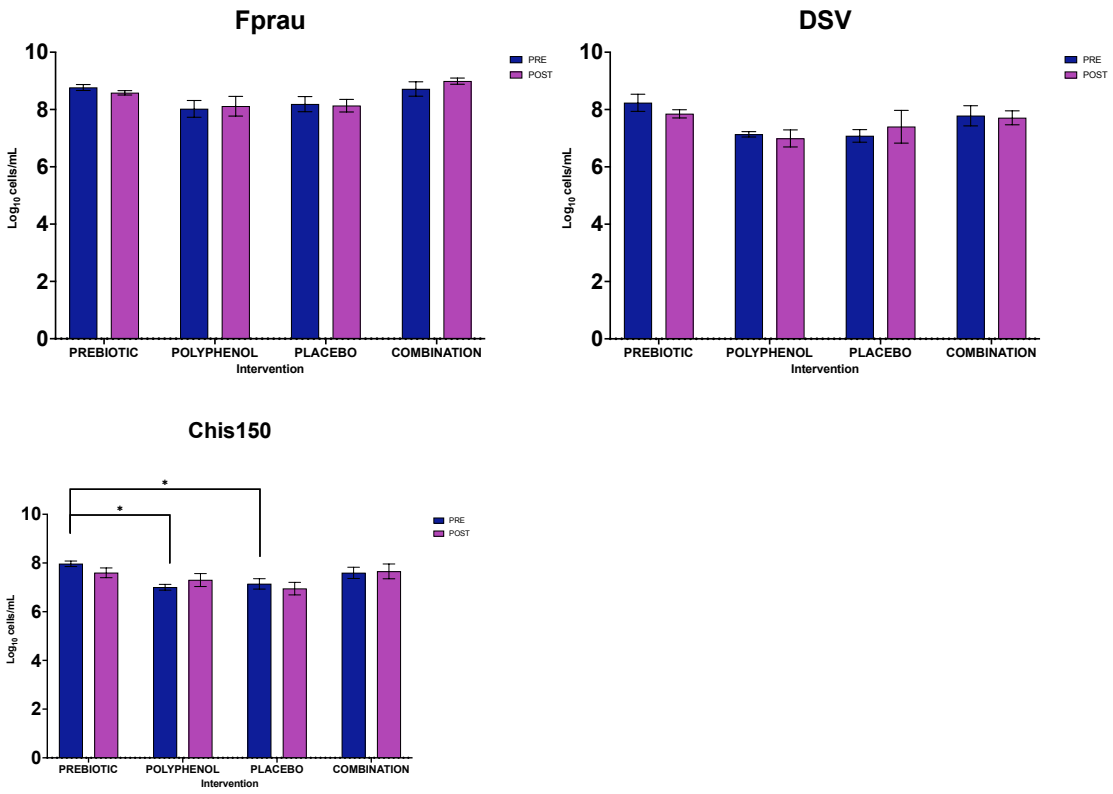


Fig. 1. Enumeration of bacteria in faecal sample by Flow-FISH at Pre and Post intervention. *Target bacteria: Bifidobacterium spp.*(BIF), *Lactobacillus* spp. (LAB), most Bacteroidaceae and Prevotellaceae (BAC), *Clostridium coccoides–Eubacterium rectale* group (EREC), *Roseburia* subcluster (RREC), *Faecalibacterium prausnitzii* (FPRAU), *Clostridium* cluster IX (PROP), *Atopobium- Coriobacterium* spp. (ATO), *Desulfovibrio* (DSV) and *Clostridium histolyticum* (CHIS). Values are presented as mean ± standard error. * denotes significant difference where $p < 0.05$. n=20

Qualitative analysis of mood states

Figure 2 (A, B and C) represent the results from various mood state questionnaires. A - the perceived stress scale (PSS) (full copy of this found in Appendix A(i)), B – GAD-7 (Appendix A(ii)) and C – PHQ-9 (Appendix A(iii)).

Overall, the scores decreased for all questionnaires and all groups. This may be a 'placebo effect' - as all participants were aware of the purpose of the study and that what they were taking had had proven effects in modifying mood states. As a note, whilst there is not evaluations of these speficic questionnaires in terms of susceptibility to the effects of a placebo, self-reporting measures are the

most strongly influenced by placebo (Hróbjartsson et al., 2011, Hodgins et al., 2018), including those measures for depression and anxiety. In order to counteract this and improve the efficacy of the primary aim of measuring stress, more comprehensive stress tests should be utlised, for example, measures of biomarkers such as cortisol, or even EEGs to indicate brainwave action (Crosswell et al., 2020).

In the PSS (Fig. 2 (A)) there was a significant difference only seen within the control group (pre and post supplementation – p=0.007).

For GAD-7, the indicator for anxiety moods, -both the control group and the combination group significantly decreased post supplementation – both p=0.006. For PHQ-9 – the prebiotics group showed a significant decrease post supplementation, p=0.023.

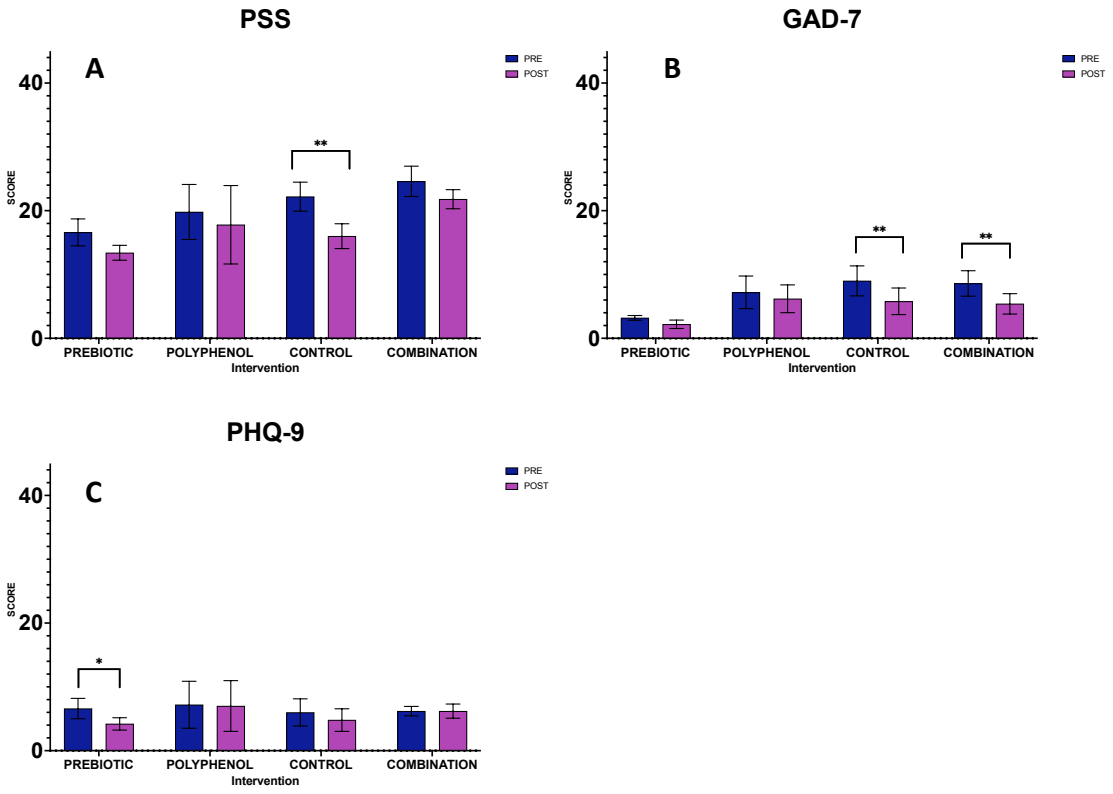


Fig 2. Analysis of mood states using the PSS(A), GAD-7 (B)and PHQ-9 (C) at pre and post intervention. Values are presented as mean ± standard error. * denotes significant difference where p <0.05. n=20

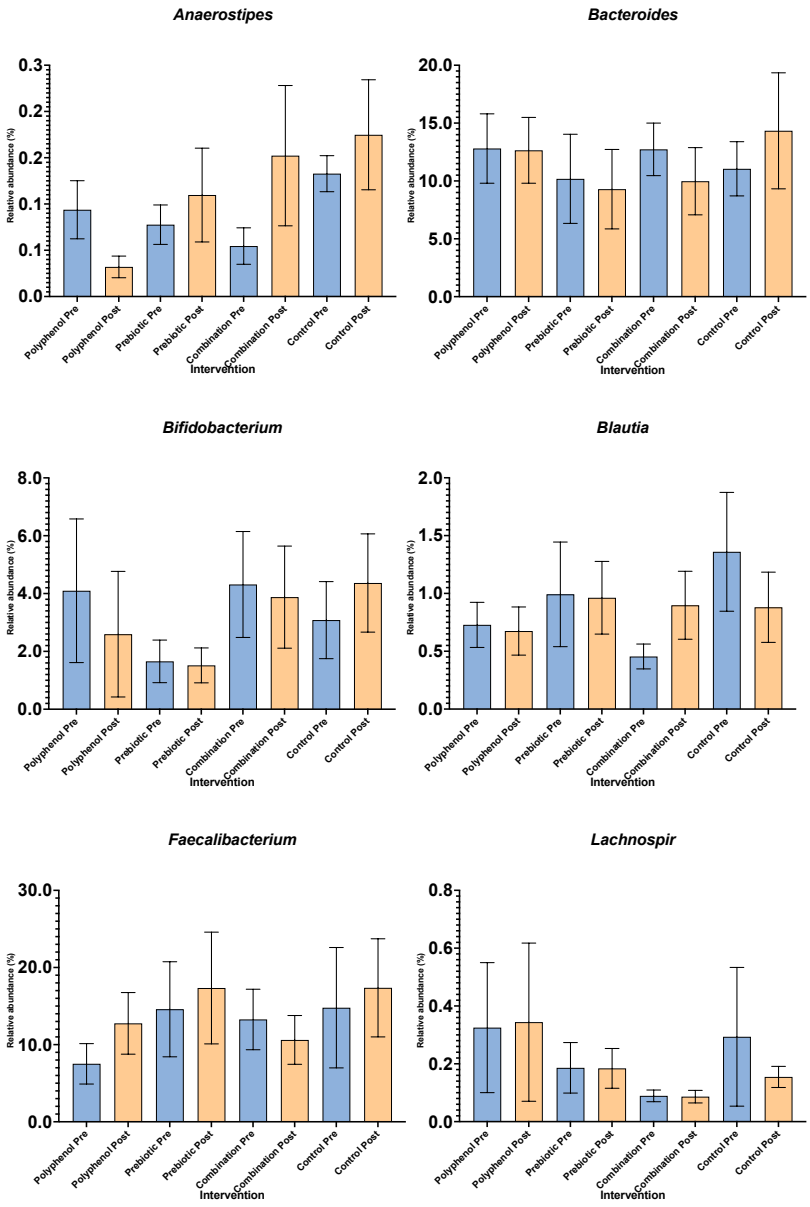
16S rRNA partial gene sequencing – relative abundance.

Figure 2 shows a selection of the primary bacterial groups explored through 16S rRNA partial gene sequencing. The relative abundance of these is displayed below. These were selected to align with the Flow-fish probes as best as possible.

Significant changes were seen within *Prevotella*, as well as *Lactobacillus*/enterococcus. *Prevotella* showed a significant difference from the prebiotic and the control groups at the Post supplementation sample ($p=0.043$). *Lactobacillus* showed a significant within substrate decrease from pre-post supplementation ($p=0.043$).

Interestingly, bifidobacteria decreased in relative abundance in all but the control group. This would suggest a growth of other bacterial groups stimulated by prebiotic and polyphenol supplementation

No significant differences were seen in any other group. There do not seem to be any specific trends within this analysis- most changes can be explained as transient changes, possibly skewed by the small sample size.


Anaerostipes
Relative abundance (%)
0.3
0.2
0.2
0.1
0.1
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention
Bacteroides
Relative abundance (%)
20.0
15.0
10.0
5.0
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention
Bifidobacterium
Relative abundance (%)
8.0
6.0
4.0
2.0
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention
Blautia
Relative abundance (%)
2.0
1.5
1.0
0.5
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention
Faecalibacterium
Relative abundance (%)
30.0
20.0
10.0
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention
Lachnospir
Relative abundance (%)
0.8
0.6
0.4
0.2
0.0
Polyphenol Pre
Polyphenol Post
Prebiotic Pre
Prebiotic Post
Combination Pre
Combination Post
Control Pre
Control Post
Intervention

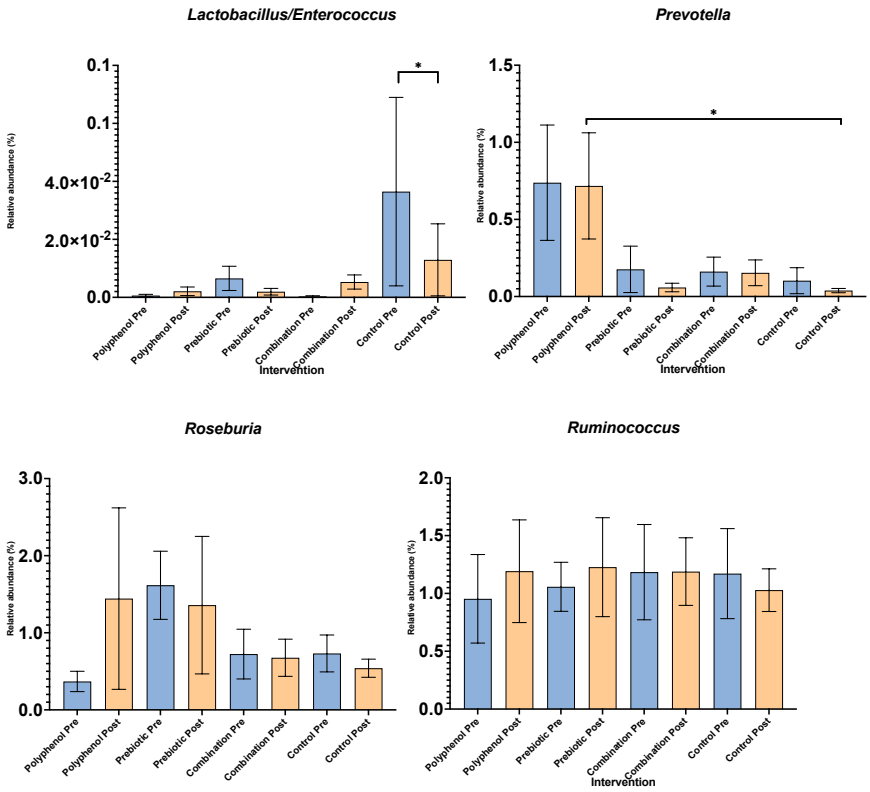


Fig 3 – Illustration of changes of relative abundance for roseburia, ruminococcus, prevotella, lactobacilli/enterococcus, fecalibacteria, lachnospiracea, bifidobacteria, blautia, *Bacteroides* and anaerostipes. Values are presented as mean ± standard error. * denotes significant difference where p <0.05. n=20

Blood Pressure Data

Systolic, diastolic and heart rate measurements were taken, at least three times per week, plus one at baseline and one on the final day, throughout the study. Systolic data is presented below. Missing values were calculated as an average of the result before and after. No significant data is was shown, calculated using an area under the curve measurement, followed by a comparative one way anova (using SPSS software).

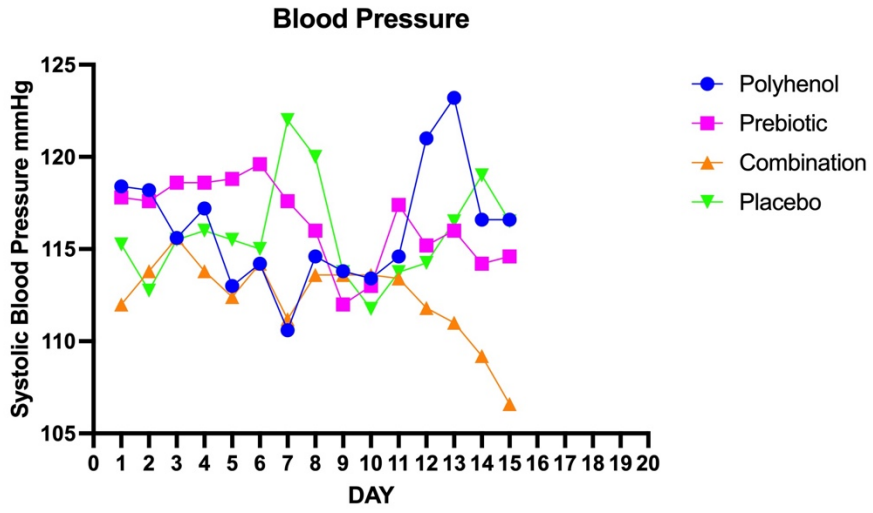


Fig. 4 – Illustration of data taking systolic blood pressure measurements over the course of a two week supplementation period. Values are presented as mean. No error bars included for clarity. n=20.

Fig 5.

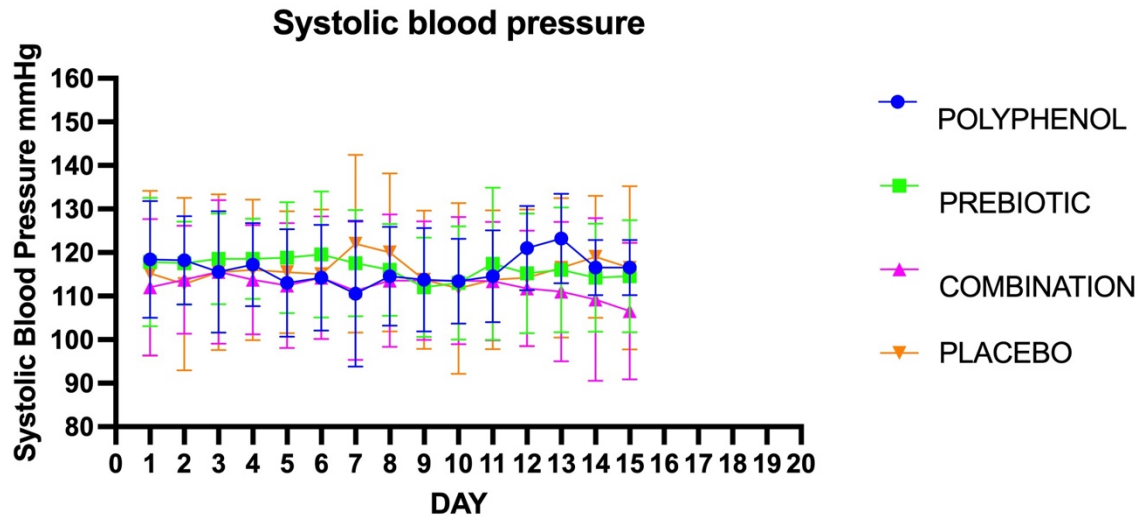


Fig. 5. Illustration of data taking systolic blood pressure measurements over the course of a two week supplementation period. Values are presented as mean ± standard error. n=20.

3.4 Discussion

The aim of this work was to explore whether a novel combination of prebiotics and polyphenols, administered a short chronic 2 week supplementation period could positively modify the gut microbiome, as well as reduce perceived stress levels and improve mood in healthy adults who feel

stressed. Outcome measures included changes in bacterial composition, as explored through Flow-FISH, as well as cognitive outcome measures including stress anxiety and depression. This was a placebo controlled study.

As this was a pilot study with a small cohort of participants, this discussion will look at both statistically significant data, and visual inspection of trends within the results.

The data presented above did not show any predictable significant changes within mood states, nor within the bacteriology. As this pilot study was low in participant numbers, this presents a particular challenge when interpreting the results. This does not mean, however, that there is no scope for this novel combination to be a putative nutraceutical to benefit gut and cognitive health, more that it is not significantly better than the sum of its parts, the prebiotics and polyphenols. This does not mean that this novel combination will not be of benefit. There is minimal research into supplementation of combined prebiotics and polyphenols, thus data from this study can be considered novel. One study investigated the effects of a polyphenol combination, similar to the one used in this study, and found that the consumption of these for two weeks did not significantly affect gut inflammation or diversity of the gut microbiome (Kung et al., 2020).

Polyphenols also have a putative prebiotic effect, with the low bioavailable compounds within polyphenols reaching the colon undigested or altered. One mechanism of action that polyphenols exert their effects is through bidirectionally modulating the intestinal microbes, thus the interest in polyphenols as a prebiotic, with much preclinical evidencing supporting this (Rodriguez-Daza et al., 2020; Plamada et al., 2021; Makarewicz et al., 2021). Again, this effect may be the reason that significant differences were not always seen between the polyphenols/prebiotics and the polyphenol combination, if both compounds were exerting similar outcomes and metabolite production.

Flow-FISH analysis showed only small changes when comparing pre and post intervention - Total bacteria showed a significant increase in the combination group post study, when compared to the polyphenols and the placebo control. Visual inspection of the data confirms that levels of total bac were also higher than the prebiotic group. The changes seen within the bacterial analysis, overall, are

less dramatic than expected when looking at the well researched correlation between inulin and modification of the gut microbiome (Wang et al., 2021).

The data also showed a significant difference at baseline between prebiotic and polyphenol group for RREC (*Roseburia*), as well as a significant increase at the post time point for the novel combination group. Interestingly, this occurred more so with this mixture of prebiotics and polyphenols compared to the separate components. *Roseburia* has been shown to have anti inflammatory properties, within the intestines, and is also associated with a potential reduction in neuroinflammation (Nie et al., 2021). The general consensus is that *Roseburia* may be considered a beneficial microbe. This is often attributed to the role of *Roseburia* in the production of beneficial microbes, such as butyrate, as discussed in Chapter 2 (Nie et al., 2021). Butyrate is associated with reduced inflammation, and has also been assicated with the amelioration of poor mood states, like depression (Suda et al., 2022). Some studies have also linked the intake of Roseburia with an improvement in positive mood (Hao et al., 2023). Further to this, much research has linked *Roseburia* and gastrointestinal issues, with reduced levels being associated with IBD (Nie et al.,2021).

Faecalibacteria also increased on the polyphenol and combination arms, as seen in Figure 1. This group is touted as a next generation probiotic owing to its anti-inflammatory properties (Martin et al., 2017). This aligns with research showing that the specific prebiotics used within this combination increase faecalibacteria (Park et al., 2022; Teichmann et al., 2021). As above, decreases in faecalibacteria are associated with gastrointestinal illness. The intake of both prebiotics and polyphenols has been associated with increases in *Fecalibacterium prausnitzii* (Wang et al., 2022), so this finding does align with current research. It is interesting that there was not an increase on the prebiotic arm, however there is much that can be explained through interindividual variation in response to prebiotic intake (Holmes et al., 2022).

Lactobacilli, another probiotic group, decreased on every group except from the novel combination. This is interesting, and warrants further exploration into the interactions between polyphenols and prebiotics when consumed in tandem. This finding is surprising, however, as research highly associates prebiotic intake with increases in lactobacilli (Hernández-Hernández et al., 2012). Dietary

polyphenols have also been shown to increase lactobacilli, with a meta-analysis demonstrating that polyphenol supplementation may increase *Lactobacillus* by 220% (Ma et al., 2020). However, what is interesting in regards to the novel combination increasing is that some research posits that there is a mutual relationship between polyphenols and lactobacilli – where not only polyphenols increases lactobacilli, but enzymatic action aids in the transformation of phenolic compounds (Piekarska-Radzik et al., 2021). Thus, the combination of prebiotics and polyphenols may be interacting with one another in order to increase *Lactobacillus* levels and exert positive systemic changes. Though usually in probiotic studies, an increase in lactobacilli is thought to alleviate stress levels and exert positive mood changes (Mindus et al., 2021; Johnson et al., 2021).

Bacteroides increased on the polyphenols but not any other tests. However, polyphenol intake has been associated with the increased of *Bacteroides*, so this was a fairly expected result (DOlara et al., 2005; Queipo-Ortuño. Et al., 2012). One interesting thing to note is that *Bacteroides* is often found to be reduced in those with chronic stress conditions (Gao et al., 2022). As such, the fact that polyphenols seemingly mitigate this is useful. Moreover, much evidence suggests that higher levels of *Bacteroides* are associated with higher amounts of GABA- likely to do with the presence of the glutamate decarboxylase (GAD)-encoding gene (Otaru et al., 2021). Again, polyphenols are also linked to increased GABA, thus making sense when considering the known action of polyphenol intake (Reba et al., 2020). It may, however, be expected that the novel combination would see the same effects as this. It may be that there was too much variation between donors to see overall significant changes.

Bifidobacteria, in the combination group, also increased post intervention in the combination group. This was likely due to the prebiotic component of the mixture (by comparing effects on the prebiotic and polyphenol arms), though polyphenols such as cocoa, have been linked to the increase of bifidobacteria also (Fogliano et al., 2011; Tzounis et al., 2011). Levels of bifidobacteria were also seen to be highest within this combination group. Bifidobacteria is associated with attenuated strsss levels and improved stress tolerance (Yoda et al., 2022), thus demonstrating that the novel combination may be suitable for the reduction in perceived stress levels.

A recognised pathogen desulfovibrio (Dsv) increased on placebo but this was not the case for the other interventions. This is a pro-inflammatory pathogenic bacterial group, associated with a compromised gut barrier integrity (Murros et al., 2021; Nie et al., 2023; Singh et al., 2022). Research has also shown that pro inflammatory microbes are enriched in individuals who develop Adverse post-traumatic neuropsychiatric sequelae – like PTSD (Zeamer et al., 2023). These data also correlate with the results section in the following chapter.

These data, together, show the potential for the novel combination to have more of a beneficial effect on the gut microbiome and resultant host health than either prebiotics or polyphenols alone.

Wellbeing questionnaires.

Scores decreased post intervention (visually) for all questionnaires and groups. The PSS demonstrated a significant difference between pre and post for the control group. Whilst no specific research has looked at a combination of prebiotics and polyphenols with this measure, some studies have shown that polyphenol intake could lower stress levels using PSS. When looking at the increase in beneficial bacteria within the group supplementing with the novel combination, it would have been expected to have a more stark response in mood states between pre and post for this group. Research has shown that even a moderate intake of polyphenols may promote a reduction in stress (Golmohammadi et al,.2023), thus potentially explaining reduction throughout the control groups as polyphenols were not excluded from the participants regular diet. Further exploration is needed to address the small study participant number.

GAD-7 showed both the control and the combination groups significantly decreased post supplementation. The control group showed significant changes, however with such a small participant group size it is to be expected that there would not be much difference between groups. When looking at the PHQ-9, the prebiotic group showed a significant decrease post supplementation. This improvement in mood state aligns with current research (Zhang et al., 2023).

The blood pressure data, as seen Figs 4 and 5 demonstrated that systolic blood pressures did seem to be lower within the combination groups - theoretically, - the novel combination may provide

favourable metabolic profiles. This aligns with research suggesting that intake of both prebiotics and polyphenols may reduce blood pressure (Godos et al., 2019; Yeo et al., 2009). As the same outcome was not seen for the individual components of the combination, it might be acceptable to hypothesise that the novel combination has a more pronounced effect on blood pressure than either alone. The data were too sporadic to use normal ANOVA statistical analyses – so an area under the curve model was used to ascertain differences in the groups, followed by a one way anova.

The primary limitation of this research was the small sample size. Though intended to be a pilot study, the study was highly affected by the covid pandemic, thus participant uptake was low. Not only does this limit the statistical power, but it also means that we cannot generalise the results, or assume that hey have an effect. The results may be highly skewed by a small subsection of the participants, and this sample is less likely to be representative of a population.

To conclude, the data presented above suggest that, throughout all measures bar wellbeing questionnaires, the combination group may prove more beneficial than either of the other supplementation groups, and when compared to the placebo. In order to further explore the mood states it may be beneficial to have a larger cohort. If this pilot study had further exploration with a larger participant group it may be evident that the novel combination provides an opportunity to reduce stress, improve mood, and improve GI health.

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Chapter 4 - Efficacy of a gut microbiota targeted nutritional intervention for combat soldiers during short term exposure to hypobaric hypoxia.

4.1 Introduction

Active military personnel are subject to extreme stressors, whether psychological or physical. This often results in soldiers having severe gastrointestinal diseases and cognitive perturbations such as Post Traumatic Stress Disorder (PTSD) (Armenta et al., 2018; Riddle et al., 2015). One of the physiological stressors affecting military personnel specifically is High Altitude (HA) stress. Whilst many populations exist within a HA environment, they are likely acclimatised and function at near normal capacity (Luo et al., 2014). For a warfighter, who will have to acclimatise fast and be expected to function at the top of their capacity in a new environment, adapting successfully to HA is a necessity.

HA environments have been shown to negatively affect wellbeing and neurological, physical and immune function of military personnel who have not yet acclimatised (TB MED 505).
Though some of these detriments are negated by slow acclimation, this is not an ideal approach for the combat lifestyle. Pharmaceutical treatments are available, they are not always the most viable option, either because of poor efficacy, side effects, availability or economic detriment. By exploring the potential of beneficial nutritional interventions, it may be possible to establish whether increased intake of certain nutraceuticals (such as polyphenols and prebiotics) can improve psychological and gut health in combat soldiers, and reduce the effects that PTSD and related gastrointestinal issues may have on health and wellbeing.

Acute mountain sickness is one of the symptoms seen when hypoxemia occurs, this can include gastrointestinal distress, like nausea, vomiting and diarrhoea (Clarke et al., 2006). As research shows, the gastrointestinal tract can have a major impact on neurological function (Mayer et al., 2022), it may be that by targeting GI symptoms with a nutrition based approach, we can potentially also alleviate neurological symptoms too.

Research in animals has demonstrated that at HA, the GI tract demonstrates inflammation and oxidative stress – leading to increased intestinal permeability (Zhang et al., 2015). Research by Karl et al showed a 71% increase in intestinal permeability at HA (unacclimatised) within a 36h ascent (Karl et al., 2018).

A ‘leaky gut’ is theorised to be involved in the pathogenesis of many diseases – including immune/autoimmune conditions and neurological disorders (Grochowska et al., 2019; Mu et al., 2017). Whether the gut influences the brain through co-opting the HPA axis (Carabotti et al., 2015), or through hormonal secretion (Sun et al., 2020), negative mood states have been associated with increased GI permeability (Madison et al., 2019). There is also a putative bidirectional relationship between stressors and gut permeability (Foster et al., 2017). Dietary interventions have been shown to potentially reverse intestinal permeability in certain negative mood states (Bear et al., 2020). It

therefore follows that there may be an association between GI tract damage at high altitude and resulting decrements to health at high altitude.

Thus, regardless of mechanism, the association provides an exciting opportunity to influence neurological and physical function within war fighters through nutritional interventions. Whilst there is much research exploring gut barrier function, there is not enough that specifically explores the effects of hypobaric hypoxia on intestinal permeability. Prebiotics have been shown to impact intestinal barrier function, having a stablilising effect. This can be through the enhanced production of SCFAs (Nogal et al., 2021), or through the modulation of intestinal tight junction (TJ) proteins, with studies demonstrating the upregulation of TJ genes after consumption of inulin (Uerlings et al., 2020).

Research has been able to link specific gut bacterial changes with increased GI permeability and inflammation in a specific military training exercise (Karl et al., 2017) Whilst not at HA, these changes are still relevant, as there were physiological stressors involved that the warfighter will undoubtably face, regardless of altitude. In this study, decreases in Bacteroidetes and increases in Firmicutes were seen – a ratio accepted to influence intestinal homeostasis. Increases in Firmicutes proportionally to Bacteroidetes has been associated with dysbiosis, as well as inflammation (Karl et al., 2017) . This study also was able to discover that those soldiers with a higher relative abundance of *Bifidobacterium* spp. and seemed to have less GI permeability during training. Lower butyrate concentrations have also been associated with intestinal permeability (Peng et al., 2007), and a decrease in lactobacilli was seen within this study – this are positive bacteria with potentially beneficial effects (Dempsey et al., 2022).

Certain nutraceuticals that modulate the human GI tract may be able to modify the response to HA in warfighters. Though probiotics are of course a suitable option for delivering beneficial microbes to the host, even improving the gut barrier (Kocot et al., 2022), this study proposes that a more efficient approach would be to use a prebiotic based intervention to promote the growth of positive bacteria. Polyphenols will also be included in this, as not only have they been shown to improve gut barrier

integrity (Gil-cardoso et al., 2016; Aires et al., 2019), but they also have potential anti-inflammatory properties (Truong et al., 2022). They are also thought to have a putative prebiotic effect (Alves-Santos et al., 2020). Though the idea of polyphenols being 'antioxidants' is thought to be out of date (Croft et al., 2016), they certainly do seem to present with some anti-oxidative effects, again which may be beneficial to the host, even in reduction of cognitive decline (Naomi et al., 2023).

In this study, three prebiotic sources were used: Inulin, GOS and resistant starch (RS2). Inulin has been shown to increase beneficial bacteria such as *Bifidobacterium*, *Lactobacillus* and *F. prausnitzii*, and to improve gut barrier integrity (Dewulf et al., 2013; SO et al.,2018; Uerlings et al., 2020). GOS has also been shown to reduce colonic permeability (Gao et al., 2021), as well as potentially improving indices of pre-clinical anxiety (Johnstone et al., 2021). RS2 has been associated with increased butyrate, a health promoting short chain fatty acid. The doses given were: Inulin (5 g/d); GOS (5 g/d); RS2 (20 g/d). These doses were decided on based on previous research, and decided in Natik.

The polyphenols sources chosen were cocoa, green tea, blueberry and cranberry, selected not only for their putative prebiotic effect, with certain studies demonstrating increased lactobacilli, bifidobacteria and decreasing pathogenic bacteria (Tzounis et al., 2011), but also (in some animal studies) they reduced GI inflammation (Romier et al., 2009). Selected polyphenol sources have also evidenced improved memory, cognitive performance, as well as reduced stress levels (Naomi et al., 2023; Carillo et al., 2019; de Vries et al., 2022; Micek et al., 2023)). 500mg/day of each polyphenol was given. These polyphenol sources were selected to provide a broad representation of the myriad different polyphenol families found in plant-based foods, and at an intake level consistent with the upper end of dietary intakes.

This trial did not take place within the University of Reading but at the USA Army Research Station in Natick. It was a randomised, double-blind, placebo-controlled crossover clinical trial consisting of

three 2-week experimental periods each separated by a 1-week washout from the diet intervention (3-week washout for the altitude exposure). The following conditions were examined:

1) placebo + sham HA (PL+SHAM); (low altitude conditions)

2) placebo + HA (PL+HA); (high altitude)

3) fiber & polyphenol supplementation + HA (FP+HA) (high altitude)

High altitude was created by the use of a hypobaric chamber. During one phase the chamber environment will mimic low-altitude conditions (SHAM). During two phases the chamber environment mimiced the barometric pressure of 460 mmHg; to create the high altitude.

Participant numbers were as follows: Intention-to-treat cohort (n = 22) // Complete case cohort (n = 6).

Please see the figure below for intended study participation throughout each phase.

PPT	Phases completed	Sequence Order (A|B|C)	PL + LA	FP+HA	PL + HA
1	ABC	(PL+LA|FP+HA|PL+HA)	X	X	X
2	ABC	(PL+LA|FP+HA|PL+HA)	X	X	X
3	AC	(PL+LA|FP+HA|PL+HA)	X		X
4	ABC	(PL+LA|FP+HA|PL+HA)	X	X	X
5	A	(PL+HA|PL+LA|FP+HA)			X
6	A	(PL+HA|PL+LA|FP+HA)			X
7	A	(PL+HA|PL+LA|FP+HA)			X
8	A	(PL+HA|PL+LA|FP+HA)			X
9	none	(FP+HA|PL+HA|PL+LA)			
10	none	(FP+HA|PL+HA|PL+LA)			
11	none	(FP+HA|PL+HA|PL+LA)			
12	none	(FP+HA|PL+HA|PL+LA)			
13	A	(PL+HA|PL+LA|FP+HA)		X	
14	A	(PL+HA|PL+LA|FP+HA)		X	
15	A	(FP+HA|PL+HA|PL+LA)		X	
16	A	(FP+HA|PL+HA|PL+LA)		X	
17	A	(FP+HA|PL+HA|PL+LA)		X	
18	none	(FP+HA|PL+HA|PL+LA)			
19	AB (1d only of B)	(FP+HA|PL+HA|PL+LA)		X	X
20	none	(FP+HA|PL+HA|PL+LA)			
21	none	(FP+HA|PL+HA|PL+LA)			
22	ABC	(FP+HA|PL+HA|PL+LA)	X	X	X
23	ABC (1d only of B)	(PL+LA|FP+HA|PL+HA)	X	X	X
24	ABC (1d only of B)	(PL+LA|FP+HA|PL+HA)	X	X	X
25	ABC	(PL+LA|FP+HA|PL+HA)	X	X	X
26	ABC	(PL+LA|FP+HA|PL+HA)	X	X	X
27	A	(PL+HA|PL+LA|FP+HA)			
28	ABC (1d only of C)	(PL+HA|PL+LA|FP+HA)	X	X	X
29	A	(PL+HA|PL+LA|FP+HA)			

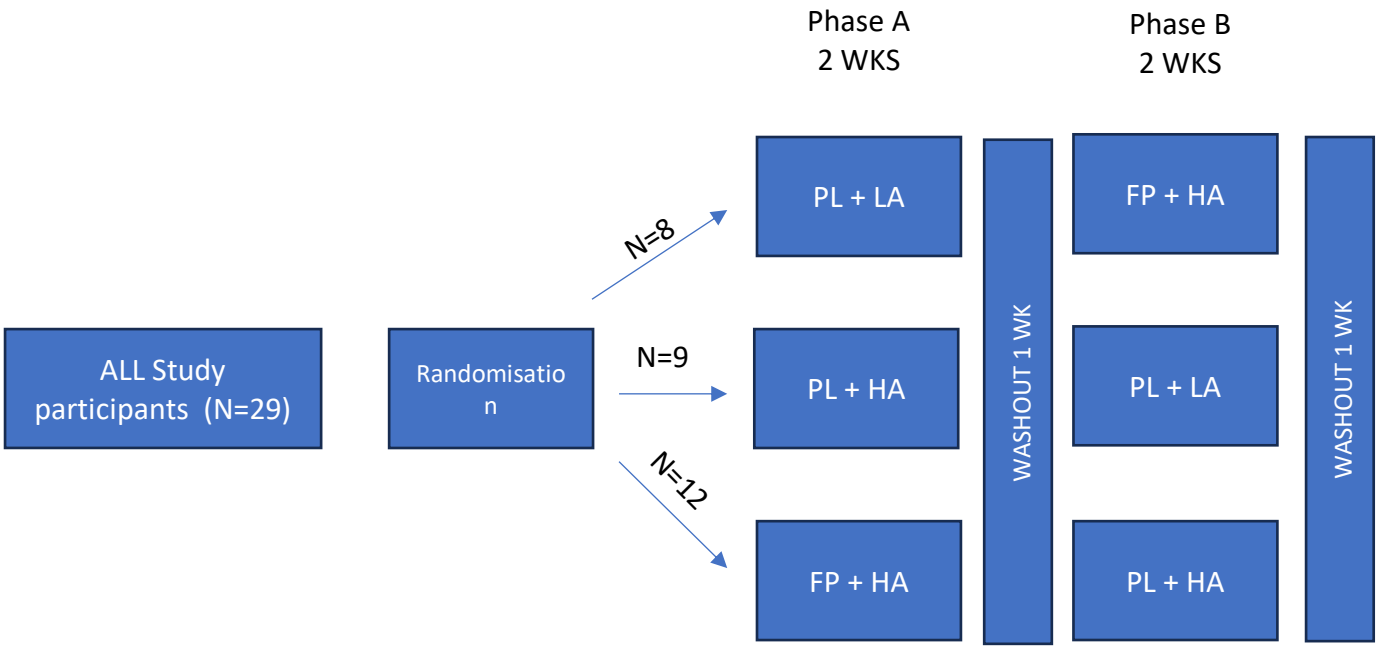

Figure 1. Illustrating the completion of each phase by study participants. Each phase, A, B or C is a week study phase separated by ≥1 week w ashout. 6 participants completed the study (complete case cohort), the remaining 22 were included in the intention to treat cohort.

This study did not meet it's required enrolment for a power calculation, however, please see this below:

Power analyses for primary outcomes were performed using GLIMMPSE v.2.2.7 (http://glimmpse.samplesizeshop.org/#/) and using data obtained during USARIEM protocol 16-02HC. The primary outcome of this study is intestinal permeability. It was previously documented that a ~60% increase in small intestinal permeability within 36hr of ascent to HA (4300m) with mean ± SD lactulose:mannitol ratio increasing from 0.0085 ± 0.0041 at sea level to 0.0135 ± 0.0060 at HA (Karl et al., 2018). It was also reported that an increase in intestinal permeability of this magnitude coincides with increased inflammation (Karl et al., 2018) and gastrointestinal discomfort (Karl et al., 2018). Using these means, the more conservative SD of 0.006, and a 1-factor (phase) repeated measures ANOVA design, n=15 would provide 82% power to detect a main effect of study phase with a medium effect size for the FP treatment (mean = 0.0105 ± 0.0060) at α=0.05. Previous studies have reported larger effect sizes when examining the effects of nutrient supplementation on intestinal permeability during exercise (Davison et al., 2016)

4.4 Methods

Faecal, spot urine and serum samples were collected in this trial. Faecal samples were taken at the following time points: Baseline (pre-supplementation); Week 2, day 5 (pre-chamber residence); Week 2, day 7 (chamber residence); and Week 3, day 2 (washout). One urine sample was taken per treatment (Low altitude, HA and placebo, HA and supplement). Serum was taken on Day 6 – the 1st morning of chamber residence (fasted sample) and Day 7 – the 2nd morning of chamber residence (fasted sample).

Supplementation doses were as follows:

Inulin (5 g/d); GOS (5 g/d); RS2 (20 g/d), Cocoa – 500mg/day, Cranberry 500mg/day, Green tea 500mg/day and Green Tea, 500mg/day. These were all combined within a first-strike ration bar,

Faeces was taken for flow-FISH analysis of microbiota changes by thawing after -80 storage, and faecal sample was placed in a 10 mL falcon tube with glass beads (3mm) with phosphate buffered saline (0.1 mol l/1 anaerobically prepared PBS (pH 7.4)) with appropriate volume to dilute to a 1:10 ratio. Labelled oligonucleotide probes were used to hybridise genus specific targets with fluorescent markers. Samples were screened using a BD Accuri™ C6 flow cytometer, measuring at 488 nm and 640 nm and analysed using Accuri CFlow Sampler software.

75 µL of the sample was added to 500 µL 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) in an Eppendorf tube (1.5 mL), vortexed and centrifuged at 13000 × g for 3 min. The supernatant was removed, and 100 µL Tris-EDTA buffer containing lysozyme added to the tube, mixed using a pipette and incubated in the dark for 10 minutes at room temperature. Samples were then vortexed and centrifuged for 3min at 13000xg. The supernatant was once again discarded, and the pellet washed with 500 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4). This was then vortexed gently and centrifuged for 3 minutes at 13000xg.The supernatant was discarded a final time and the pellet resuspended in 150 µl of hybridisation buffer (0.9 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.01% sodium dodecyl sulphate, 30% formamide). Samples were vortexed and centrifuged at 13000xg for 3 minutes, and the supernatant discarded. The pellet was resuspended in 1ml hybridisation buffer.

Four µL(50 ng/µl^{-1}) of the selected oligonucleotide probe solutions (Table 1) was added to 50 µL of sample in Eppendorf tubes, vortexed and incubated at 36◦C overnight. After the incubation period, 125 µl of hybridisation buffer was added to each tube and they were subsequently vortexed, then centrifuged for 3 minutes at 13000rpm. Supernatants were removed and the each pellet resuspended in 175 µl washing buffer solution (0.064 M NaCl, 0.02 M Tris/HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 0.01% sodium dodecyl sulphate). This was then incubated for 20 minutes, covered, at 38°C in a

heating block. Following this, samples were centrifuged for 3 minutes at 13000xg, and the supernatant discarded. 300 µl of 0.1 mol l/1 anaerobically prepared PBS (pH 7.4) was added to each sample, and this was vortexed. Samples were held at 4 ◦C in the dark before flow cytometry was used. Bacteriology measurements were taken by a by a BD Accuri™ C6 flow cytometer, BD, Erembodegem, Brussels, and analysed used Accuri CFlow Sampler software.

Probes used were: Bif164 for *Bifidobacterium* spp, Lab158 for *Lactobacillus/Enterococcus*, Bac303 for *Bacteroides–Prevotella* group, Erec482 for *Eubacterium rectale–Clostridium coccoides* group, Rrec584 for *Roseburia–E. rectale* group, Ato291 for *Atopobium* cluster, Prop853 for clostridial cluster IX, Fprau 645 for *Faecalibacterium prausnitzii* spp, Dsv687 for *Desulfovibrio* genus and Chis 150 for most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II). Total bacteria were enumerated by use of the Eub 338 probe mix (Eub338‡, Eub338II‡, Eub338III‡), and Non-Eub was used as a negative control. Table 1 shows individual probe sequences.

Probe name	Sequence (5’ to 3’)	Target groups
Non Eub	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338
Eub338‡	GCTGCCTCCCGTAGGAGT	Most Bacteria
Eub338II‡	GCAGCCACCCGTAGGTGT	Planctomycetales
Eub338III‡	GCTGCCACCCGTAGGTGT	Verrucomicrobiales
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*
Bac303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae
Erec482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)
Rrec584	TCAGACTTGCCGYACCGC	*Roseburia* genus
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster
Prop853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX
Fprau655	CGCCTACCTCTGCACTAC	Feacalibacterium prausnitzii and relatives
DSV687	TACGGATTTCACTCCT	*Desulfovibrio* genus
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II)

Table 1: Oligonucleotide probe sequences.

Nuclear magnetic resonance spectroscopy:

Urine samples were thawed, and a phosphate buffer (pH 7·4 sodium phosphate with 0.2 mol/L disodium phosphate (Na*2*HPO*4*), 0.04 mol/L monosodium phosphate (NaH*2*PO*4*) in deuterium oxide (99·9 %) was prepared, with 1 mmol/L 3-(trimethylsilyl) propionic acid-d*4* sodium salt (TSP) and 3 mmol/L sodium azide in the solution. 400 µL of each urine sample was mixed with 200 µL buffer. 550 µL of supernatant were aliquoted into 5 mm NMR tubes.

Plasma samples were thawed and inverted prior to sample preparation. 250ul of plasma was aliquoted into an Eppendorf tube. These Eppendorf's were then spin for 10 minutes at 1300xg. A saline extender ((20ml D2O; 80 mml H2O; 0.9g NaCL; 0.02g 99% sodium azide) was then added to a new Eppendorf, and 200ul of the centrifuged plasma was added to this. This was then vortexed for 10 seconds, and 500ul of the prepared samples was added into the 5mm NMR tube.

*1*H-NMR spectroscopy analysis was carried out using a Bruker DRX 500 MHz NMR spectrometer (Bruker Biospin, Germany). The spectrometer was operated at 500.13 MHz. Urine water spectra were acquired using a standard 1D pulse sequence [recycle delay (RD)-90◦-t1-90◦-Tm-90◦-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time (Tm) of 100 ms and a 90*o* pulse set at 7.70 µs. Per spectrum, a total of 128 scans were carried out with a spectral width of 14.0019 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. Acquired spectroscopic data were processed using the TopSpin 3.6.5 software package (Bruker Biospin, Rheinstetten, Germany)

4.3 Results

Results from flow cytometry, seen in Table 2, showed little change in either cohort. This results section will primarily focus on the intention to treat cohort as it is more representative given the comparative cohort numbers. Statistically significant data are indicated in detail on the table above. As the cohorts were small, visual inspection of the data will be described here. Total bacterial

populations showed little change between baseline and pre-chamber (high altitude) entry but controls decreased after 2 weeks. During high altitude exposure, these populations were decreased to levels more reflective of low altitude, with similar trends at washout. Bifidobacterial populations increased on the intervention prior (compared to placebo at both low and high altitude) to high altitude confirming its prebiotic effect. These then declined during chamber residence but were still higher with the intervention compared to placebo at either high or low altitude - suggesting an amelioration of the effects of high altitude by the supplementation. This represents a very promising result. Another probiotic genus lactobacilli, showed little change between baseline and week 2 day 5 (pre chamber entrance). High altitude decreased numbers and this was also the case for the intervention – showing that this arm of the study preferred fermentation by bifidobacteria. This was also the case for *Bacteroides*; erec (eubacteria), rrec (*Roseburia*), Ato (*Atopobium*), Pro (mainly propionibacteria), Fprau (*Faecalibacterium prausnitzii* and relatives), Dsv (sulphate-reducing bacteria) and Chis (clostridia).

Lower levels of both DSV and CHIS were seen within the chamber residence when supplemented, when compared to both placebo and low altitude. This is a promising result as often pathogenic bacteria are associated with high altitude exposure, or rather associated with acute mountain sickness. However, this was not selective as commensal populations showed similar effects. That is, for most populations investigated, a similar effect occurred in that high altitude decreased numbers of bacteria up to the 5 day assessment but this was repressed by the intervention after 7 days.

Bacterial group (log_{10} cells/g feces wet wt.)	Intention-to-treat cohort (n = 22)[2]						Complete case cohort (n = 6)[2]					
	PL+LA	PL+HA	FP+HA	P-condition	P-time	P-interaction	PL+LA	PL+HA	FP+HA	P-condition	P-time	P-interaction
Total bacteria (Eub338)	A	B	B	**0.01**	**0.07**	0.71	A	B	A,B	**0.03**	**0.10**	0.76
Baseline (pre-supplementation)	9.82 ± 0.37	9.75 ± 0.40	9.97 ± 0.28		A		9.86 ± 0.23	9.91 ± 0.38	9.94 ± 0.29			
Week 2, day 5 (pre-chamber residence)	9.56 ± 0.50	9.78 ± 0.39	9.96 ± 0.40		A,B		9.50 ± 0.56	9.63 ± 0.37	9.89 ± 0.44			
Week 2, day 7 (chamber residence)	9.56 ± 0.74	9.71 ± 0.32	9.72 ± 0.35		B		9.47 ± 0.89	9.86 ± 0.20	9.64 ± 0.35			
Week 3, day 2 (washout)	9.44 ± 0.84	9.66 ± 0.48	9.80 ± 0.49		B		9.91 ± 0.22	9.98 ± 0.45	9.86 ± 0.61			
Bifidobacterium spp. (Bif164)	A	A,B	B	**0.004**	**0.07**	0.82	A	B	B	**0.04**	0.92	0.62
Baseline (pre-supplementation)	8.58 ± 0.60	8.55 ± 0.59	8.77 ± 0.51		A,B		8.54 ± 0.55	8.86 ± 0.53	8.91 ± 0.38			
Week 2, day 5 (pre-chamber residence)	8.62 ± 0.64	8.73 ± 0.54	9.16 ± 0.51		A		8.59 ± 0.79	8.65 ± 0.61	9.29 ± 0.48			
Week 2, day 7 (chamber residence)	8.73 ± 0.88	8.60 ± 0.58	8.86 ± 0.75		A,B		8.63 ± 1.04	8.69 ± 0.64	8.87 ± 0.61			
Week 3, day 2 (washout)	8.46 ± 0.80	8.45 ± 0.60	8.75 ± 0.61		B		8.85 ± 0.53	8.82 ± 0.58	8.78 ± 0.67			
Lactobacillus-Enterococcus group (Lab158)	A	B	B	**0.01**	**0.02**	0.35	A	B	A,B	**0.07**	**0.005**	0.68
Baseline (pre-supplementation)	7.96 ± 0.51	8.19 ± 0.45	8.19 ± 0.45		A		7.93 ± 0.43	8.21 ± 0.47	8.11 ± 0.43		A,B	
Week 2, day 5 (pre-chamber residence)	7.49 ± 0.56	8.16 ± 0.56	8.16 ± 0.56		A,B		7.43 ± 0.44	7.74 ± 0.52	8.08 ± 0.63		B,C	
Week 2, day 7 (chamber residence)	7.56 ± 0.96	7.69 ± 0.63	7.69 ± 0.63		B		7.32 ± 1.10	7.76 ± 0.51	7.49 ± 0.56		C	
Week 3, day 2 (washout)	7.65 ± 0.76	7.95 ± 0.57	7.95 ± 0.57		A,B		8.10 ± 0.18	8.21 ± 0.59	8.02 ± 0.66		A	
Bacteroides-Prevotella group (Bac303)	A	B	B	**0.002**	0.50	0.87	A	B	A,B	**0.03**	**0.02**	0.55
Baseline (pre-supplementation)	8.12 ± 0.58	8.29 ± 0.46	8.36 ± 0.54				8.11 ± 0.52	8.29 ± 0.50	8.34 ± 0.36		A,B	
Week 2, day 5 (pre-chamber residence)	7.91 ± 0.53	8.32 ± 0.39	8.42 ± 0.45				7.66 ± 0.50	8.16 ± 0.21	8.33 ± 0.49		A	
Week 2, day 7 (chamber residence)	7.95 ± 0.70	8.25 ± 0.45	8.09 ± 0.31				7.83 ± 0.81	8.31 ± 0.36	7.96 ± 0.30		A	
Week 3, day 2 (washout)	8.02 ± 0.82	8.38 ± 0.38	8.34 ± 0.44				8.46 ± 0.15	8.50 ± 0.38	8.48 ± 0.53		B	
Eubacterium rectale-Clostridium coccoides group (Erec482)	A	B	A,B	**0.03**	**0.02**	0.76				0.14	**0.03**	0.77
Baseline (pre-supplementation)	9.37 ± 0.42	9.28 ± 0.44	9.48 ± 0.33		A		9.36 ± 0.38	9.44 ± 0.40	9.39 ± 0.35		A	
Week 2, day 5 (pre-chamber residence)	9.03 ± 0.53	9.27 ± 0.48	9.35 ± 0.42		A,B		8.94 ± 0.52	8.99 ± 0.51	9.25 ± 0.45		B	
Week 2, day 7 (chamber residence)	8.93 ± 0.72	9.13 ± 0.33	9.10 ± 0.33		B		8.89 ± 0.74	9.26 ± 0.19	9.03 ± 0.35		B	

Week 3, day 2 (washout)	8.93 ± 0.91	9.16 ± 0.50	9.25 ± 0.57		B		9.45 ± 0.25	9.38 ± 0.46	9.34 ± 0.68		A	
Roseburia spp. (Rrec584)	A	B	B	**0.01**	0.18	0.74	A	B	B	**0.02**	0.44	0.55
Baseline (pre-supplementation)	8.32 ± 0.70	8.25 ± 0.62	8.42 ± 0.38				8.26 ± 0.59	8.39 ± 0.70	8.28 ± 0.26			
Week 2, day 5 (pre-chamber residence)	8.04 ± 0.61	8.38 ± 0.47	8.55 ± 0.52				7.96 ± 0.55	8.10 ± 0.27	8.53 ± 0.63			
Week 2, day 7 (chamber residence)	7.94 ± 0.90	8.13 ± 0.45	8.19 ± 0.53				7.81 ± 1.03	8.39 ± 0.33	8.26 ± 0.64			
Week 3, day 2 (washout)	7.92 ± 1.06	8.23 ± 0.59	8.37 ± 0.69				8.47 ± 0.39	8.25 ± 0.39	8.60 ± 0.74			
Atopium cluster (Ato291)	A	A,B	B	**0.09**	**0.03**	0.33				0.23	0.52	0.54
Baseline (pre-supplementation)	8.08 ± 0.50	7.71 ± 0.83	8.11 ± 0.69		A		8.27 ± 0.36	8.00 ± 0.90	8.24 ± 0.84			
Week 2, day 5 (pre-chamber residence)	7.83 ± 0.82	7.90 ± 0.65	8.24 ± 0.69		A		7.98 ± 0.86	8.11 ± 0.46	8.45 ± 0.54			
Week 2, day 7 (chamber residence)	8.00 ± 1.04	7.75 ± 0.86	7.84 ± 0.85		A,B		8.02 ± 1.29	8.14 ± 0.79	8.05 ± 0.64			
Week 3, day 2 (washout)	7.70 ± 0.90	7.69 ± 0.84	7.82 ± 0.72		B		8.10 ± 0.64	8.42 ± 0.67	8.02 ± 0.65			
Clostridial cluster IX (Prop853)	A	A,B	B	**0.04**	0.18	0.57	A	B	A,B	**0.06**	**0.04**	0.95
Baseline (pre-supplementation)	8.21 ± 0.45	8.08 ± 0.64	8.33 ± 0.43				8.18 ± 0.28	8.21 ± 0.61	8.20 ± 0.36		A,B	
Week 2, day 5 (pre-chamber residence)	7.87 ± 0.73	8.13 ± 0.41	8.29 ± 0.66				7.89 ± 0.70	8.00 ± 0.42	8.26 ± .73		A	
Week 2, day 7 (chamber residence)	7.96 ± 0.82	7.97 ± 0.43	7.99 ± 0.73				7.90 ± 1.04	8.01 ± 0.48	8.02 ± 0.73		A	
Week 3, day 2 (washout)	7.87 ± 0.89	8.02 ± 0.73	8.35 ± 0.55				8.39 ± 0.22	8.52 ± 0.67	8.34 ± 0.68		B	
Faecalibacterium prausnitzii (Fprau655)	A	B	B	**0.01**	0.24	0.40				0.15	**0.04**	0.63
Baseline (pre-supplementation)	8.94 ± 0.47	8.86 ± 0.53	8.95 ± 0.43				8.91 ± 0.39	9.00 ± 0.42	8.80 ± 0.39		A,B	
Week 2, day 5 (pre-chamber residence)	8.49 ± 0.66	8.89 ± 0.50	9.06 ± 0.53				8.40 ± 0.62	8.57 ± 0.55	8.85 ± 0.46		A	
Week 2, day 7 (chamber residence)	8.47 ± 0.79	8.80 ± 0.38	8.78 ± 0.38				8.39 ± 0.82	9.00 ± 0.33	8.66 ± 0.22		A	
Week 3, day 2 (washout)	8.52 ± 0.79	8.87 ± 0.44	9.00 ± 0.67				8.98 ± 0.25	9.07 ± 0.41	9.12 ± 0.82		B	
Desulfovibrio spp. (Dsv687)	A	B	A,B	**0.003**	**0.08**	0.64	A	B	A,B	**0.05**	**0.06**	0.94
Baseline (pre-supplementation)	7.44 ± 0.59	7.52 ± 0.39	7.43 ± 0.57		A		7.57 ± 0.38	7.52 ± 0.56	7.57 ± 0.37		A	
Week 2, day 5 (pre-chamber residence)	7.24 ± 0.59	7.52 ± 0.40	7.44 ± 0.61		A,B		7.23 ± 0.75	7.40 ± 0.47	7.44 ± 0.52		A,B	
Week 2, day 7 (chamber residence)	7.20 ± 0.88	7.45 ± 0.37	7.00 ± 0.46		B		7.18 ± 1.12	7.28 ± 0.58	6.96 ± 0.24		B	
Week 3, day 2 (washout)	7.08 ± 0.87	7.38 ± 0.51	7.37 ± 0.54		A,B		7.44 ± 0.32	7.64 ± 0.46	7.52 ± 0.57		A	
Clostridium clusters I and II (Chis150)				0.11	**0.002**	**0.07**				0.11	**0.01**	0.23
Baseline (pre-supplementation)	7.31 ± 0.56	7.34 ± 0.61	7.24 ± 0.49		A		7.61 ± 0.31	7.68 ± 0.34	7.30 ± 0.38		A	
Week 2, day 5 (pre-chamber residence)	6.79 ± 0.63[a]*	7.26 ± 0.60[b]	7.51 ± 0.67[b]		A,B		6.87 ± 0.65	7.18 ± 0.81	7.51 ± 0.66		A,B	

Week 2, day 7 (chamber residence)	6.93 ± 0.77*	7.01 ± 0.52	6.69 ± 0.73*^	C	7.08 ± 0.89	7.18 ± 0.57	6.66 ± 0.68	B
Week 3, day 2 (washout)	6.97 ± 0.79	7.11 ± 0.70	7.10 ± 0.49[#]	B,C	7.35 ± 0.32	7.66 ± 0.57	7.29 ± 0.47	A

Table 2. Effects of dietary supplementation with a blend of fermentable fibers and polyphenol sources on faecal bacterial abundances measured by fluorescence in-situ hybridization before, during and after hypoxic stress for both the full intention to treat cohort and the complete case cohort.

FP, fiber and polyphenol intervention; HA, high altitude conditions; LA, low altitude conditions; PL, placebo intervention. Data are mean ± SD. Analyzed by general linear model with correlated errors. Uppercase letters indicate main effects of condition and time. Columns and rows not sharing a superscript uppercase letter are significantly different ($P < 0.05$). Lowercase letters and symbols indicate differences following condition-by-time interaction. Within a row, values not sharing a superscript lowercase letter are significantly different ($P < 0.05$). *Different from baseline; ^different from Week 2, day 5; #diffrent from Week 2, day 7. [2]n = 4 did not have samples available for analysis..

NMR

Metabonomic approaches using NMR-spectroscopy based metabonomics was used for high-throughput detection and quantification of metabolites. Both urine and plasma fluids were analysed in this study in order to uncover alterations in system-level molecular pathways, as well as providing functional assessment of the gut microbiota.

One urine sample was collected from each volunteer during each study phase (A, B, C) giving a total of 38 urine samples. Two plasma samples were collected at the end of each study phase, when the volunteers resided for ~36hr in a hypobaric chamber simulating low altitude or high altitude environment (Day 6 and Day 7). There were a total of 80 plasma samples collected for analysis. Selected complete data sets are presented below.

Plasma Metabolic Profiles

Plasma samples were initially analysed to assess the difference in Day 6 and Day 7 samples (Figure 1). Plasma samples contain information relating to circulating metabolites, and in this analysis we

observed a decrease in plasma lactate levels whilst lipoprotein levels increased, following residence in the hypobaric chamber.

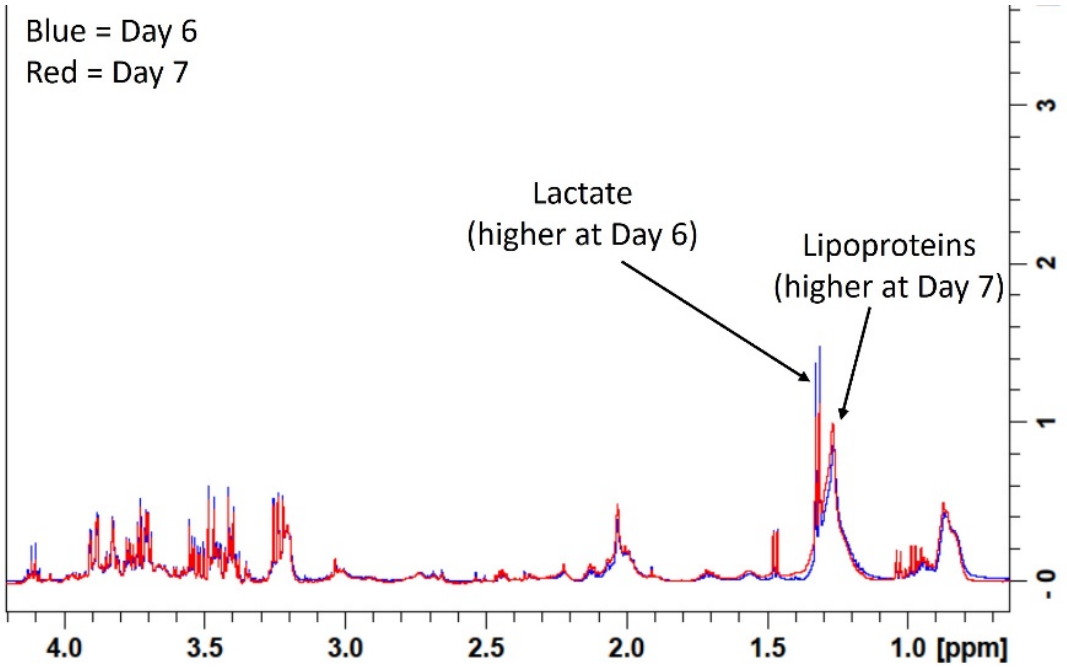

Figure 1: Overlay of plasma metabolic profiles at Day 6 and Day 7

Using spectral data from Day 7 only, plasma samples were then compared according to study phase (Figure 2). This analysis revealed that the dietary intervention lowered circulating lipoprotein levels compared to the placebo. n=1

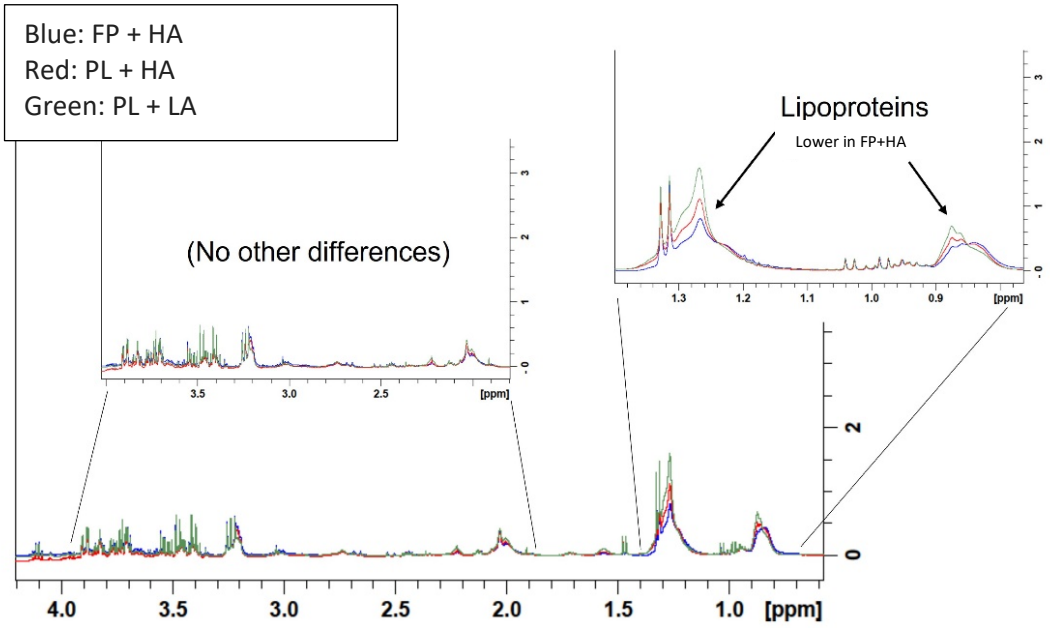

Figure 2: Differences in plasma untargeted metabolic profiles according to study intervention. n=1

Urine samples carries information on chemical signals relating to intermediate and end products of endogenous metabolism, as well as additional information on microbial co-metabolites. When we compared the untargeted metabolic profiles from each intervention, we observed alterations to endogenous metabolic pathways (citrate; energy metabolism, creatinine; muscle turnover) as well as differences in metabolites produced as a result of microbial metabolism (acetate, hippurate).

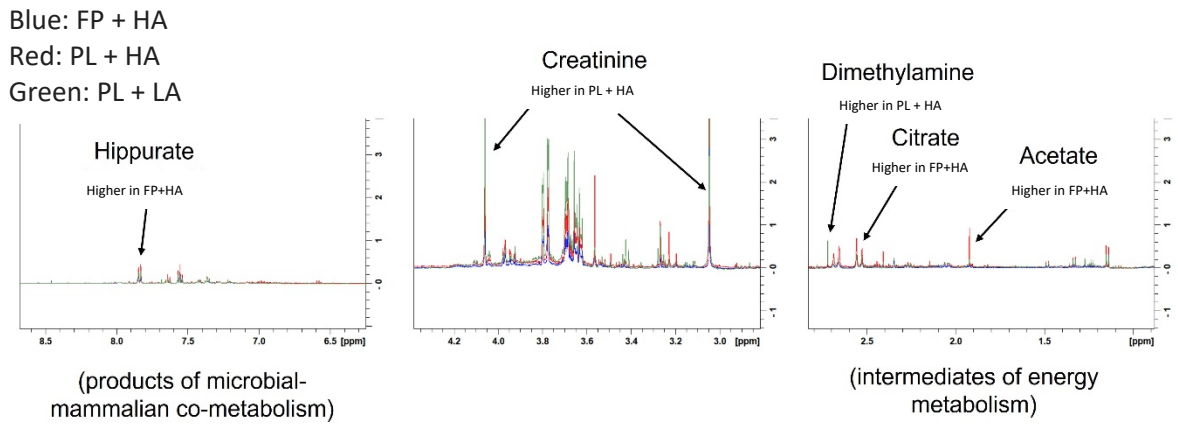

Figure 3: Differences in urine untargeted metabolic profiles according to study intervention. n=1

Taken together, these results demonstrate that the dietary intervention had a positive impact on the system altering mechanisms mediated by the gut microbiota. However, this is with the caveat that this is an incredible small sample size, with only 6 people completing the full duration of the study. This should not go unnoticed. Even though it may be interesting that we see a general trend towards a positive effect of this combination at high altitude, the results cannot be generalised because of this small sample size.

4.4 Discussion

As a note, in this discussion, as the cohorts are so small, both a visual interpretation of the data and a statistical analysis will be discussed. Visually, total bacterial populations showed little change between baseline and pre-chamber (high altitude) entry but controls decreased after 2 weeks. During high altitude exposure, these populations were decreased to levels more reflective of low altitude, with similar trends at washout. This does seem to align with current research suggesting that HA can have detrimental effect on the diversity of the gut microbiome (Zhang et al., 2018; Suzuki et al., 2019).

One of the most promising outputs from these bacterial data is that bifidobacteria, a health promoting genus, increased on the intervention prior (compared to placebo at both low and high altitude) to high altitude - confirming a prebiotic effect. These then declined during chamber residence but were still higher with the intervention compared to placebo at either high or low altitude. This represents a very promising result, as if there is a possibility that the supplementation of prebiotics and polyphenols will attenuate high altitude related illness, and cognitive detriment through potentially providing more stability to the gut microbial composition – essentially increasing the resilience of the communities- this could be incredibly helpful. Potential attenuation of decrease in bifidobacteria at HA may reduce the detrimental GI symptoms like nausea, diarrhoea etc (Pratt et al., 2020). Bifidobacteria is also associated with mood improvements, reduced anxiety and decreased psychological distress (Li et al., 2023; Wang et al., 2019).

Lactobacillus, another probiotic genus, showed little change between baseline and week 2 day 5 (pre chamber entrance). High altitude decreased numbers and this was also the case for the intervention – showing that this arm of the study preferred fermentation by bifidobacteria. This was also the case for *Bacteroides* (commensals that are both saccharolytic and proteolytic), erec (eubacteria), rrec (*Roseburia*), Ato (*Atopobium*), Pro (mainly propionibacteria), Fpra (*Faecalibacterium prausnitzii* and relatives), Dsv (sulphate-reducing bacteria) and Chis (clostridia). Arguably the influence on a major pathogenic groups (desulfovibrio – a producer of toxic H_2S) was a positive outcome in the study. However, this was not selective as commensal populations showed similar effects. That is, for most populations investigated, a similar effect occurred in that high altitude decreased numbers of bacteria up to the 5 day assessment but this was repressed by the intervention after 7 days. More importantly, the health positive bifidobacteria were an outlier to this observation.

Bifidobacteria is not only associated with improved gastrointestinal health (which can be damaged through high altitude exposure (McKenna et al., 2022) but also an amelioration of cognitive impairment (Zhu et al., 2023), which can be affected at high altitude also (Koester-Hegmann et al., 2019). There is also an association between higher bifidobacteria levels and a modified, ameliorated physiological stress response (Yang et al., 2017). The psychobiotic functions of bifidobacteria have also been demonstrated in the mood states of anxiety and depression, showing an improvement of both negative states (Li et al., 2023; Sakar et al., 2016). Colonisation of germ free mice with bifidobacteria have been able to normalise the HPA axis mediated stress response (Sudo et al., 2004) – giving more evidence fot the gut brain axis as well as potentially suggesting protective mechanisms against PTSD, of which a dysregulated HPA is a risk factor (Shumacher et al., 2019).

In terms of statistically significant data, there was, as expected, a trend towards a main event of condition in CHIS – in the two high altitude stress groups there was an increase, though this was reduced within the high altitude supplement group. The reduction in this potentially pathogenic group

is of course of benefit to host health – as this group has previously been associated with diseases states such as IBD (Poveda et al., 2020).

There also, as with visual inspection, seemed to be a main effect of condition for bifidobacteria in both analyses – whilst not significant, it seems to be that this increased and then decreased whilst in the HA chamber. Bifidobacterial levels then decreased again, though only to a similar level as pre supplementation. This may suggest that, again, as research shows bifidobacteria decreases at HA (Kleesen et al., 2005), there is some attenuation of this through the supplementation. Ato demonstrates a similar trend to bifidobacteria. This is particularly interesting as we also saw significant increases in Ato in chapter 2. Interestingly, Atopobium has been shown to decrease at high altitude (Kleesen et al., 2005), so we clearly do see the amelioration of this HA resultant decrease through supplementation. Not much is known about the function of *Atopobium* within the gut microbiome – though this research may suggest that it could be of benefit to explore further, especially if it correlates with other health conferring changes.

NMR analysis, in plasma (Figure 1 and 2), revealed that the dietary intervention (G) lowered circulating lipoprotein levels compared to the placebo within plasma. LDLs are not only increased at high altitude (Gonzales et al., 2013), but also have been shown to be associated with increased stress levels (Steptoe et al., 2016). We see the increased of LDLs within many disease states, such as increased risk of coronary heart disease (Fernandez et al., 2008). However – what is particularly interesting about these increased LDL levels is that some research suggests that increased LDL levels is actually a protective mechanism within the body against acute mountain sickness (Harrison et al.,2015). This would suggest that having these lowered is not necessarily of benefit. However, this relationship of Acute mountain sickness (AMS) and circulating LDLs is not without its complexities – a secondary analysis exploring this showed that statins were able to reduce the acute mountain sickness symptoms, theoretically through anti-inflammatory properties (Harrison et al.,2015). Statins do lower LDLs, but in this case a reduction in AMS symptom was still apparent. This may suggest that the elevated LDLs are not necessary for the protection against AMS symptoms, and the anti-

inflammatory effects of the prebiotics and polyphenols in the novel combination may be a suitable alternative, without putting those at high altitude at risk of the pathogenesis of other diseases (Mortensen et al., 2020). Statins themselves also present with some issues – studies have reported side effects such as musculoskeletal symptoms, increased diabetes risk and higher rates of stroke (Pinal-Fernandez et al., 2018), if it is possible to maintain this protective effect against AMS without having to intake these, it would be preferable to have a nutraceutical rather than a pharmaceutical option.

The NMR overlays also showed higher levels of circulating lactate, however these were ameliorated by the administration of the dietary intervention. Though thought to settle with acclimatisation, higher levels of lactate or lactate accumulation is expected, as often seen in those at high altitude due to the coexisting hypoxia (Hochachka et al., 2002). These data are as expected.

Urine analysis (Figure 3) compared the untargeted metabolic profiles from each intervention, and alterations to endogenous metabolic pathways as well as differences in metabolites produced as a result of microbial metabolism were seen.

Hippurate was seen to be higher within the intervention arm of the study. The microbial co-metabolite Hippurate is associated with increase microbial gene richness and greater diversity of the gut microbiome (Brial et al., 2021; Pallister et al 2017). These re two factors often associated with a 'healthier more resilient' gut microbiome, thought to be a good indicator of a reduction in cardiomatabolic disease risk (Jin et al., 2021).

What is interesting, as that as well as being associated with the gut microbial outputs in general, Hippurate is also specifically associated with the metabolism of polyphenols – derived from the microbial metabolism of polyphenols to benzoates (Pallister et al., 2017). High urinary Hippurate concentrations are also linked to inulin consumption, again as a metabolic product (Carlson et al., 2018). Hippurate in urine is a marker of good metabolic health in general - and increased polyphenols are also correlated to good metabolic health. Studies have demonstrated that, specifically in soldiers, a more diverse microbiota and higher *Bifidobacterium* levels were protective against increases in GI

permeability during training (Karl et al., 2017; Li et al., 2013). As high altitude is associated with increased levels of permeability - a reduction in this through the supplementation and subsequent increases in diversity and bifidobacteria is beneficial (Mckenna et al., 2022).

Hypoxic stress on the kidney can result in higher creatinine levels. In fig 3., we see that the urinary creatinine levels are higher in HA with placebo than HA with supplementation. If it is the case that he hypoxia is causing elevated creatinine levels (Chhabra et al.,2018), this suggests an amelioration of damage done by the high altitude through supplementation. As previously mentioned, Acute Mountain Sickness is often relieved by acclimatisation (Clarke., 2006), and research suggests that this acclimatisation may be influenced by action of the kidneys – so if it possible to optimise kidney health this would be ideal (Goldfarb-Rumyantzev et al.,2014). We also see another disease state associated metabolic product within the urine - increased levels of dimethylamine were seen only in the high altitude and placebo group, again suggesting an amelioration of potential illness and disease markers by the novel combination (Tsikas, 2020).

The research presented above presents a strong case for the use of nutraceuticals in place of pharmaceutical interventions when considering the acclimatization and reduction of AMS within military personnel. This also, of course, applies to those visiting high altitude environments, though less relevant to those who have already acclimatized. The use of a nutraceutical provides an option for a less expensive, potentially more broadly efficacious, and potentially safer (less side effects) intervention for those looking to optimize performance and health in a high altitude environment. Taken together, these results demonstrate that the dietary intervention has a positive impact on the system altering mechanisms mediated by the gut microbiota.

4.5 References

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Chapter 5 – Final Discussion

5.1 – Discussion of the current work

The aims of this thesis were as follows: 1) to explore whether a novel combination of prebiotics and polyphenols would have an effect on both bacterial composition and microbially derived neurotransmitters in vitro, 2) to explore how a novel combination of prebiotics and polyphenols could affect perceived stress levels and mood in healthy adults, as well as assess whether there are any key metabolite changes associated with mood states and 3) to investigate the effects of this novel combination on active military personnel under high altitude stress.

Initially, batch culture fermentation models were used to explore the effects of inulin, GOS, resistant starch, cocoa, green tea, blueberry and cranberry on the bacterial composition, neurotransmitters and short chain fatty acids in vitro. These substrates were explored individually and also as a combination. Bacterial enumeration was analysed through Flow-FISH, SCFAs were analysed through GC and the neurotransmitters were explored through LC/MS. Samples were analysed at 0 hours (baseline), 8hours, 24 and 48 hours of fermentation.

Whilst there was not much significant change, the novel combination seemed to outperform the prebiotics and the polyphenols alone, through stimulating beneficial bacteria. Some of the most

interesting results came from SCFA analysis where it was seen that the novel combination was superior in production of acetate and lactate. Interestingly, much of the beneficial action of the novel combination was seen at the 48-hour mark. Often, there is a marked decrease within in vitro work at this time point, with some studies not even taking samples at this time. This may suggest that there is a synergistic action between the prebiotics and polyphenols – providing the better longevity //efficacy.

Microbial production of neurotransmitters was also evident, with GABA being present, and significantly increasing within the combination vessel. Lower concentrations of dopamine, kynurenic acid, and norepinephrine presented, with epinephrine and serotonin being below detection.

However, it is important to note that it is not possible to separate out whether the success of the combination was because of the combined prebiotics, or through interactions between the prebiotics and polyphenols.

As this set of in vitro experiments was promising in showing the putative beneficial effects of a more combinatorial approach to supplementation, a further, slightly more complex set of in vitro experiments was undertaken, this time combining the prebiotics, combining the polyphenols, and then fermenting the same novel combination as in previous experiments.

Whilst the principle of a 'pH modulated batch' in vitro method is similar to standard batch, manual adjustments of the pH to better mimic the intestinal tract - the proximal, transverse, and distal regions of the colon - make it, perhaps a more suitable option for the exploration of metabolic outputs and micribial effects. This experiment was undertaken primarily to explore the combinations of prebiotics, and polyphenols separately, as well as in a combination, to draw out if the different prebiotics might have an effect on eachother, or if it was likely that the combination of prebiotics and polyphenols interacted with one another. Of course, it is important to note that the sheer amount of substrate/nutrients within the full combination may also play a role in the increased activity seen. It is important to optimise procedures throughout the scientific process, and I believe that better reflecting the transit times and the pHs of the intestinal tract is highly beneficial when looking at a static batch culture.

Bacterial outputs from these two in vitro experiments were similar – we saw in both that the combination of polyphenols and prebiotics was successful – the pH modulated batch cultures showed that at 48 hours, bifidobacteria began to increase again, which supports findings from the previous fermentations that there may be better longevity when the polyphenols and prebiotics are combined. Further to this, the polyphenol combination alone saw significant increases in bifidobacteria, following a very similar trend to the prebiotics. This is interesting, as polyphenols are a putative prebiotic themselves (Singh et al., 2019; Rodriguez-Daza et al.,2021). There is also thought that crosstalk between polyphenols and prebiotics could increase efficacy – primarily through the bidirectional relationship that polyphenols have in the gut microbiome. Polyphenols are able to exert benefit through both acting as a prebiotic – increasing the bacteria such as bifidobacteria, and also reducing pathogenic bacteria (Alcves-Santos et al., 2020; Zhang et al., 2022; Kasprzak-Drozd et al., 2021). Polyphenols also rely on the gut microbiome to transform their low bioavailable form to useable metabolites that improve host health (Pasinetti et al., 2018; Corrêa et al.,2019).

Studies in animals where combinations of polyphenols and prebiotics have been utilised have shown improved polyphenol activity – a study that added dietary fibre to soy milk demonstrated better antioxidant status of the polyphenols, likely to be related to gut microbial activity (Lee et al., 2015). Whilst not much is known about the specific mechanisms by which we may see crosstalk between prebiotics and polyphenols, it can be suggested that combinations of the two may improve efficacy of both (Edwards et al., 2017). Whether this is literally as they both have a prebiotic function is unknown, however it is also likely that the metabolic breakdown of polyphenols into its components that benefit the gut microbial environment (Correa et al,. 2019). Another study has demonstrated that when polyphenols and dietary fibre are combined together, the dietary fibre is able to enhance the breakdown of the polyphenol compounds – increasing positive effects on human health, including the elevated production of butyrate and reduction in the inflammatory TNF-a - an inflammatory marker increased in conditions such as IBD and, more pertinently, increased in plasma in those with PTSD. (Guo et al., 2018;. Hussein et al., 2017; Dib et al., 2021; Genaro et al., 2021). In the present study, an

increase in some of the SCFAs was seen in the combination vessel, potentially due to this interaction between the prebiotics and polyphenols.

The SCFAs themselves provided some interesting results – specifically in the case of lactate. In both in vitro models, in the combination vessel, we saw the highest levels of acetate, propionate, and lactate.

Lactate, as discussed in previous chapters, is thought to make valuable contributions to host health. For example, research suggests that intraluminal levels of lactate (produced by lactobacilli or bifidobacteria) can have modulatory effects on inflammation of the intestinal lining (Zhou et al.,2022; Iraporda et al., 2015; Wang et al., 2020). This proves beneficial in terms of modulating gut symptoms. As well the as benefits seen in terms of mood state and neurological health - chronic psychological stress has been shown to increase intestinal mucosal mast cells (Yang et al., 2006)– which are key effectors of the gut-brain axis. These are important players within the HPA axis and respond to stressors by way of altering permeability of the gastrointestinal tract (Carabotti et al., 2015; Appleton. 2018).

Propionate, associated with various bacterial increases, such as Bacteroidetes/Prevotella (Hughes et al., 2008), is an anti-inflammatory SCFA, and one of the three most commonly occurring SCFAs within the gut environment (Silva et al., 2020). It is thought to be anti-inflammatory through various actions, such as the reduction of the inflammatory response via the NF-κB pathway(Usami et al., 2008). Propionate has also been associated with various mood states – increases in this SCFA are positive, as lower levels of propionate have been related to depression (CAspani et al., 2019) .

Higher acetate levels within this study are also of note – as acetate is linked to, not only physiological changes like reduced inflammation, but also alteration of neurotransmitter levels, like GABA within the hypothalamus (Frost et al., 2014).

SCFAs, in general, are related to protection against oxidative stressors, as well as in mouse studies, been shown to mitigate the effects of psychosocial stress, reducing the heightened stress response.

This same study also demonstrated that the SCFAs were able to reduce stress related damage to the intestinal barrier (Van de Wouw et al., 2018) again, something that within the present study were aiming to do, as the intestinal barrier is damaged under all different types, including psychological, stress (Ilchmann-Diounou et al., 2020).

Therefore, increases in these major SCFAs within this novel combination not only align with previous research on prebiotics, but contribute a novel finding that a combinatorial approach of phenols and prebiotics may have the most beneficial effect when it comes to short chain fatty acid production.

Further to this, what is potentially the most interesting is that, whilst there was not a correlation to specific bacterial increases in this research, the potential outputs of SCFAs are the same – so regardless of individual differences in bacteria or reaction to substrate, which as we know can vary enormously based on previous diet, individual bacterial composition etc, the output of the positive, health promoting metabolites remain similar, or at least have similar trends.

When considering the bacterial derivatisation of neurotransmitters in Chapter 2, It is evident that the most abundant neurotransmitter across both sets of fermentations was GABA. Interestingly in the batch culture fermentations, the highest levels of GABA were seen in the cocoa vessel. Cocoa is associated with an increase in lactobacilli and bifidobacteria (Jang et al., 2045; Tzounis et al., 2011) both bacterial groups that are suspected to synthesise GABA from dietary glutamate through action of the GAD operon (Monteagudo-Mera et al., 2023). Then, in the pH modified batch, it was shown that the highest levels were seen within the prebiotic combination. This is an interesting difference and does pose a question as to the different response of individuals to substrates.

One of the suspected routes of communication for the gut brain axis is the vagus nerve – which is thought to be stimulated by neuroactive compounds (Bonaz et al., 2018; Strandwitz, 2018), such as GABA – these can be produced, as mentioned, by action of the gut microbiota on these dietary sources. GABA is a very beneficial metabolite to focus on the promotion of, as it has been associated

with a reduction of anxiety and stress (Tette et al., 2022) – as well as being a promising target for therapeutic interventions in depression (Fogaça et al., 2019).

This increase in GABA does provide further support to the gut-brain axis pathway, however it was not shown that for GABA at least, the novel combination was superior in production than individual components.

Other neuroactive metabolites were also seen in the two in vitro experiments, albeit at lower concentrations, however one of the most interesting results was production of serotonin in the pH modulated batch cultures. This was not expected as serotonin was at too low a level to be detected within the original batch culture. The combination and polyphenol vessels were the only substrates that produced serotonin, with levels higher within the combination – suggesting again an improvement of efficacy. However, it is important to note that some plant sources to contain tryptophan (Vitalini et al., 2020), so the production/synthesis of serotonin may be no through breakdown of existing tryptophan rather than an organic production by bacteria. Tryptophan can produce both serotonin and kynurenic acid – and the control of tryptophan metabolism is thought to be stress responsive (Roth et al., 2021; Chojnaki et al., 2023; Miura et al., 2008)). Chronic stress is known to dysregulate feedback of the HPA axis (Herman et al., 2016) – specifically though dysregulation of glucocorticoid receptors (Cohen et al;., 2012; Wang et al., 2017). Elevated glucocorticoid levels, caused by chronic stress, also activate pathways which promote the production of kynurenic acid from tryptophan instead of serotonin (Kennedy et al., 2017) – this reduction in serotonin levels is thought to be a key player in the onset of stress related major depressive disorder (Porter et al., 2021).

Whilst we can by no means confirm that this is the case, the potential for polyphenols to reduce inflammation in the gut (Zhang et al., 2016), as well as modulate glucocorticoid receptors (Donoso et al., 2019) may suggest that it is an optimal substrate to counteract this stress related dysregulation and influence production of serotonin, hence the polyphenol containing vessels had superior production of serotonin.

Regardless of the low concentration of these bacterially derived metabolites, and whether the production of this be through downstream production, as mentioned above, this does not negate the fact that the addition of nutraceuticals shown in these in vitro experiments may have an influence on the production of these neuroactive compounds.

Though significance between condition changes did not prove evident, trends seen in this research, specifically within the short chain fatty acid production, suggest that a novel combination of prebiotics and polyphenols may benefit host health, as well as potentially modifying neuroactive compounds. Overall, the production of SCFAs, and neuroactive compounds, whilst not significant, provide scope for further exploration through models that are not acellular, and less highly controlled to optimise production of metabolites.

The second and third aims in this thesis were explored through two in vivo studies. These were undertaken to assess the effect of this novel combination on different populations - firstly, a human trial in healthy adults who considered themselves to be stressed, and secondly, a population of active military personnel who were exposed to artificial high-altitude stress.

The first study explored the effects on perceived mood and wellbeing through questionnaires (PSS, GAD-7 and PHQ-9), as well as the effects on blood pressure throughout the study. Bacteriology and 16s rRNA sequencing were carried out for pre and post stool samples.

Initially, focusing on bacteriology, the novel combination demonstrated good prebiotic qualities. The novel combination was the only group to produce a significant increase at the post time point for *Roseburia* – a bacterial group associated with reduced inflammation, increased SCFAs and reduced neuroinflammation (Nie et al., 2021)., it is also associated with 5-HT production within the gut (Song et al., 2021). Other groups, such as *Faecalibacterium* also increased with the novel combination – this is another group associated with anti-inflammatory and is an interesting putative next generation probiotic (Martin et al., 2017). Bifidobacteria and lactobacilli also increased in the combination group, despite lactobacilli decreasing in all other arms. What is also very interesting is the placebo group saw the increase of a pathogenic bacteria, desulfovibrio – which was not the case on any other

intervention. Whilst the goal, of course, for prebiotics is often focused solely on the increase of positive bacteria, a reduction of pathogens can only be a good thing.

As this novel combination did contain prebiotics, results do align with previous research into prebiotics, however we see that the novel combination (including polyphenols) consistently improved efficacy as a prebiotic. It is likely therefore that there is some sort of either prebiotic effect from the polyphenols or a symbiotic relationship.

Blood pressure data, though sporadic, was visually inspected to show that systolic blood pressures did seem to be lower within the combination groups. This aligns with research that suggests lower levels of *Roseburia* and *Faecalibacterium* are linked to hypertension (Yan et al., 2017). In this case, we see a reduction in systolic pressure correlated with higher *Roseburia* and *Faecalibacterium prausnitzii*. This is likely due to the fact that these are two bacterial groups associated with the production of SCFAs. If we can extrapolate from the in vitro work, which suggested that the novel combination did, in fact, increase aetate, propionate and lactate comparatively, then this also confirms superior action of the novel combination. Polyphenols are also, independently, associated with reduced blood pressure (Onakpoya et al., 2015).

Wellbeing questionnaires showed a reduction in scores for all groups, including the placebo. This is contrary to the expectations, as these were all participants who had said they had higher perceived stress levels. It would have been expected, when looking at previous research into stress and specifically prebiotics that there would be a marked difference with supplementation groups than the placebo (Schmidt et al., 2015; Zhang et al., 2020). That being said, there may of course have been a placebo effect (Colagiuri et al., 2015) at play here, as these results do not necessarily correlate with the physiological changes seen above. Prebiotics and polyphenols have both been associated with a reduction in stress in vivo (Zhang et al., 2020; Micek et al., 2023) – perhaps this study required a longer supplementation period to see a marked difference, or a larger sample size in order to confirm results.

There is clearly an interaction between the novel combination and bacterial changes, as well as potentially the blood pressure data. This would suggest that consuming a combination of prebiotics and polyphenol provided more of a benefit than either alone – however more research is needed on this. There is very minimal, if any, research that looks not only at combinations of supplements – especially prebiotics and polyphenols. In the case of this research, not only were these two nutraceuticals combined, but also multiple prebiotics were used in order to optimise outcomes. It would also be interesting to develop a more systematic approach into exploring the prebiotic and polyphenol combination – as the interplay between these two supplements is under investigated.

The second human trial in active military personnel, showed fairly similar outcomes. This was also a placebo-controlled trial, with also two-week experimental period, as in the study above. Participants were all under the low altitude condition, the high altitude and placebo condition and the supplementation and high altitude.

It was expected that at high attitude there would be a decrease in bacterial counts, as well as decrease in specific bacterial groups like bifidobacteria. This was of course, seen, however the health positive bifidobacteria (O'Callaghan et al., 2016) were actually reduced less in the group with the supplementation - this is an interesting finding as the supplementation seemed to ameliorate high altitude stress induced dysbiosis.

As with the previous in vivo study, what is interesting is the reduction of pathogenic desulfovibrios (DSV) within the group under HA stress, who were consuming the supplement. This is beneficial, as many people who are under HA have severe gastrointestinal issues (Anand et al., 2006). Though not always related to bacteriology itself, IBS is highly associated with increases in DSV (Bennet et al., 2015), as well as it being implicated in cases of depression (Barandouzi et al., 2020; Lowe et al., 2023). Military personnel need to optimise their memory, cognition, and reaction times, even at sudden altitude exposure. Therefore, both decreasing pathogenic, disease associated bacteria and increasing positive bacteria is crucial, and in this case the DSV was potentially mitigated by the increased bifidobacteria, or rather an attenuated reduction in bifidobacteria. This study shows that,

again, the novel combination proves potential superior efficacy in terms of modifying the gut bacteria when compared to a placebo.

NMR analysis, in plasma and urine, revealed that the dietary intervention lowered circulating lipoprotein levels compared to the placebo within plasma. LDLs are known to be increased at high altitude (Gonzales et al., 2013)– as well as being increased by stress (Steptoe et al., 2016). A lower LDL level is beneficial within the body - higher LDL levels are linked to many diseased states including a higher risk of coronary heart disease (Fernandez et al., 2008).

Within the plasma, at high altitude, higher levels of circulating lactate were seen, however these changes were not apparent when the dietary intervention was administered. Higher levels of lactate or lactate accumulation is expected, as often seen in those at high altitude due to hypoxia (Hochachka et al., 2002). This is thought to settle with accumulation (West et al., 2007).

Urine analysis provided information on chemical signals relating to intermediate and end products of endogenous metabolism, as well as additional information on microbial co-metabolites. When we compared the untargeted metabolic profiles from each intervention, we observed alterations to endogenous metabolic pathways as well as differences in metabolites produced as a result of microbial metabolism. The microbial co-metabolite Hippurate is associated with increase microbial gene richness and greater diversity of the gut microbiome (Brial et al., 2021; Pallister et al 2017). These are two factors often associated with a 'healthier' more resilient gut microbiome (Hollister et al., 2014). Again, this was higher within the intervention study.

Creatinine was higher in the high altitude but without the intervention – potentially resultant of hypoxic stress on the kidneys (Chhabra et al.,2018). If this is the case, we do not see the elevated hypoxic state at HA with the supplementation, suggesting an amelioration of damage done by the high altitude through supplementation. It is thought that kidneys play a crucial role in acclimatisation and Acute Mountain sickness so optimising kidney health would be beneficial (Goldfarb-Rumyantzev et

al., 2014). It is also thought that the increased levels of dimethylamine may be associated with a disease state (Tsikas et al., 2020) – again this is not seen in the high-altitude group with supplementation, only the high altitude and placebo group.

Taken together, these results demonstrate that the dietary intervention has a positive impact on the system altering mechanisms mediated by the gut microbiota. However, as previously stated, throughout both studies, the warfighter and the civilian study, these were both extremely small sample sizes. The calculation for the warfighter study suggested that at least 15 complete participants were needed in order to have statistical power. Future considerations for similar work must include an appropriate number of participants.

Whilst there may not be a gold standard for exploring diversity of the microbiome, human, or in vivo studies are considered to be some of the most robust research techniques for exploring human health, nutraceuticals and the gut microbiome (Swann et al.,2020; Sibbald et al., 1998). However, these are not without their limitations, as it is expensive, complex, and difficult to separate out confounding mechanisms from the host (Vujkovic-Cvijin et al., 2020; Swann et al., 2020).

To counter these limitations whilst still exploring the human gastrointestinal tract, models have been developed to replicate, and explore this ecosystem, whether these be in vitro, in silico or animal systems. As with all non-human studies, there are limitations by way of applicability and transferability to humans, but in demonstrating proof of concept, or exploring potential mechanisms in tandem, or before in vivo studies, they are immensely useful (Williams et al., 2015).

Whilst in vitro models such as those employed within this work do of course provide useful insights into the activity of these substrates on microbial activity and metabolite production, one of the primary limitations of these in vitro models is the lack of human tissue. This is not a methodological limitation, as these models are fit for purpose, but rather a limitation in terms of how the research outputs can be generalised. It is not possible to see the interactions between metabolites and human tissue, and how they may influence a stress response. A secondary limitation is the use of the faecal samples – though a widely used and accepted model, they do not represent the gut mucosal microbial community.

A final limitation of the human study is the aspect of self reported stress scores. It would be useful to accompany this data with quantifiable absolute data, such as levels of circulating stress hormones.

By combining in vitro and in vivo work, we are able to overcome some of the limitations to each – as one of the primary limitations of an in vitro model is the lack of colonic human tissues. By following this work with in vivo study, it is possible to compare and explore the novel combination across, not only human tissue, but two subgroups of participants – with varying stressors. It is important, however, to note that both of these studies were limited in participant numbers, both were heavily impacted by the covid pandemic – this this limits them in power and applicability.

5.2 Future work

The primary takeaway from this work is twofold – one, that the novel combination of prebiotics and polyphenols does seem to be superior when given as a supplement, both in favourably modifying the gut microbiome, and in influencing metabolic outputs, like SCFAs. Further in vivo studies, or expansions on the current research are needed to confirm the effect on mood states and wellbeing. Secondly, that further research is needed in vitro to elucidate the mechanisms by which polyphenols and prebiotics may act in the environment of the gut microbiome, as this is an underexplored area, and, if a combination does produce better results on host health, there are clearly some interactions and crosstalk that are as yet not understood.

It may also be interesting to use, perhaps in tandem with a more expansive in vivo study, a more complex continuous culture model that replicates the state of a disease state, or a common AMS affected gut. It would be interesting to see the effects of supplementation on this.

A further human study, involving perhaps those with diagnosed stress disorders, or further in the future, military personnel designated as at risk for PTSD, would be very interesting to undertake. It would be very useful perhaps to also replicate the current study in a larger, ergo better powered,

cohort. Including the prebiotic combination and the polyphenol combination would still be useful, as though proof of concept has been suggested, it is still important to show that the combination supplement is more beneficial that either supplement alone.

It would also be useful to further explore metabolites specifically related to stress – perhaps through more plasma analysis, for example, circulating cortisol which is a key biomarker for stress. In terms of military personnel specifically, it may be interesting to also look at modifying the amounts of glycophospholipids within the blood, as elevation of these is also associated with an increased risk of PTSD. Using NMR more extensively would be a useful analytical tool, especially for exploring correlations between circulating metabolites and those within the stool.

To conclude, the in vitro work in this thesis was able to provide putative evidence that a novel combination of prebiotics and polyphenols may be beneficial towards human health, including through modifying the gut microbial composition, and through the metabolite production. What is not known, however, is the interplay between polyphenols and prebiotics, and this would be a very interesting topic to explore further. The in vivo work was able to suggest that, in a stressed cohort, the novel combination reduces stress associated dysbiosis, as well as upregulate positive metabolites, therefore it is a useful supplement and would be of benefit for both 'civilian' populations as well as active military personnel. Future studies should absolutely focus on increasing the participant numbers, as this research was in a very small population with no power calculations. Overall, the experimental work within this thesis may contribute to the body of research exploring the ways in which various nutraceuticals are able to positively modulate the gut microbiome, and, therefore, how that affects host health.

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Chapter 6 - Dissemination of research

6.1 Overview

Good dissemination of research is important for many reasons. The ability to communicate research to the lay public is crucial, thereby increasing understanding and interest in people who are not involved in the field will ultimately benefit everyone. Much research can be applied to every day life and allowing people to read information themselves and form their own ideas on accessible research can only benefit the field. It is also important to disseminate research to those in policy development – so that important research breakthroughs can be communicated and put into practice outside of a laboratory setting.

Below are two pieces of writing that were distributed to members of parliament, through an APPG*, in order to further understanding and increase interest around the gut microbiome.

6.2 The use of probiotics in the treatment mix for COVID-19

Two recent peer-reviewed studies provide the first evidence of the potential benefit of probiotics in the fight against COVID-19. Both studies show significant improvements in COVID-19 with administration of probiotics, both in the mortality rate, and in the risk of developing respiratory failure.

In two recent Italian trials of COVID-19 patients, a mixture of probiotics was added to existing best available therapy including antibiotics, hydroxychloroquine and tocilizumab. A control group took the same medication without probiotics.

Gastrointestinal disorders are frequent in COVID 19. In the first study of 70 (matched) patients, within 72 hours, nearly all patients treated with probiotics showed remission of diarrhoea and other symptoms compared to fewer than half in the control group.

The risk of developing respiratory failure was eight- fold lower in patients receiving probiotics. Fewer patients taking probiotics entered ICU, and no patients in the probiotic group passed away compared to 10% in the drugs only group.

In the second study, the researchers looked retrospectively at 200 patients with severe COVID-19 pneumonia who were treated just with drugs (112 patients) or drugs plus the probiotic mix (88 patients).

While overall mortality was 22%, risk of mortality was significantly lower in the probiotic patients, with only 11% of those dying compared with 30% of the patients who were not given the probiotic formulation alongside the best available treatment. Moreover, there was a reduction in progression to severe disease for those patients who received the probiotic formulation alongside the best available treatment.

References

d'Ettorre et al. Challenges in the Management of SARS- CoV2 Infection: The Role of Oral Bacteriotherapy as Complementary Therapeutic Strategy to Avoid the Progression of COVID-19.

Cecarelli et al. Oral Bacteriotherapy in Patients With COVID-19: A Retrospective Cohort Study.

Walton et al. Mechanisms linking the human gut microbiome to prophylactic and treatment strategies for COVID-19.

The probiotic formulation used in these studies is marketed as Vivomixx in the UK and Sivomixx elsewhere.

It contains eight strains of probiotics: *Streptococcus thermophilus* DSM 32245 *Bifidobacterium lactis* DSM 32246 *Bifidobacterium lactis* DSM 32247 *Lactobacillus acidophilus* DSM 32241 *Lactobacillus helveticus* DSM 32242 *Lactobacillus paracasei* DSM 32243 *Lactobacillus plantarum* DSM 32244 *Lactobacillus brevis* DSM 27961

The formulation was administered in three equal doses per day, for a total of 2,400 billion bacteria per day.

6.3 Improving the performance of military personnel

Recently published research on military personnel indicates that their decision-making is bettered by improving their gut microbiomes

Whether the gut microbiome can be manipulated to improve the physical and mental health of active military personnel is an important area of microbiome research. A recent study investigated whether consumption of foods containing both a prebiotic (food for positive bacteria) and probiotics (positive bacteria) could improve the wellbeing and composition of the gut microbiome of young military participants in field training.

The study looked at mood and wellbeing; sleep quality; the participants' susceptibility to contracting infection; and the gut microbiome. After 30 days, the group who consumed the supplemented diet showed reductions in tenseness and sleepiness, as well as improvements in the gut microbiome when compared to a placebo group. This suggests that communication between the brain and gut (the microbiome–gut-brain axis) was responsible for these improvements in wellbeing. There were no significant findings for infection levels.

As this specific population is required to make important decisions in highly stressed environments, reducing tenseness and sleepiness is beneficial. These results may also be applied to other high-stress professions, such as those in the medical field, politicians, police officers, firefighters, prison officers and athletes.

Reference

Valle, M., Vieira, I., Fino, L., Gallina, D., Esteves, A., Da Cunha, D., . . . Antunes, A. Immune status, well-being and gut microbiota in military supplemented with synbiotic ice cream and submitted to field training: A randomised clinical trial.

British Journal of Nutrition, 1-15. Published 17th February 2021.

*In 2019,University of Reading helped set up an All Party Parliamentary Group (APPG) on the microbiome. This influential body includes a group of MPs and peers that have a growing interest in the field. We have secured funding from 10 industry partners to support the initiative. The aim is to increase knowledge among parliamentarians on the potential benefits of the human microbiome and its modulation to improve health. The APPG will influence national health services, the media, key opinion leaders, treasury, legislation, government and consumers. We are working with the APPG on the following:

- An inquiry into the most promising health interventions that can arise from altering the gut microbiome through diet

- A series of briefings and expert presentations

- PhD students produce regular research bulletins and newsletters (sent to all members of both UK houses of parliament)

- Appropriate health regulation approaches and product labelling

- Drafting house questions to relevant ministers

- Developing working groups on particular items

- Producing a series of talks for MP staff

- Identification of specific constituency relevance and working with appropriate MPs

- Influence party manifestos as appropriate

APPENDIX A

(i) PSS

(ii) GAD-7

GAD-7 Anxiety

Over the last 2 weeks, how often have you been bothered by the following problems? *(Use "✔" to indicate your answer"*	Not at all	Several days	More than half the days	Nearly every day
1. Feeling nervous, anxious or on edge	0	1	2	3
2. Not being able to stop or control worrying	0	1	2	3
3. Worrying too much about different things	0	1	2	3
4. Trouble relaxing	0	1	2	3
5. Being so restless that it is hard to sit still	0	1	2	3
6. Becoming easily annoyed or irritable	0	1	2	3
7. Feeling afraid as if something awful might happen	0	1	2	3

Column totals: ___ + ___ + ___ + ___

= *Total Score* _____

If you checked off any problems, how difficult have these problems made it for you to do your work, take care of things at home, or get along with other people?

Not difficult at all	**Somewhat difficult**	**Very difficult**	**Extremely difficult**
☐	☐	☐	☐

(iii) PHQ-9

PHQ-9 Depression

Over the last 2 weeks, how often have you been bothered by any of the following problems? *(Use "✔" to indicate your answer)*	**Not at all**	**Several days**	**More than half the days**	**Nearly every day**
1. Little interest or pleasure in doing things.....	0	1	2	3
2. Feeling down, depressed, or hopeless.....	0	1	2	3
3. Trouble falling or staying asleep, or sleeping too much.....	0	1	2	3
4. Feeling tired or having little energy.....	0	1	2	3
5. Poor appetite or overeating.....	0	1	2	3
6. Feeling bad about yourself — or that you are a failure or have let yourself or your family down.....	0	1	2	3
7. Trouble concentrating on things, such as reading the newspaper or watching television.....	0	1	2	3
8. Moving or speaking so slowly that other people could have noticed? Or the opposite — being so fidgety or restless that you have been moving .around a lot more than usual.....	0	1	2	3
9. Thoughts that you would be better off dead or of hurting yourself in some way.....	0	1	2	3

Column totals ___ + ___ +____ + ___

= ***Total Score*** _____

Appendix B - Abbreviations

5-HT	Serotonin or 5-hydroxytryptamine
ABC	ATP-binding cassette transporters
AMS	Acute Mountain Sickness
APPG	All-Party Parliamentary Group
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CREB	cAMP response element-binding protein
DNA	Deoxyribonucleic acid
DSS	Dextran Sulfate Sodium
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated kinases
Flow-FISH	luorescence in-situ hybridization
FOS	Fructooligosaccharide
FP	Fibre/Polyphenol
GABA	γ-Aminobutyric acid
GBA	Gut-brain axis
GC	Gas Chromatography
GI	Gastrointesinal
GOS	galactooligosaccharide
GTE	Green tea extract
HA	High Altitude
HCL	Hydrochloric
HPA	hypothalamic-pituitary-adrenal (axis)
HPLC	High-performance liquid chromatograpy
IBD	Irritable Bowel Disease

IBS	Irritable Bowel Syndrome
IFN-γ	interferon gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
ISAPP	International Scientific Association for Probiotics and Prebiotics
KEGG	Kyoto Encyclopedia of Genes and Genomes
LA	Low Altitude
LC-MS	Liquid chromatography–mass spectrometry
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases
MOA	Mechanism of action
NMR	Nuclear Magnetic Resonance (spectroscopy)
Nrf2	nuclear factor erythroid 2–related factor 2
0.1 mol l/1 anaerobically prepared PBS (pH 7.4)	Phosphate buffered saline
PL	Placebo
PSS	Perceived stress scale
PTSD	Post traumatic stress disorder
ROS	Reactive oxygen species
RS2	Resistant Starch
SCFA	Short chain fatty acid
TNF-α	Tumor necrosis factor alpha