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Unravelling the Role of Tryptophan Metabolism in the Microbiota-Gut-Brain Axis: Focus on the Effects of Stress and Exercise

Thesis presented by

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Declaration

This thesis submitted is my original work and has not been submitted for any other degree, either at University College or elsewhere.

Author Contributions

All the work included in this thesis was performed independently by the author with the following exceptions:

Chapter 2: JML conducted mice restraint stress procedures, HPLC, ELISA and some of the PCRs on murine tissues.

Chapter 3: SJL assisted with Ussing chamber and AhR reporter cell experiments and analyses. CS and AG performed metagenomic sequencing. TFSB carried out the bioinformatic processing of the metagenomic data and constructed tailored R functions for data analysis.

Chapter 4: UV, FC and PDC were provided metagenomic sequencing services. TFSB and BV carried out bioinformatic processing of the metagenomic data. FM and AA performed GC-MS/MS experiments.

Signed

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Publications and presentations

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Jason A Martin, CE Gheorghe, Francisca Villalobos-Manriquez, Michael Molloy, Ken O'Halloran, Eric Kelleher, Timothy G Dinan, John F Cryan, Gerard Clarke **The effect of exercise on gastrointestinal permeability: acute and chronic measures**. Irish Society of Gastroenterology – November 2020

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CE Gheorghe, Joshua M. Lyte, Loreto Olavarría-Ramírez, Michael S. Goodson, Nancy Kelley Loughnane, Gerard M Moloney, Fergus Shanahan, Timothy G. Dinan, John F. Cryan, Gerard Clarke **Microbial Regulation of Tryptophan Metabolism: Focus on Gastrointestinal and Hepatic Gene Expression**. ECNP congress 2020 – September 2020

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Science communication

CE Gheorghe, *FameLab competition*: "**Physiology of stress** February 2020" (https://www.youtube.com/watch?v=RIiykL_eN40)

Abbreviations

5-HT : 5-Hydroxytryptamine

5-HIAA: 5-hydroxyindoleacetic acid

AANAT: Aralkylamine N-acetyltransferase

Abx: Antibiotic

ACTH: Adrenocorticotropic hormone

AHR: Aryl Hydrocarbon Receptor

Amy: Amygdala

ANS: Autonomic Nervous System

ASMT: N-Acetylserotonin O-methyltransferase

ATD: Acute Tryptophan Depletion

AUCg: Area Under the Curve were calculated with respect to ground

AVP: Arginine Vasopressin

BBB: Blood Brain Barrier

BDNF: Brain Derived Neurotrophic Factor

BDI: Beck Depression Inventory

BMAL1: Brain and muscle Arnt-like protein-1

BOLD: Blood-Oxygen-Level-dependent

CAR: Cortisol Awakening Response

CLOCK: Circadian locomotor output cycles kaput

CNS: Central Nervous System

CRH/F: Corticotropin-Releasing Hormone/Factor

CSF: Cerebrospinal fluid

CUMS: Chronic Unpredictable mild stress

CRY: Cryptochrome

CYP1a1: Cytochrome P450, family 1, subfamily A, polypeptide 1

DGBI: Disorders of gut–brain interaction

EC: Enterochromafin cells

FITC : Fluorescein-5-isothiocyanate-dextran

FMT : Faecal Microbiota Transplantation

GC: Glucocorticoid

GF: Germ-free

GI: Gastrointestinal

GPR35: G Protein-Coupled Receptor 35

HPA: Hypothalamus-Pituitary-Adrenal

HPLC: High performance liquid chromatography

Hip: Hippocampus

Hyp: Hypothalamus

IBS: Irritable Bowel Syndrome

IDO: Indoleamine 2,3-dioxygenase

I-FABP: Intestinal fatty acid binding protein

IFN: Interferon

IgA: Immunoglobuline A

IL: Interleukin

ILC3: innate lymphoid cells

KAT: kynurenine aminotransferase

KYNA/KA: Kynurenic Acid

KYN: Kynurenine

LBP : Lipopolysaccharide Binding protein

LC: Locus coeruleus

LNAA: Large Neutral Amino Acid

MDD: Major Depressive Disorder

MT1/2: melatonin receptor

NAc: Nucleus accumbens

NAD: Nicotinamide adenine dinucleotides

NFkB: Nuclear factor kappa-B

NMDA: N-methyl-D-aspartate

NMDA: N-methyl-D-aspartate (NMDA)

NTS: Nucleus tractus solitarius

PANAS: Positive Affect and Negative Affect Schedule

PGC-1α1: peroxisome proliferator-activated receptor-γ coactivator-1α1

PER: Period

PFC: Prefrontal Cortex

PVN: Paraventricular Nucleus of the Hypothalamus

PXR: Pregnane X receptor

QUIN/QA: Quinolinic Acid

RER: Respiratory Exchange Ratio

Rev-Erbα/NR1D: nuclear receptor subfamily 1 group D

Rorα: RAR-related orphan receptor alpha

SAM: Sympathetic-Adreno-Medullar

SCFA: Short-Chain Fatty Acid

SCN: Suprachiasmatic nucleus

SCZ: Schizophrenia

SPF: Specific Pathogen Free

SNS: Sympathetic Nervous system

SSRI: Selective Serotonin Reuptake Inhibitor

TDC: Tyrosine Decarboxylase

TDO: Tryptophan 2,3-dioxygenase

TEER: Transepithelial electrical resistance

TLR: Toll-like receptor

tnaA: Tryptophanase

TPH: Tryptophan Hydroxylase

TRP: Tryptophan

Trpa1: transient receptor potential ankyrin A1

VIP: Vasoactive Intestinal Peptide

VO_2 max: Maximum (max) rate (V) of oxygen (O_2)

ZT: Zeitgeber

Abstract

The involvement of the microbiota-gut-brain axis communication in brain function and behaviour has enabled a paradigm shift in neuroscience, neurogastroenterology and psychiatric research. Two key routes of this bidirectional communication are impaired in stress-related disorders: the hypothalamic-pituitary-adrenal axis and tryptophan metabolism. This crosstalk has been widely characterised following chronic stress, in stress-related disorders and gastrointestinal disorders comorbid with psychological distress. The acute transitory reaction to an acute stressor and the associated adaptation over time, however, is not well understood.

In this work, we investigated this neglected topic by evaluating the response to a psychological and a physical s stress exposure in terms host and microbial tryptophan metabolism and other pillars of the microbiota-gut-brain axis. For this purpose, we used well-recognised pre-clinical models (germ-free and antibiotic-treated mice) to decipher the role of gut microbes in the regulation of tryptophan metabolism and availability at baseline and following hypothalamic-pituitary-adrenal axis activation. We found that a 15-min acute stressor was sufficient to upregulate colonic 5-HT concentrations in conventional and re-colonised male mice while serotonin level was unaltered in GF male mice. We have identified novel region- and sex- dependent effects of the microbiota in modulating the expression of host rate-limiting enzyme of tryptophan metabolism following acute stress at both terminals of the gut-brain axis communication network (***Chapter 2***).

We found that acute stress induced functional alterations visible as increased intestinal permeability in the ileum (ex vivo and in vitro) depending on time of day. In the colon, alterations in the rate-limiting enzyme of tryptophan breakdown towards serotonin synthesis, *TPH-1*, was reduced by stress in conventional mice but increased in antibiotic treated mice following stress (***Chapter 3***). Furthermore, both microbial and host tryptophan metabolism were altered in the caecum of conventional mice following an acute stressor, which was disturbed in germ-free mice and partially restored following colonisation of germ-free animals (***Chapter 3***). Then, we assessed

in sedentary humans transient and adaptive changes to exercise along the microbiota-gut-brain axis at different exercise intensities. We discovered intensity-dependent effects on various pillars of the microbiota-gut-brain axis. While high-intensity training increased the cortisol awakening response chronically, it also led to an increase in peripheral serotonin level directly following a maximal exhaustion test. Light-intensity exercise lead to marked compositional alterations at the species level. Lastly, our work provides the first piece of evidence that the relative abundance of exercise-responsive bacteria with saccharolytic potential decreases with exercise intensity. Together, these results contribute to a deeper comprehension of the host-microbe dialogue driving the spatial and temporal dynamics of the physiological response and adaptation to various types of stress exposure. These observations open future research avenues and encourages a move towards the integrated physiological perspectives essential for the development of precision treatment options in stress-related disorders.

Table of Contents

Chapter 1: General Introduction

I. Stress, HPA axis and stress-related disorders

A. Definition of stress

Overtime, the definition of "stress" has evolved and to this day there is no universally accepted definition. Stress can broadly be described as a state of disturbed homeostasis due to physical, mental or emotional factors causing various somatic and mental adaptive responses (Chrousos, 2009). The key function of the stress response system is to release and produce energy for immediate use by depleting the body's energy reserves and inhibiting energy-intensive processes that are not essential for immediate survival (Harrell et al., 2016). This highly conserved physiological response is essential to maintain homeostasis by influencing the overall function of the body to adapt to a new state of energy demand. This adaptive process is referred to as "Allostasis" (McEwen & Akil, 2020). However, this adaptive process is costly, leading to "allostatic load", a concept which refers to the cost the body incurs when it must adapt to either repeated, pro-longed, inadequate or non-adaptive response to adverse psychosocial or physical circumstances that disturb homeostasis (McEwen, 2000; McEwen & Akil, 2020). The concepts of allostasis, allostatic load and overload, provide a framework to understand the positive and negative side of stress (McEwen, 2000, 2019) and its role as a common component in major mental illnesses (McEwen, 2003).

Understanding the consequences of stress exposures for health and wellbeing requires an appreciation of the underlying biological mediators. Stress involves physiological responses that can be pro-adaptive or maladaptive depending on the duration (acute or chronic) or frequency and nature of exposure (de Kloet et al., 2005), early-life stress (McEwen, 2008), genetics (Ising & Holsboer, 2006), resilience, vulnerability/susceptibility and ability to cope (Musazzi et al., 2017). Even though the adaptive response to stress is beneficial, maladaptive responses can significantly alter the body's homeodynamic state (hyper- or hypo-activation of the stress system) thereby compromising mental and physical health (Agorastos & Chrousos, 2022) and leading to stress-related neuropsychiatric disorders and contributing to metabolic

disorders. Importantly, early life stress has been associated with an increased risk in adulthood of many stress-related psychiatric disorders such as anxiety, depression and post-traumatic stress disorder (Syed & Nemeroff, 2017).

B. Physiological stress response: SAM and HPA axes as mediators of adaptation

The physiological response to stress is mediated by two main pathways: the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1**). These pathways are also highly interconnected with neuromodulatory brain circuits, regions and systems such as the amygdala (involved in the fear response), the circadian system (involved in 24h synchrony of our body) and the endocannabinoid system (involved in cognitive and physiological regulation) (Agorastos & Chrousos, 2022). Although activation of these pathways is essential to the stress response, sustained activity over longer time intervals or repeated exposures can accelerate disease processes due to "chronic exposure to fluctuating or heightened neural or neuroendocrine response" in the body that can be referred to as "allostatic load" (McEewen, 2000).

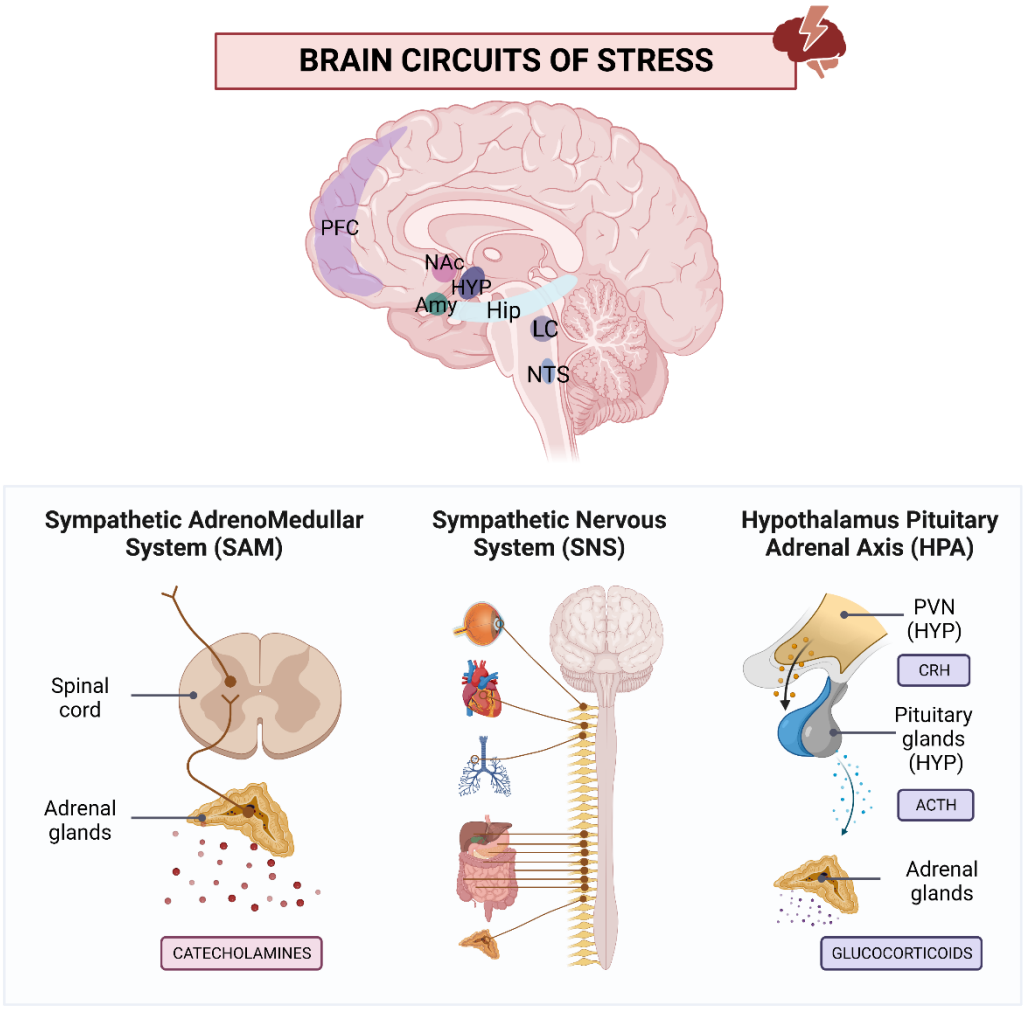

Figure 1. Brain circuits of stress: SAM, SNS and HPA axes
Following a stressor, the autonomic nervous system (ANS) will immediately activate the sympathetic nervous (SNS) and sympatho-adrenomedullary (SAM) system to stimulate the release of catecholamines (adrenaline, noradrenaline). The autonomic nervous system activation primes the body for a "flight or fight" response by regulating involuntary physiological processes including heart rate, blood pressure, respiration while inhibiting non-vital functions such as digestion and immune functions. On the other hand, HPA axis activation in the hypothalamus will trigger the release of CRF which in turn will trigger the release of ACTH from the pituitary, leading to the activation of the adrenal cortex to release glucocorticoids in the body – the main one being cortisol (Agorastos & Chrousos, 2022).Abbreviations: Prefrontal Cortex (PFC), Amygdala (Amy), Nucleus Accumbens (NAc), Hypothalamus (HYP), Hippocampus (Hip), Locus Coeruleus (LC), Nucleus Tractus Solitarius (NTS), Paraventricular Nucleus of the Hypothalamus (PVN), Corticotropin-Releasing Hormone (CRH), Adrenocorticotropic Hormone (ACTH). Figure created with BioRender.

We often view the response to stress as mediated by the HPA and SAM axis, because it is the first line response to an acute stressor. However, the response to stress evolves and adapts to create a new state of homeostasis in the whole body. In our society, stress has become undesirable and the nuances underappreciated, although the

response to stress exposures is an important coping and survival mechanism. The effect of long-term stress can be devastating but short-term stress appropriately activates important pathways to cope with the nature of the exposure, enhance chances of survival and protects against infection (Dhabhar, 2014). However, the physiological response to acute stressors can become maladaptive (Musazzi et al., 2017). The key is to have an equilibrium to stay on the good side of the stress spectrum by having a good lifestyle (sleep, nutrition, exercise) and good coping mechanisms (activities, psychosocial environment etc) to deal with the short-term stresses (Dhabhar, 2014). Obviously, when we talk about chronic stress or trauma, stress becomes deleterious (PTSD for example). In this work, we aim to understand how a stressor will impact not only the brain's response but also how it will affect the multiple pathways involved in the bidirectional communication framework of the gut-brain axis.

C. HPA axis activation by circadian cues

1. Circadian rhythm: definition

Circadian rhythms are investigated in multiple fields, having metabolic (Marcheva et al., 2013), immune (Scheiermann et al., 2013), mental (Walker et al., 2020) and behavioural (Walker et al., 2020) implications. Circadian rhythms are coordinated by a master clock located in an ancestral part of the brain called the hypothalamus - more specifically in the suprachiasmatic nucleus (SCN) (Mohawk et al., 2012). One major factor in circadian regulation is light cues from the sun across a 24-hour period. Once the suprachiasmatic nucleus (SCN) of the hypothalamus receives light information from the retina (Dibner et al., 2009), it communicates with the PVN which can then activate the HPA axis (Buijs et al., 2003).

The master clock in the SCN, our body timekeeper, sends central and peripheral signals to modulate behavioural, autonomic, and neuroendocrine circadian functions (Hastings et al., 2018). This tightly regulated system is controlled by a complex set of feedback loop mechanisms to regulate sleep/wake cycle, body temperature, energy metabolism and even behavioural functions (Mohawk et al., 2012). The central clock

orchestrates a common rhythm across the whole organism by synchronising with peripheral clocks through feedback loop of circadian genes expression in molecular clocks (**Figure 2**). These peripheral clocks are important for rhythms of cardiovascular, metabolic, endocrine, immune, digestive and reproductive systems (Richards & Gumz, 2012).

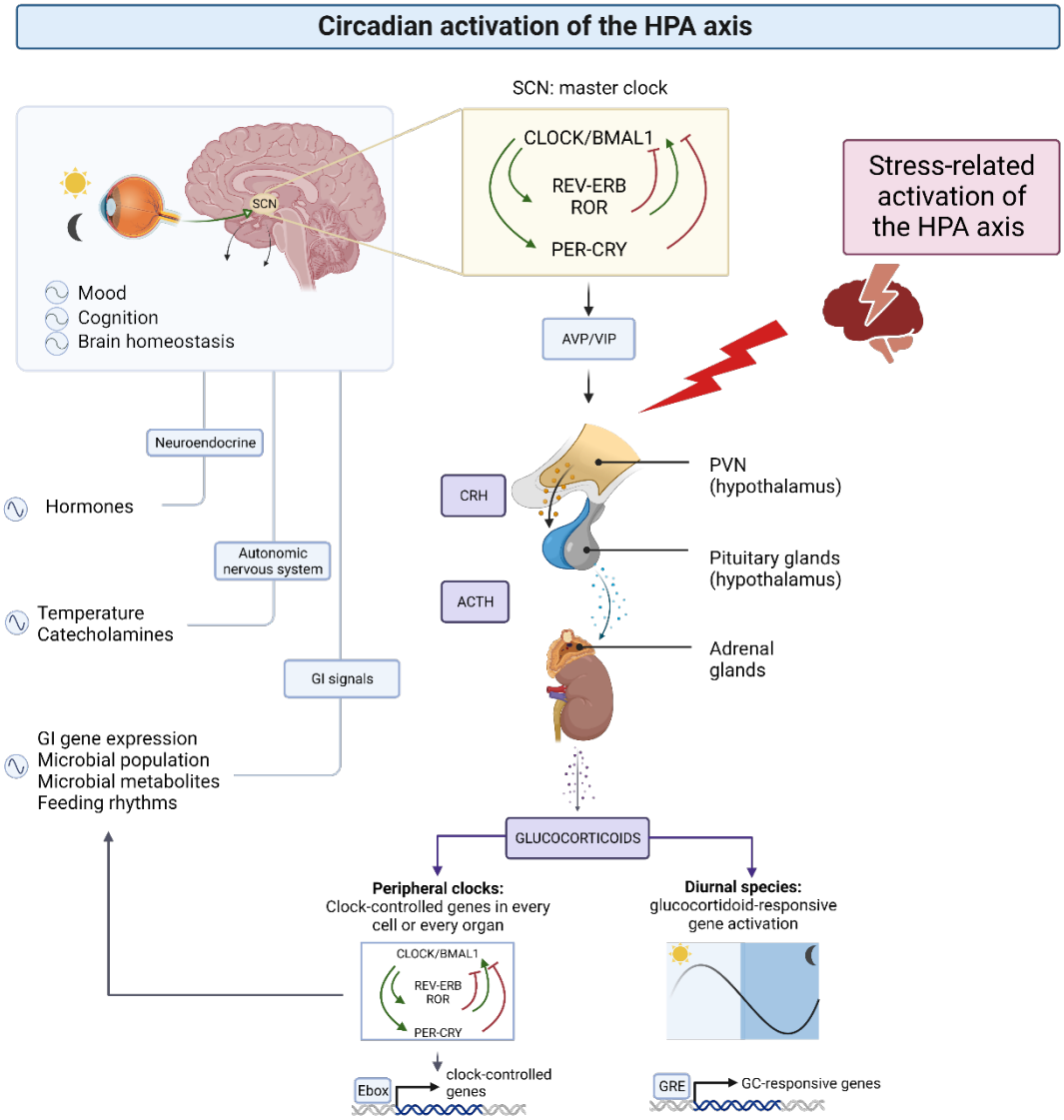

Figure 2. HPA axis activation under circadian and stress control

The master clock in our brain, located in the SCN of the hypothalamus, is activated by light cues to regulate our circadian rhythm. This ancestral mechanism is tightly regulated by negative feedback loops: the primary feedback loop consists of heterodimerisation of CLOCK with the transcription factors BMAL1 which activate the transcription of their own repressor: Period and Cryptochrome genes (Per1, Per2, Per3, Cry1, Cry2) (Ketchesin et al., 2020). Another circadian-controlled feedback loop consists in activation and transcription of Rev-Erbα and Rorα who will control BMAL1 transcription. Ultimately, this system will regulate the transcription of many circadian-controlled genes in the brain and periphery (Androulakis, 2021). Once activated, the SCN will release AVP and VIP into the PVN of the hypothalamus to activate the HPA axis cascade to produce systemic glucocorticoids for daily circadian function. Similarly, stress can activate the PVN directly to generate an increase in glucocorticoids production to cope with a physical, perceived, or psychological stressor. Circadian control of HPA axis activation leads to rhythmicity in systemic glucocorticoid production across 24 hours during the active phase (day for diurnal species like mammals and night for nocturnal species like rodents). Glucocorticoid release will lead to the transcription of glucocorticoid responsive gene and activate peripheral clock for the transcription of clock-controlled genes, this mechanism is ubiquitous. Abbreviations: Hypothalamus-Pituitary-Adrenal (HPA), Suprachiasmatic Nucleus (SCN), Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and muscle Arnt-like protein-1 (BMAL1), Arginine Vasopressin (AVP), Vasoactive Intestinal Peptide (VIP), Paraventricular Nucleus of the Hypothalamus (PVN). Figure created with BioRender.

2. Circadian variation of glucocorticoid release

The circadian rhythmicity of the HPA axis is one of the best documented cyclic endocrine activities, with daily variations in circulating glucocorticoids (Moreira et al., 2018). Interestingly, each component of the HPA axis displays a unique rhythmicity of functional and clock gene expression (Girotti et al., 2009). Glucocorticoids released are key players in peripheral circadian communication from the SCN to the periphery (Balsalobre et al., 2000). Circadian signals from the SCN in the hypothalamus are propagated in other regions of the hypothalamus: the paraventricular nucleus (PVN) and the pituitary gland from where the HPA axis cascade can start to release glucocorticoids in the periphery in a rhythmic fashion. In response to circadian signals, the main glucocorticoid increases during the active phase: cortisol in the morning in humans and corticosterone in the evening in rodents.

Glucocorticoids released in the circulation bind to glucocorticoid and mineralocorticoid receptors – to regulate essential processes such as body temperature, energy balance, water and electrolytes balance, digestion, immune activation and circadian rhythmicity (Timmermans et al., 2019). Therefore, glucocorticoids play a key role in fundamental aspects of circadian rhythmicity as well as during stress exposures when the body must adapt to an increase in energy demand. Cortisol has a higher affinity for mineralocorticoid receptors; therefore, in the daily rhythm of cortisol production, mineralocorticoids receptors will be solicited. However, when a high concentration of cortisol arises, as in the biological response to a stressful situation, cortisol will bind to glucocorticoid receptors to respond to this situation (Stephens & Wand, 2012). GR can be found in most tissues whereas MR are in the brain, kidney, colon, heart and sweat glands (Funder, 2005). Studies using adrenalectomised animals suggests that adrenal glands are the main source of GC production (Cima et al., 2004). However, glucocorticoids can be produced in the thymus (Talaber et al., 2015), brain (Gomez-Sanchez 1996) and epithelial cell barriers (Cima et al., 2004).

D. Circadian rhythmicity, HPA axis reactivity and the acute stress response: focus on clinical Studies

In clinical studies, HPA axis activation triggered by circadian signals, can be measured by evaluating the cortisol awakening response (CAR) in saliva samples during the first hour of awakening (Stalder et al., 2016a). A healthy CAR response is characterised by an increase in cortisol secretion 30-45mins post-awakening which returns to baseline after 30 mins (Kudielka & Kirschbaum, 2003). Deviations from typical CAR pattern can indicate dysfunctional neuroendocrine signals (Stalder et al., 2016b). The CAR response is highly influenced by time of awakening, age, and overall health status. CAR has been extensively used as a psychoneuroendocrinological marker in psychosocial, physical and mental health research (Stalder et al., 2016b). Indeed, CAR is often used in clinical studies as a measure of HPA axis reactivity in psychiatric conditions although its regulation differs from cortisol secretion across the day (Clow, Hucklebridge and Thorn et al 2010; Dedovic, Katarina; Ngiam, Janice (2015)) and reflects mainly circadian regulation of cortisol secretion (Stalder et al., 2016b).

In clinical research, the impact of psychological stress is often studied by generating an acute stress response in participants (A. P. Allen et al., 2014). The most used tests involve social evaluation that are frequently coupled with a physical stressor (cold, noise, exercise). Chronic, traumatic, or repeated stressors are often reported through questionnaires and many studies focus on perceived or reported stress (Crosswell & Lockwood, 2020). It is unsurprising to see differences in HPA axis reactivity depending on time of day in response to a psychological (Yamanaka et al., 2019) or physical stressor (Bonato et al., 2017), suggesting a circadian component in the response to a stressor. However, conflicting results exists on differences in psychological response to a stressor in clinical studies (TSST) where some studies suggest a difference in stress response (A. P. Allen et al., 2014; Yamanaka et al., 2019) and others suggests a similar response to stress but an increased adrenal gland sensitivity to ACTH during the active phase (morning) (Kudielka et al., 2004). Similarly, perceived stress has been shown to be increased in a clinical population during acute sleep deprivation with an increase

in cortisol levels whilst circadian misalignment increased systemic immune markers but reduced 24-hour cortisol levels (Wright et al., 2015).

Depressed individuals (Bhagwagar et al., 2005) or patients with depression in remission (Bhagwagar et al., 2003) showed an increase in the CAR response. On the contrary, PTSD patients showed a significantly reduced CAR response (Wessa et al., 2006). A meta-analysis showed that CAR response was positively associated with stress whereas it was negatively correlated with fatigue and exhaustion (Chida & Steptoe, 2009). Interestingly, a reduced CAR response was also found in patients with irritable bowel syndrome (IBS) (Groeger et al., 2023; P. J. Kennedy et al., 2014), a gastrointestinal disorder of gut-brain axis interactions often coupled with psychiatric symptomatology. These findings underscore the importance of the links between circadian rhythm and stress-induced activation of the HPA axis in stress-related disorders.

E. Stress-related disorders and gastrointestinal comorbidities

Severe or repeated stress exposure can result in a maladaptive response, and when challenges exceed one's ability to cope, it is referred to as allostatic overload (Guidi et al., 2021), which can induce and exacerbate disease processes throughout the body. An essential question that remains open in the field is to understand when and how the response to a prolonged or repeated acute stress becomes maladaptive; and when does a drastic change in allostatic load transition into stress-related disorders (Musazzi et al., 2017). In this work, we will focus on the effect of acute and chronic stress along the microbiota-gut-brain axis. A stress can be defined as acute if it lasts for a period of minutes to hours whilst chronic stress refers to a stress that persists from several hours per week to months (Dhabhar, 2014). Studying both acute and chronic stress has important implications in stress-related mental disorders (i.e. anxiety disorders, major depressive disorder (MDD), post-traumatic stress disorder (PTSD), obsessive-compulsive disorder, chronic fatigue syndrome, premenstrual syndrome and attention deficit/hyperactivity disorder (ADHD) (Stefanaki et al., 2021)). There are also important examples of gastrointestinal disorders with psychiatric comorbidities (**Figure 3**) such as IBS (Wilmes et al., 2021).

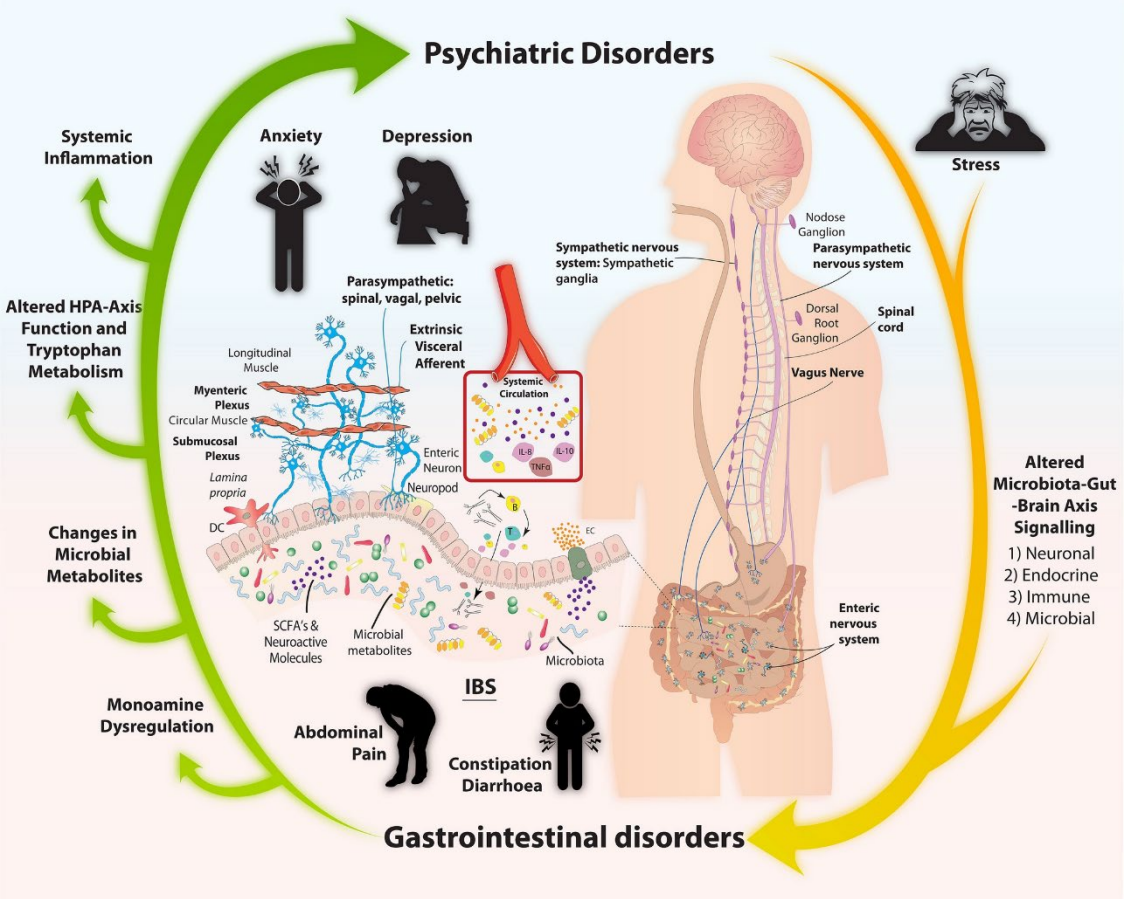

Figure 3. Psychiatric disorders and gastrointestinal comorbidities: signalling pathways of the microbiota-gut-brain axis. Several routes of communication of the microbiota-gut-brain axis are impaired in both psychiatric disorders and gastrointestinal disorders such as IBS. Stress is a major predisposing factor of the development of irritable bowel syndrome (IBS) and stress-related disorders are often comorbid with gastrointestinal symptoms; both having well-documented changes in gut microbial composition and function. Therefore, the (1) Neuronal, (2) Endocrine, (3) Immune and (4) Microbial bidirectional communication between the gut and the brain contribute to psychiatric and gastrointestinal symptoms. Figure from (Wilmes et al., 2021)

1. Acute stress

Over fifty years ago, Seyle defined acute stress as being "a general alarm of the organism when suddenly confronted with a critical situation" (Selye, 1936). Whether the threat is real (physical or psychological stressor) or anticipatory (psychological stressor), this will elicit a series of physical, emotional and behavioural responses to cope adequately with the situation. This disturbance in body's homeostasis following a stressor is referred as "allostasis" (Agorastos & Chrousos, 2022). Three potential outcomes have been identified following an acute stressor: return to basal functional homeostasis, decrease in homodynamic balance (negative adaptation) or improved

homodynamic capacity (Agorastos & Chrousos, 2022). While stress-related disorders and co-morbid conditions associated with an elevated or dysfunctional stress response have been extensively studied, research about complex pathways triggered by acute stress is limited, with a majority of studies focusing on its effect in the brain only – specifically the hippocampus (Floriou-Servou et al., 2018, 2021; von Ziegler et al., 2022).

The biological consequence of acute stress is a physiological response, often called "fight of flight", designed to mobilise energy allowing an organism to respond to the stressful situation. Short-term adaptations to a stressor are mediated by the Autonomic Nervous System (ANS) and will lead to an increase in blood glucose and pressure, increase in heart rate, stimulation of the immune system and shutdown of the digestive and reproductive systems. In the brain, 10 mins of acute restraint stress in mice is enough to alter the levels of glucocorticoids and mineralocorticoid receptors in the prefrontal cortex, the hippocampus, and the hypothalamus (Gadek-Michalska et al., 2013). Acute stress also activates the HPA axis which triggers an increase in plasma ACTH and corticosterone levels for up to 1-2 hours after stress (Gadek-Michalska et al., 2013). However, many species often habituate to the same repeated acute stress exposure applied over several days, reflected in a blunted increase in plasma corticosterone (Gadek-Michalska et al., 2013).

2. Chronic stress

Despite the well-established link between stress and psychiatric diseases, ongoing research is needed to better understand the intricate mechanisms by which stress triggers pathological changes that increases susceptible to psychiatric illnesses. Acute and chronic stress exposure can result in higher risk for mental disorders – particularly depression, anxiety disorders and post-traumatic stress disorder (PTSD)- and physical co-morbidity. Chronic stress is studied to understand this shift between stress and pathological changes. Chronic hyperactivation of HPA and ANS can lead to hyper- or hypo-corticotisolism which affects central and peripheral mechanisms through glucocorticoid and mineralocorticoid receptors (Agorastos & Chrousos, 2022). A prolonged or repeated increase in HPA axis activity can lead to conditions such as

chronic stress, melancholic depression, anorexia nervosa, obsessive-compulsive disorder and metabolic syndrome (Chrousos, 2009). The initial adaptive response can transition to a compensatory HPA axis downregulation. Several conditions have been associated with a decrease in HPA axis activity such as fibromyalgia, chronic fatigue syndrome, adrenal insufficiency, post-traumatic stress disorder, asthma, and hypothyroidism (Chrousos, 2009).

Chronic stress can be viewed as a cascade of physiological dysfunction due to an overactivation of SAM and HPA axes, which plays an important role in stress-related disease development and progression (McEwen, 2000). Our bodies can compensate for this overactivation leading to a modification of the basal functioning to maintain body functions. Ultimately, chronic stress can be characterised by changes in metabolic (insulin, glucose, cholesterol), immune (IL6, CRP, TNFα) and cardiovascular (blood pressure, heart rate) parameters (Juster et al., 2010). Interestingly, HPA axis activation can also be triggered by immune activation, since there is an important bidirectional communication between the HPA axis and the immune system (A. J. Dunn, 2007).

In acute conditions, cortisol – the main glucocorticoid secreted – is meant to rise and then decrease, inducing transient activation of the immune system (Dhabhar, 2014). However, chronic stress exposure can lead to an over- or under- production of cortisol (Juster et al., 2010) and desensitization of immune cells to glucocorticoids signalling leading to a state of "glucocorticoid resistance" (Zefferino et al., 2021). Glucocorticoid resistance in the periphery leads to an increase in peripheral inflammatory mediators (Pariante & Lightman, 2008; Rohleder, 2012) leading to a state of low-grade inflammation often observed in stress-related disorders. Hyper- and hypocortisolemia associated with chronic stress has been linked to many disorders (i.e. cardiovascular disease hypo/hypertension, fibromyalgia, hyper/hypothyroidism, asthma, eczema, osteoporosis, diabetes mellitus (Agorastos & Chrousos, 2022)) and various functional gastrointestinal diseases often liked to immune dysregulation (Barnes & Adcock, 2009). Following stress, immune responses can be immunoprotective, immunopathological or immunoinhibitory (Dhabhar, 2014),

chronically leading to stress-related disorders and influencing metabolic, immune, endocrine, gastrointestinal and cardiovascular dysfunction in different disorders (Agorastos & Chrousos, 2022).

Chronic stress exposure can have a profound impact on brain immunity and homeostasis. In the brain, microglial cells – the brain's resident macrophages - oversee immunity and help maintain brain homeostasis (Thion et al., 2018). Microglia can respond to changes triggered by chronic stress response with increased neuroinflammation and might contribute to the development and progression of stress-related disorders (Picard et al., 2021). Chronic stress has also been linked to important changes in neuronal network and structural remodelling in brain regions highly susceptible to stress such as the prefrontal cortex, the amygdala, the hippocampus (McEwen et al., 2016) and the hypothalamus (Bains et al., 2015). Similarly, chronic stress exposure can disrupt the blood brain barrier, which facilitates the passage of immune cells and mediators from the periphery to the brain (Menard et al., 2017). Ultimately, chronic stress can lead to a profound homeostasis disruption resulting in an imbalance in nervous system functions leading to structural and functional changes especially in the hippocampus and the amygdala (Yaribeygi et al., 2017).

3. Stress- related disorders

Over time, chronic stress can result in significant and long-lasting alterations in baseline HPA axis function and stress reactivity (Herman et al., 2016). These changes are a key driver in the development of stress-related disorders such as depression (Pariante, 2003). Repeated exposure to stress can lead to a habituation, sensitization, or exhaustion of HPA axis activation (Herman et al., 2016). Chronic stress produces significant habituation in corticosterone responses mainly through a feedback loop involving mineralocorticoid receptors (Cole et al., 2000). In the brain, chronic stress exposure alters the shape and number of neurons and glia and stress-related disorders are associated with decreased volumes of limbic brain regions (Duman, 2009). Glucocorticoid receptors (GR) expression, key in mediating stress-related HPA axis activation, were found to be reduces in post-mortem analysis of psychiatric

patients in a region-dependent manner. In MDD, mRNA expression of GR was decreased in the frontal cortex and in the dentate gyrus of the hippocampus, suggesting altered GR functioning in a region-dependent manner in psychiatric disorders (Webster et al., 2002). All these structural and functional changes in specific brain regions and nuclei (hippocampus, prefrontal cortex, cingulate cortex, nucleus accumbens and amygdala) controlling mood, anxiety, cognition and fear lead to the emergence of stress-related psychiatric disorders (Duman, 2009). Similarly, immune and endocrine changes in response to chronic stress have important implications along the gut-brain axis, leading to psychiatric illnesses with gastrointestinal symptomatology or gastrointestinal disorders with psychiatric comorbidity (Gracie et al., 2019).

4. Circadian rhythm disruption in stress-related disorders

Both stress and circadian rhythm cues activate HPA axis release of glucocorticoids to mobilise energy or synchronise rhythm across tissues (Kling & Landgraf, 2021). Just as there is a dysregulation of the circadian rhythm in stress-related disorders (Walker et al., 2020), endocrine diseases such as Addison's or Cushing's – represented by adrenal gland insufficiency and endogenous cortisol excess, respectively – are associated with disturbed rhythms (Alachkar et al., 2022). Even healthy individuals experienced symptoms associated with circadian rhythm disruption due to jet lag following travel: headache, insomnia, fatigue, gastrointestinal discomfort and irritability (Choy & Salbu, 2011). A systematic review reported that a flatter diurnal cortisol rhythm across the day is associated with poorer mental and physical health (Adam et al., 2017). Shift workers reported circadian changes (insomnia, sleepiness) and affective symptoms (irritability, depression) (Walker et al., 2020). A recent meta-analysis pointed out an increased risk for depression in shift workers (A. Lee et al., 2017). Similarly, seasonal affective disorder is characterised by the emergence of depressive symptoms occurring during the winter months as the days shorten (R. W. Lam & Levitan, 2000).

In 1988, the social zeitgeber theory suggests that a disruption of social rhythms could cause an instability in biological rhythms that could trigger depressive episode in

vulnerable individuals (Ehlers et al., 1988). Since then, dysregulation of circadian rhythmicity in mood and stress-related disorders have been increasingly studied (Walker et al., 2020). Abnormal or blunted circadian rhythms can alter body temperature, plasma cortisol, dopamine, norepinephrine, thyroid stimulating hormone, blood pressure and melatonin levels (McClung, 2007). It is unsurprising to see such a tight implication of circadian rhythm in stress- and mood- related disorders since important neurotransmitters involved in these disorders have a strong rhythm.

Some scientists even proposed a new clinical phenotype called "circadian depression" based on the observation that circadian dysregulation is very prevalent in mood disorders and particularly in depression (Carpenter et al., 2021). Indeed, according to the DSM-5, MDD is characterised by a depressed mood, loss of interest or pleasure, weight loss/gain, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue amongst other (American Psychiatric Association, 2013). Circadian dysregulation could play an important role in core symptoms by influencing overall changes in metabolism, sleep patterns, appetite and, most importantly, levels of important circadian neurotransmitters such as serotonin (Carpenter et al., 2021). Conversely, it has also been suggested that stress-induced disturbances of the serotonergic system may cause circadian rhythm disturbances, thereby increasing susceptibility to depression (Daut & Fonken, 2019) This hypothesis puts serotonin as a central player linking stress-related disorders and circadian rhythm disruption.

Some pharmacological treatments for stress-related disorders, such as fluoxetine (a common selective serotonin reuptake inhibitor (SSRIs), play an important role in restoring rhythmicity of serotonin in the brain (Tan et al., 2007). Similarly, molecules acting on melatonin receptors demonstrated antidepressant properties by modulating circadian disruption in depression (Carpenter et al., 2021; Daut & Fonken, 2019). A new field of research, called chronopharmacology, is interested in circadian influences on drug handling and efficacy (M. Li & Stewart, 2022), which is particularly relevant for psychiatric disorders. Chronopharmacological strategies were used to investigate chronotherapy for the treatment of depression (Silva et al., 2021) and to normalise circulating glucocorticoids levels for a variety of physical and mental

disorders (Oster et al., 2017). Similarly, some specific type of drugs, called chronobiotics, are used to synchronised or increase amplitude of circadian rhythms (Cardinali, 2019).

5. Gut permeability dysfunction and stress-related disorders

The primary function of stress is to focus on energy release and production for immediate use, resulting in the cessation of all non-immediate survival energy expenditure processes. This includes digestive processes, highly energy consuming. Not surprisingly, chronic or maladaptive stress can have a significant impact on the gut and bidirectional gut-brain communication. Recently, disorders of the gut-brain axis (DGBI), formerly known as functional gastrointestinal disorders, have received new insights. DGBI are often characterised by a disruptions or imbalances in gut microbial community composition, an alteration in mucosal immune function, an alteration in gut signalling leading to visceral hypersensitivity as well as CNS dysregulation leading to changes in gut signalling and motility (Drossman & Hasler, 2016) This includes several disorders from the oesophagus to the anorectal ring (Drossman & Hasler, 2016; Person & Keefer, 2021). As an example, we will focus on IBS, a well characterised and well-studied DGBI (Simons et al., 2022; Sperber, 2021). IBS is characterised by abdominal pain or discomfort and altered bowel habits manifesting as diarrhoea and constipation (Longstreth et al., 2006) divided in different subtypes depending on the predominant GI symptoms (Drossman & Hasler, 2016).

Notably, disorders of gut-brain interaction are associated with impaired quality of life (Knowles et al., 2022). More specifically, IBS severity is impacted by maladaptive copying style such as catastrophizing and somatization (Hunt et al., 2009; van Tilburg et al., 2013) and has been shown to be comorbid with psychiatric conditions in 80% of cases in a systematic review (Singh et al., 2012). Indeed, stressful (O'malley et al., 2011) or early adverse life events (Park et al., 2016) are associated with an increased risk of developing or exacerbating IBS (Person & Keefer, 2021). Mechanistically, this could be explained by an increase HPA axis responsiveness in IBS (Videlock et al., 2009) and an overactivation of the sympathetic arm of the ANS (Gracie et al., 2019). A systematic review and meta-analysis confirmed that IBS patients had increased level

of anxiety and depression compared to controls, without any specific subtype of IBS being identified as being more likely to be associated with higher psychiatric comorbidities (Fond et al., 2014). This raises the question of whether the intestinal symptoms associated with IBS are the cause of psychiatric comorbidities, or whether, conversely, the anxiety and depressive symptoms induces GI symptoms.

Indeed, both acute and chronic stress modulate different layers of the gastrointestinal tract from microbiota composition to the physical barrier (mucus, epithelium cells), and of course the immune barrier and enteric nervous system function. This bidirectional communication between the brain and the gut can act directly (vagus nerve) or indirectly (metabolites secretion, modulation of gut microbiome and immunity) causing changes in gut permeability, function, and motility in response to stress. It is thus not surprising that stress-related disorders such as anxiety and depression have comorbid gastrointestinal symptoms (Mussell et al., 2008). Recently, an important study has been conducted to disentangle shared genetic aetiology between four GI diseases and six different psychiatric disorders demonstrating genome-wide distribution of genetic factors linking psychiatric and gastrointestinal disorders (Gong et al., 2023), suggesting an important link between the two, that could be explained by the bidirectional communication between the brain and the gut.

II. Systemic and CNS tryptophan metabolism: A key player in stress-related disorders

A. Tryptophan metabolism: overview

Tryptophan is an essential amino acid available in the gut after digestion, where it can be absorbed through the intestinal epithelium to enter the bloodstream. Tryptophan is utilised for protein synthesis by every living cell but is also the sole precursor of important neuroactive molecules – the most widely known being serotonin and melatonin, both highly relevant for stress-related disorders and circadian rhythms. However, tryptophan metabolites that become available in the systemic circulation can migrate to target sites both peripherally and, in the CNS, where they play crucial roles. In the circulation, 90% of tryptophan is found bound to albumin; only the rest, found in free form, can cross the blood brain barrier although there may be some dissociation to consider at the cerebral microvasculature (Ruddick et al., 2006). Outside of protein synthesis, tryptophan can be metabolised by three major pathways: serotonin, kynurenine, and indole. Molecules generated from these three pathways greatly influence the gut-brain axis communication (**Figure 4**). The indole pathway is exclusively microbial, meaning that gut bacteria can have a direct or indirect control on gut tryptophan metabolism thereby changing tryptophan availability to the host. Microbial metabolism of tryptophan generates a range of indoles with diverse biological activity (A. Allison et al., 2018). The combination of microbial and host gastrointestinal metabolism of tryptophan (Lavelle & Sokol, 2020) is thus likely an important factor in the systemic availability of tryptophan, as well as indoles, kynurenine and serotonin produced locally (Roager & Licht, 2018). This may have important implications for the treatment of both gastrointestinal and psychiatric disorders (Gheorghe et al., 2019; Michaudel et al., 2022).

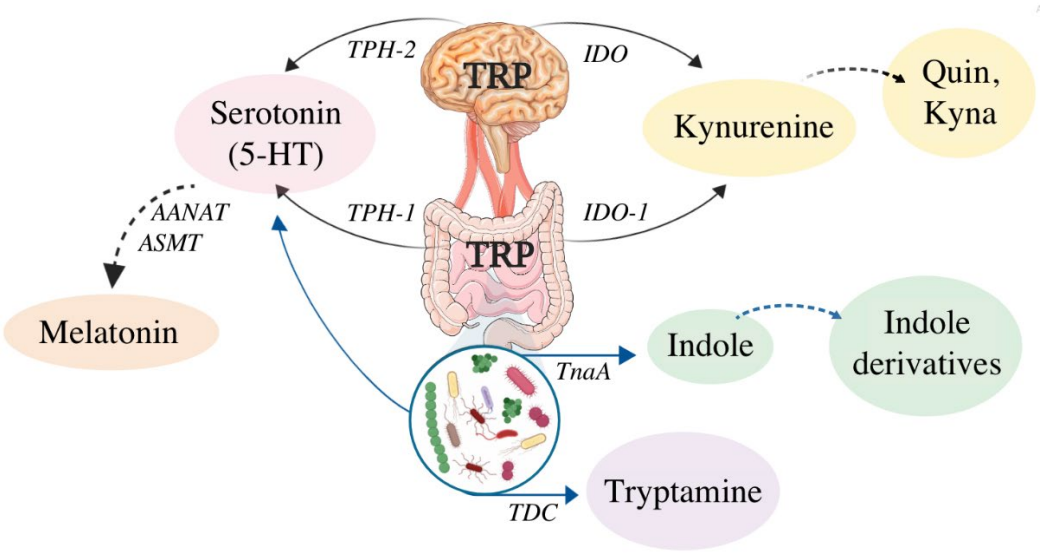

Figure 4. Tryptophan metabolism and the Microbiome-gut-brain axis.
An essential amino acid obtained from dietary proteins, tryptophan serves as a precursor to a variety of imperative bioactive molecules, some generated by the host and some fabricated by gut microbes (indicated by blue arrows). The most widely known fate of tryptophan is conversion into 5-HT and melatonin downstream. Nevertheless, a large majority of tryptophan is metabolised along the kynurenine pathway giving rise to molecules often collectively referred to as "kynurenines". The availability of tryptophan is also altered by gut microbes generating either indole and its derivatives, tryptamine or even 5-HT which can impact on gastrointestinal function via GPCR activation. An increasing number of studies highlight the importance of this pathway in metabolic and psychiatric disorders. Figure from (Gheorghe et al., 2019)Abbreviations: 5-hydroxytryptamine (5-HT aka serotonin), Aralkylamine N-acetyltransferase (AANAT), N-Acetylserotonin O-methyltransferase (ASMT), Tryptophan Hydroxylase (TPH), Indoleamine 2,3-dioxygenase (IDO), G Protein-Coupled receptor (GPCR), Quinolinic Acid (Quin), Kynurenic Acid (Kyna), Tryptophanase (tnaA), Tyrosine Decarboxylase (TDC)

B. Tryptophan metabolism in the brain

1. Tryptophan

Several factors influence circulating tryptophan levels such as diet, stress, immune activation, exercise (**Figure 5**) which ultimately influence central level of tryptophan (Höglund et al., 2019). Ultimately, tryptophan availability in the brain depends on free circulating tryptophan levels and on circulating levels of other large neutral amino acids (valine, isoleucine, leucine, tyrosine, phenylalanine and methionine) since they enter through the same transporter (Fernstrom & Wurtman, 1972; Höglund et al., 2019). Therefore, a reduction in TRP/LNAA ratio is a common method to estimate brain tryptophan availability (van Donkelaar et al., 2011). Activation of the SNS, and the release of its mediators, increases free tryptophan levels in the circulation by influencing lipolysis (Chaouloff, 1993). Once in the brain, tryptophan is available for protein synthesis in all cells. However, some cells require a tryptophan supply to

generate important neuroactive compounds, such as serotonin and kynurenine metabolites. Serotonergic neurons and mast cells can generate serotonin from tryptophan. On the other hand, kynurenine and its metabolites are produced by brain immune cells – namely astrocytes, microglia, macrophages, and dendritic cells (Ruddick et al., 2006).

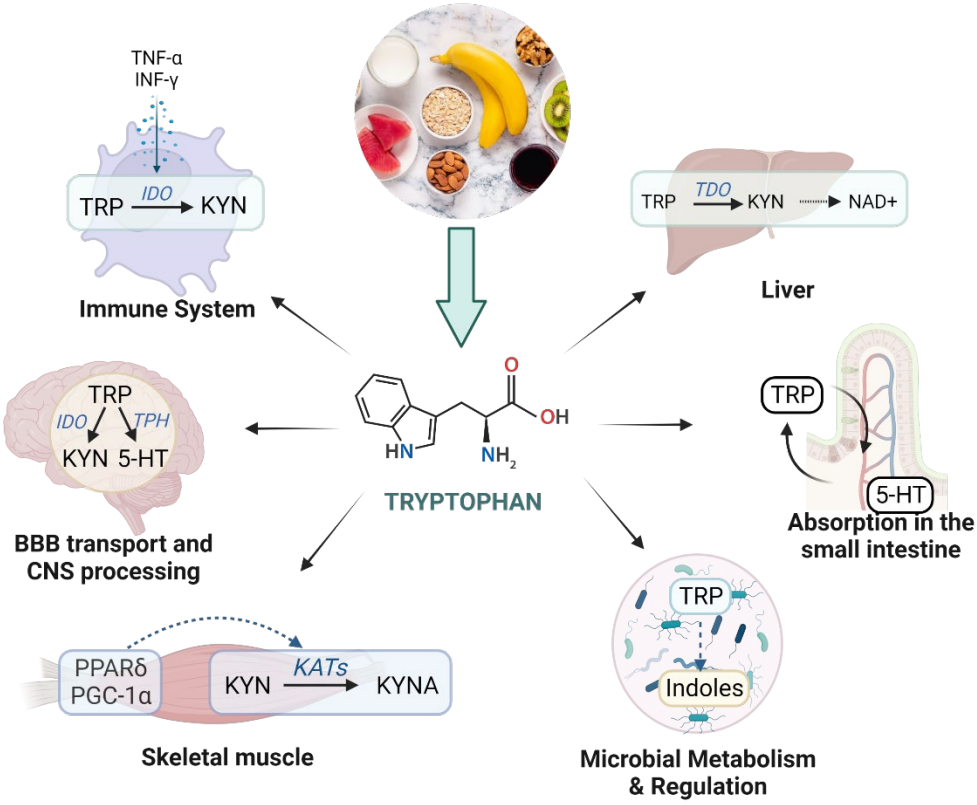

Figure 5. Factors influencing peripheral and central level of tryptophan metabolism. Several factors influence tryptophan metabolism and availability, which ultimately determine brain's ability to secrete serotonin. Tryptophan gets mostly breakdown towards the kynurenine pathway (IDO and TDO enzymes), generating bioactive metabolites. After absorption of tryptophan in the small intestine, tryptophan can be further breakdown by immune cells, skeletal muscle, enterochromaffin cells and hepatocytes. Tryptophan also degraded towards the indole pathway in the gut, exclusively microbial. Ultimately, peripheral degradation of tryptophan directly changes the availability of tryptophan in the central nervous system where it can be metabolised further towards the kynurenine (IDO) or serotonin (TPH) pathway. Figure adapted from (G. Clarke et al., 2021) Figure created with BioRender.

2. Serotonin

Serotonin is one of the best-characterised neurotransmitters and has received much attention as it regulates a variety of behavioural and cognitive functions, including mood, perception, reward, anger, aggression, appetite, memory, sexuality, and

attention (Berger et al., 2009). Serotonin is produced from tryptophan by the rate-limiting enzyme tryptophan-hydroxylase 1 (TPH1) in pinealocytes and TPH2 in neurons (Keszthelyi et al., 2009). Importantly, the rate of serotonin synthesis in the brain is dependent on its precursors crossing the BBB (Ruddick et al., 2006): tryptophan (Fernstrom & Wurtman, 1971) or 5-HTP (Ruddick et al., 2006)). Disruptions of the serotonergic system has been linked to the pathophysiology of numerous mental and neurological illnesses hence the prevalence of drugs targeting brain serotonergic system. Serotonin neurons from the raphe nuclei – where most serotonin-producing neurons are found - project throughout the brainstem and the brain providing serotonin to the rest of the CNS (Berger et al., 2009). Since all brain regions express various types of serotonin receptors, the CNS serotonin neurons affect the activity of a wide range of human brain circuits, leading to its pleiotropic effect. Serotonin receptors have been implicated in many behavioural effects in rodent and clinical studies supporting evidence of a strong influence of serotonin in the central nervous system behavioural function (Sharp & Barnes, 2020).

3. Melatonin

Melatonin is mostly produced in the pineal gland from serotonin (Zefferino et al., 2021) but can be also secreted elsewhere in minimal concentration including in enterochromaffin cells of the gastrointestinal tract. Interestingly, gastrointestinal concentrations exceed circulating levels of melatonin (Tordjman et al., 2017). In the brain, melatonin is a key molecule regulating circadian rhythms: its synthesis depends on light cues received by the SCN and is increased by darkness in diurnal species which helps synchronizing sleep-wake cycles (Tordjman et al., 2017). Its chronobiotic properties makes melatonin a key molecule to influence central and peripheral biological rhythms (Cardinali, 2019; Tordjman et al., 2017). Melatonin is also well-known for its cytoprotective and antioxidant effect – reducing low inflammation damage and preventing oxidative damage (Cardinali, 2019). Melatonin affects multiple molecular pathways including immune function, apoptosis, proliferation, angiogenesis, oxidative stress, metastasis and even sexual behaviour (Simonneaux, 2003).

4. Kynurenine

Brain kynurenine breakdown from tryptophan is mostly produced in inflammatory cells: macrophages (Chiarugi et al., 2001) and microglial cells (Guillemin· et al., 2003); and in astrocytes (Guillemin et al., 2001). Under normal conditions, CNS degradation of tryptophan through the kynurenine pathway is primarily to store glycogen and produce NAD+ from QUIN (Qin et al., 2018). Kynurenine can be further broken into important neuroactive molecules such as kynurenic acid (KYNA) (Schwarcz & Stone, 2017) and quinolinic acid (QUIN) (Guillemin, 2012; Schwarcz & Stone, 2017) – known to be neuroprotective and neurotoxic, respectively by acting on N-methyl D-aspartate NMDA receptor, KYNA being an antagonist and QUIN an agonist (Gheorghe et al., 2019; Schwarcz & Stone, 2017). KYNA and QUIN are unable to cross the BBB, only their precursor kynurenine is, therefore, kynurenine produced in the periphery is likely an important source for CNS metabolism and a key factor for brain function.

C. Brain tryptophan metabolism following stress and in stress-related disorders

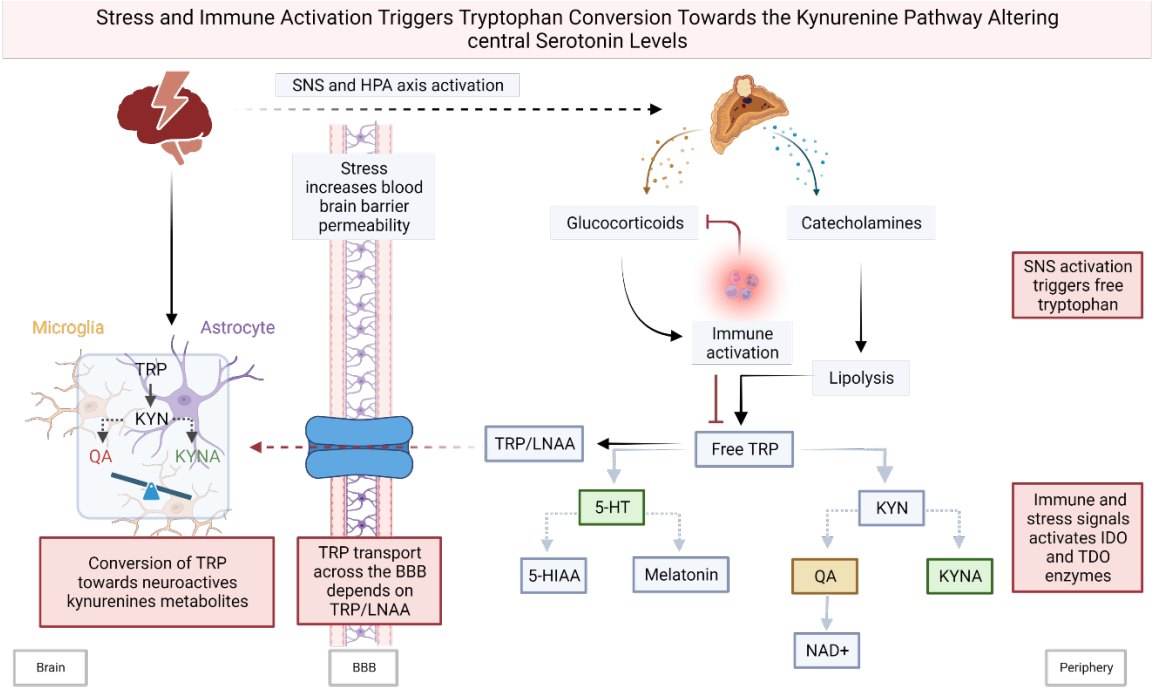

Figure 6. Stress and immune activation triggers tryptophan conversion towards the kynurenine pathway altering central serotonin levels. Following a stressor, activation of the HPA and SN causes the adrenal glands to release catecholamines and glucocorticoids, which causes tryptophan to unbind from albumin. Changes in free tryptophan in the circulation leads to the breakdown of tryptophan. Moreover, free tryptophan competes with other large neutral amino acid (LNAA) to cross the blood brain barrier. In the brain, stress triggers IDO-1 expression in astrocytes and microglia converting tryptophan into kynurenine metabolites (QUIN and KYNA). Both downstream metabolites are neuromodulators by acting on NMDA receptors. Excessive activation of the kynurenine metabolism in the brain can lead to neurotoxic consequences observed in clinical psychiatry such as depression. Abbreviations: Kynurenine (KYN), Quinolinic Acid (QA), Kynurenic Acid (KYNA), Tryptophan (TRP), Blood Brain Barrier (BBB), Sympathetic Nervous System (SNS), Hypothalamus-Pituitary-Adrenal (HPA) axis, Indoleamine-pyrrole 2,3-dioxygenase (IDO), Tryptophan 2,3-dioxygenase (TDO), Nicotinamide adenine dinucleotide (NAD). Figure created with BioRender.

1. Tryptophan

Interestingly, acute stress appears to increase tryptophan levels in the brain, as opposed to the decreases reported following chronic stress (A. J. Dunn & Welch, 1991; Höglund et al., 2019). Moreover, elevation of central tryptophan levels following acute but not chronic stress has been shown to be at least in part mediated by sympathetic activation (A. J. Dunn & Welch, 1991). Stress- or immune- challenges have shown to contribute to brain tryptophan concentration (Ruddick et al., 2006). Interestingly, cytokine therapy induces depressive symptoms that were associated with a significant decrease of peripheral tryptophan and tryptophan/LNAA ratio (Capuron et al., 2002);

suggesting that immune-mediated activation of the kynurenine pathway could be a potential mechanism that links to the symptoms of depression.

Early work demonstrated that stress-related increases in glucocorticoids induce tryptophan hydroxylase activity and that the increase in central tryptophan level following stress was mediated by the SNS potentially through an increase in the BBB permeability and by an increase in lipolysis triggering an increase in free circulating tryptophan (Chaouloff, 1993). Interestingly, recent work combining pre-clinical and clinical investigations suggests that impairment of the BBB can be found in a mouse model of chronic stress in NAc in male (Menard et al., 2017) and in PFC in female (Dion-Albert et al., 2022); with impairment seen in post-mortem tissue of MDD patients. However, it is currently unknown how these impairments affect tryptophan metabolism in the brain. Overall, a meta-analysis demonstrates that MDD patients have a reduce tryptophan level in plasma, specifically in unmedicated patients (Ogawa et al., 2014).

2. Serotonin

Unlike peripheral 5-HT, central 5-HT concentration is critical for the stress response and stress-related disorders. Although framing the neurobiological basis of depression in terms of a deficit in serotonin is probably oversimplistic (Jauhar et al., 2023) it has recently been demonstrated that MDD patients showed decreased levels in central serotonin levels (Erritzoe et al., 2023). Alterations in presynaptic and postsynaptic serotonergic receptors in the CNS have also been reported (Risch & Nemeroff, 1992). Many antidepressant drugs can act on the central serotonergic system to increase availability of serotonin (Fuller, 1980). In fact, selective serotonin reuptake inhibitors, the most frequently prescribed antidepressant, function by preventing serotonin reuptake to increase the concentration of serotonin in synapses. Importantly, central serotonergic systems modulate the two stress axes, SNS and HPA, and conversely stress mediators such as glucocorticoids and catecholamines affect central serotonergic systems (Chaouloff, 1993; Dinan, 1996). Later studies demonstrated that serotonin regulate CRH signalling through activation of serotonin

2C receptors in the PVH (Heisler et al., 2007) and that cortisol induces TPH2 expression to produce de-novo serotonin (Chen & Miller, 2012).

Since brain serotonin levels are highly dependent on peripheral levels of its precursor, a common method seen in clinical and pre-clinical studies is Acute Tryptophan Depletion (ATD) to assess brain serotonin deficiency in psychiatric disorders (Fadda et al., 2000; van Donkelaar et al., 2011) although it has been shown to affect peripheral kynurenine level as well (P. J. Kennedy et al., 2015). ATD in pre-clinical studies led to an increase in mobility time in the forced swim test (Uchida et al., 2005; L. Zhang et al., 2006) and nesting behaviour (Browne et al., 2012). Tryptophan-deficient diet in rats caused reduced serotonin in the brain (prefrontal cortex, striatum, hippocampus and brainstem) and reduced dendritic arborisation and cell proliferation in the dentate gyrus of the hippocampus indicating neuroplasticity changes that could be due to serotonin deficiency (L. Zhang et al., 2006).

In healthy volunteers, ATD alters the blood-oxygen-level-dependent (BOLD) signals - driven by localised changes in brain blood flow and blood oxygenation – in the inferior/orbitofrontal cortex, the anterior cingulate cortex and the dorsomedial prefrontal cortex (Evers et al., 2010). Furthermore, ATD modulated BOLD response in the amygdala of healthy volunteers during emotional processed (Evers et al., 2010). In healthy volunteers with family history of affective illness, ATD was associated with lower mood after tryptophan depletion, which might suggest a vulnerability to depression in healthy volunteer with family history of mental illness (Benkelfat et al., 1994). In clinical studies, changes in brain tryptophan levels can be estimated by circulating TRP/LNAA ratio (van Donkelaar et al., 2011). Similarly, 5-HT synthesis or release can be estimated by changes in 5-hydroxyindole acetic acid(5-HIAA), or the 5-HIAA/5-HT ratio (Höglund et al., 2019). In a small study of 8 non-psychiatric sudden death controls and 6 suicides, an increase in 5-HT and 5-HIAA in the brainstem in suicides; with a lower 5-HIAA/5-HT ratio in the brainstem and PFC indicating a lower 5-HT turnover of suicidal patients (Bach et al., 2014).

3. Melatonin

Aside from its potential in the regulation of circadian rhythms and sleep, melatonin was associated with psychiatric disorders due to its anti-inflammatory, antioxidant and anxiolytic properties (Maldonado et al., 2009). Similarly, its neuroplasticity capacity, demonstrated in the hippocampus (J. G. Lee et al., 2019), contributes to its known neuroprotective effects. Some preclinical studies also found melatonin (Crupi et al., 2010; Detanico et al., 2009; Mantovani et al., 2003) or agonists of its receptors in the brain MT1 and MT2 (Cardinali et al., 2012) to have anti-depressant effects. Although another study demonstrated that melatonin could exert antidepressant effect through its antagonistic activity to 5-HT_{2A} (Micale et al., 2006). Interestingly, MT1 knock-out mice displayed an increase in depressive-like behaviour and a sex-dependent alteration in a stress-related behavioural task (Weil et al., 2006). This antidepressant effect can be mediated by the ability of melatonin to increase neuroplasticity in the hippocampus (Crupi et al., 2010) or its interaction with NMDA receptors (Mantovani et al., 2003).

In clinical trials, aglometine - a melatoninergic agonist and selective antagonist of 5-HT_{2C} receptor - was found to be efficient for the treatment of MDD in clinical trials (De Berardis et al., 2011; S. H. Kennedy & Emsley, 2006; Lo et al., 2002). Ramelteon, another commercialised drug for the treatment of insomnia, was efficient against insomnia – a common symptom in psychiatric disorders - by acting as a selective agonist of MT1/MT2 without any other affinity for other receptors (Cardinali et al., 2012). Overall, melatonin and melatonin-related drugs have demonstrated their efficiency in a subset of patients with psychiatric disorders and can also be used as an adjuvant to lessen psychiatric symptoms (J. G. Lee et al., 2019).

4. Kynurenine

Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), the two-rate limiting enzyme of the kynurenine pathway, are an immune- and stress- sensitive enzymes. Therefore, immune or stress challenges can shift tryptophan conversion from serotonin production into the kynurenine pathway (Höglund et al., 2019; Qin et

al., 2018). In a meta-analysis involving 101 studies, kynurenines metabolites were shown to be altered differently in major depressive disorder, bipolar disorder and schizophrenia (Marx et al., 2021). They reported that both tryptophan and kynurenine were decreased in those 3 disorders. However, KYNA to QUIN ratio, two neuroactive metabolites from the kynurenine pathways, were decreased in mood disorders (MDD and BD); while KA to 3-hydroxykyn ratio was decreased in MDD. Finally, KYNA to kynurenine ratio was decreased and KYN to tryptophan ratio increase in MDD and SZ (Marx et al., 2021).

A subset of patients with MDD have systemic low-grade inflammation, which triggers systemic conversion of tryptophan towards the kynurenine pathway (Myint, 2012). Most brain tryptophan levels get converted into kynurenine metabolites by the rate-limiting enzyme IDO. By investigating the imbalance between circulating neuroprotective and neurodegenerative metabolites of the kynurenine pathway, Myint et al. showed that depressed individuals had a lower tryptophan availability and a higher tryptophan breakdown. Circulating tryptophan and kynurenine levels were similar when compared to controls, but tryptophan breakdown index was higher and kynurenic acid concentration was lower in depressed patients (Myint et al., 2007).

In patients with chronic hepatitis C, INF-γ treatment significantly increased circulating KYN/TRP ratio overtime suggesting an increase in IDO/TDO activity. Circulating KYN/KA ratio were also increased in these patients suggesting increase brain neurotoxicity and those results were associated with increased depression scores (Wichers et al., 2004). Interestingly, in similar patients, INF-γ treatment increased kynurenine, QUIN and KYNA levels in the CSF as well as Kyn levels in plasma – but not tryptophan (Raison et al., 2010). These clinical data suggests that peripheral inflammation could trigger the kynurenine arm of tryptophan metabolism.

Pre-clinical work using mice deficient in TDO enzyme showed that these mice had an increase tryptophan and 5-HIAA in the circulation and the hippocampus; changes that were accompanied by anxiolytic effects (Funakoshi et al., 2011). Another study demonstrated that both acute and repeated stress in rats triggered an increase in TDO activity in the liver – one of the major sites of tryptophan degradation – and in the

cerebral cortex. This was accompanied by a higher level of circulating kynurenine and a depressive-like behaviour that was reversed by a treatment with a TDO inhibitor (Gibney et al., 2014). This suggests that hepatic TDO activation by stress hormones is a main contributor to the increase of circulating kynurenine metabolites linked to depressive symptoms. Similarly, peripheral activation of IDO by an immune challenge induced depression-like behaviour in mice and changes in peripheral and central kyn/trp ratio that was alleviate by either anti-inflammatory treatment or by an IDO antagonist (O'connor et al., 2009).

Interestingly, physical exercise which also triggers HPA axis and immune activation, has been shown to mediate resilience to stress-induced depression by increasing peripheral levels of KYNA (Agudelo et al., 2014). The kynurenine pathway is also crucial for its end-product nicotinamide adenine dinucleotide (NAD+) since it is a key co-factor of many enzymes (Schwarcz & Stone, 2017). Interestingly, another clinical study compared the effect of psychological (TSST) and physical stressor (exercise) on peripheral immune and tryptophan changes and demonstrated that both stressors induced significant changes of tryptophan and immune kinetics, with more pronounced effect after exercise (Herhaus et al., 2021).

D. Circadian rhythmicity of tryptophan metabolism

Tryptophan and a downstream product, serotonin, are the precursors of melatonin synthesis; therefore, it is not surprising that brain tryptophan and its metabolites are also under circadian control in the periphery and in the CNS (Fernstrom & Wurtman, 1971; Lewczuk et al., 2014). To understand the role of essential amino acids in behavioural circadian rhythms, Petrus et al. depleted each essential amino acid separately in diet of the mouse and identified tryptophan as being a central modulator of circadian homeostasis (Petrus et al., 2022). They found that tryptophan metabolism was regulated by environmental cues and contributes to the control of central and hepatic circadian rhythms (Petrus et al., 2022). In the pineal gland, the conversion of serotonin into melatonin during the dark phase is essential for circadian rhythmicity of neuroendocrine signals in the brain and periphery

(Ruddick et al., 2006). Melatonin rhythmicity regulated important physiological and behavioural responses including sleep patterns, immune response and oxidative stress (Ruddick et al., 2006). Melatonin receptors are widely distributed around the body and contributes to the regulation of cardiovascular, immune, and endocrine systems (Slominski et al., 2012). Importantly, serotonin levels display circadian rhythmicity in several key brain regions such as the SCN (our master clock), pineal gland (the main site of melatonin synthesis), raphe nuclei (responsible for the release of serotonin in the different parts of the brain) and striatum (Versteeg et al., 2015). This is in line with the fact that TPH2, the rate-limiting enzyme of serotonin synthesis in serotonergic neurons, demonstrated diurnal rhythmicity in the brain (J. Liang et al., 2004; Malek et al., 2005).

TPH2 is also one of the many genes modulated by glucocorticoids following HPA axis activation, which not only explains its circadian variation but also its reactivity to stress and implication in stress-related disorders (Chen & Miller, 2013). Interestingly, norepinephrine and sympathetic innervation has been shown to be an important regulator of the pineal gland secretory activity in geese, including melatonin production (Ziółkowska 2021). Conversely, melatonin directly inhibits ACTH-stimulated cortisol production in adrenal glands in adult monkeys by stimulation of MT1 melatonin receptors (Torres-Farfan 2004). A study in humans demonstrated direct inhibitory effects of melatonin on ACTH response in adrenal explants (Campino 2011). Interestingly, mice with a disrupted serotonin system show altered circadian rhythms and locomotor activity, highlighting a potential role for 5-HT rhythmicity to modulate behavioural activity pattern (Paulus 2012). N-acetylserotonin, the precursor of melatonin from serotonin, exhibit antidepressant and neuroprotective effects by activating TrkB (BDNF receptor) in a circadian manner (Jang 2009). All of these lines of evidence suggest a bidirectional regulation of tryptophan metabolites and HPA axis activation, whether it is by circadian cues or stress induced.

III. Microbiota-gut-brain axis communication

A. Definition

The gut microbiota is composed of all microorganisms that are present in our gut - including bacteria, archaea, eukaryotes and viruses - interacting with our body and shaping our immune system since birth. Bacterial load increases gradually from the stomach to the jejunum to colon (Mailhe et al., 2018). In addition, different environmental conditions (pH, presence or absence of oxygen, temperature) will lead to different species in different sections of the gut (Mailhe et al., 2018). Gut bacteria participate in the process of digestion by harvesting, storing and expending energy obtained from diet (Krajmalnik-Brown et al., 2012). This process generates important molecules (Short-chain fatty acids (SCFA), bile acids, tryptophan metabolites) that can influence the bidirectional communication between the gut and the brain, often referred to as the "microbiota-gut-brain axis". This community of gut microorganisms is a dynamic entity that can change both in composition and activity and is influenced by several factors such as diet, age, genetics, environmental factors, antibiotic consumption, mode of delivery among others (Cryan et al., 2019). Over the past decades, microbiome research has expanded our understanding of its impact on human health too. A symbiotic relationship between our body and our microorganisms has existed since day one.

A deeper understanding has led us to realise that they are not only there to affect energy use and digestion but can also have a significant impact on brain function and behaviour (Cryan & Dinan, 2012). The microbiota-gut-brain axis refers to the network of connections involving multiple biological systems, direct or indirect signalling via microbial metabolites, neuronal pathways or interaction with our immune system. The microbiota-gut-brain axis is composed of the central nervous system in the brain, the enteric nervous system in the gut - often referred to as "the second brain", the immune, endocrine and digestive systems connecting them, and the trillions of microorganisms inhabiting it.

By comparing conventional rodents with germ free ones (i.e. devoid of all microorganisms), an important role of the intestinal microbiota is attributed to the symptoms expressed and prominent features of numerous diseases (Luczynski et al., 2016). However, despite the invaluable information gathered from preclinical studies, in most cases, causality remains unclear. Recently, we have begun to understand how host physiology and behaviour – in rodents – can be affected solely by the transplantation of faecal microbiota from a donor (human, rodent, etc.). This method has become common for assessing causality arising from the complex host-microbiota interactions, as it is a powerful way to understand the strong involvement of intestinal microorganisms on our overall health (Gheorghe et al., 2021). Since then, faecal microbiota transplantation (FMT) studies have implicated the gut microbiota in gastrointestinal disorders like irritable bowel syndrome (IBS) (Weingarden & Vaughn, 2017) and metabolic disorders such as Type 2 Diabetes and Obesity (Hartstra et al., 2015). Even more surprisingly, FMT studies highlighted the potential involvement of the intestinal microbiota in CNS diseases including neurodegenerative disorders and some psychiatric conditions including schizophrenia (F. Zhu et al., 2019) and depression (Kelly et al., 2016). The interaction gut microbes with host tryptophan metabolism, hypothalamus-pituitary-adrenal axis, gut permeability are important features of this bidirectional communication will be discussed below.

B. Relevance in stress-related disorders

A promising body of preclinical and clinical work is emerging showing the importance of this bidirectional communication in stress-related and psychiatric disorders. This can be explained by the fact that gut microbes, in healthy individuals, confers a broad array of benefits including induction, training and function of our immune system, enhancing permeability and function of the intestinal barrier, nutrient extraction from food, protection against infection, improvement in energy harvest amongst other (Sekirov et al., 2010). More importantly, gut microbes were found to be critical in the development and maintenance of the two most important stress-axis: the HPA (Crumeyrolle-Arias et al., 2014; Sudo et al., 2004) and the SAM (van de Wouw et al., 2020; Xiang et al., 2021) axes. Psychological stress has been shown to alter gut

microbiota composition in pre-clinical models of stress exposure (Foster et al., 2017) as well as in humans (Holdeman 1976, Lizko 1987, Knowles 2008).

1. Microbial HPA axis regulation and implication for behaviour: evidence from preclinical studies

There is a growing body evidence highlighting the important role of gut microbiota in shaping HPA axis reactivity to stress. The role of gut microbes in stress-related disorders has been extensively explored in behavioural experiments in animals lacking a microbiome (germ-free or antibiotic-treated). Germ-free mice show an increase in anxiety-like behaviours (G. Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011) that could be rescued/normalised with colonisation (G. Clarke et al., 2013) beneficial commensal microbes (Sudo et al., 2004), probiotics (Moloney et al., 2014) or microbiota transplant (Nishino et al., 2013; Schretter et al., 2018; Sudo, 2019). By comparing HPA axis reactivity to stress in GF and SPF mice, Sudo et al. demonstrated that GF mice exhibited an exaggerated stress-reactivity characterised by increased plasma ACTH and corticosterone production compared to SPF mice. Surprisingly, they also showed that colonisation with SPF microbiota could restore HPA axis reactivity to stress only at an early developmental stage suggesting the need of commensal microbes to regulate the development of the HPA stress response (Sudo et al., 2004). This evidence suggests a role for gut microbes in shaping HPA axis reactivity to stress and behaviour.

2. Functional changes in gut microbial population in stress-related disorders: Implications in humans

Patients with MDD were shown to have an altered faecal microbiota composition (Cheung et al., 2019; Jiang et al., 2015; Naseribafrouei et al., 2014). Intestinal microbes are thought to be involved in stress-related disorders as exemplified by the study of Valles-Colomer et al. (Valles-Colomer et al., 2019a) where differences in gut microbial composition were associated with lower quality of life (QoL) and depression status compared to healthy controls. More specifically, this study identifies for the first time

several functional key features of the gut microbiota in terms of neuroactive potential including tryptophan metabolism. A recent systematic review characterised the gut microbiota of patients with anxiety and depressive disorders and concluded that these patients had higher pro-inflammatory species and lower abundance in SCFAs producing-species overall (Simpson et al., 2021).

Studies have been conducted in healthy humans to understand the potential of microbial modulation or supplementation of microbial products on stress perception and related-neuroendocrine changes. In healthy humans' probiotic administration FOS but not GOS treatment for 3 weeks reduced cortisol awakening response (Schmidt et al., 2015). A psychobiotic diet (rich in prebiotic and fermented food) for four weeks had no impact on biological markers of stress but reduced perceived stress in a healthy adult population accompanied by an increase in microbial stability and an alteration in urinary tryptophan metabolites (Berding et al., 2022). Administration of colonic-delivered microbial by-products (SCFAs) were shown to attenuate cortisol-responsivity to a psychosocial stress task in healthy men with no impact on cortisol awakening response (Dalile et al., 2020). Another study demonstrated that probiotic consumptions in healthy individuals undergoing a stressful period (exams) led to a reduction in stress-induced abdominal dysfunction and feelings of stress (Kato-Kataoka et al., 2016). Some studies in patients with MDD concluded that probiotics could be useful as an adjuvant of anti-depressant treatment to ameliorate cognitive functions (Rudzki et al., 2019) or gastro-intestinal co-morbidities such as IBS (Noonan et al., 2020).

C. Tryptophan metabolism in the gut

While tryptophan metabolism has been largely studied in the brain in the context of psychiatric disease, emerging research indicates that depression is associated with tryptophan dysfunction in the gut (Lukić et al., 2022). Tryptophan is first metabolised in the gut before entering the systemic circulation or being able to reach the brain. Since 90% of tryptophan is metabolised in the gut and the liver (Mahony et al., 2015), the fate of tryptophan conversion in the periphery will determine the molecules that

enter the brain due to differences in the ability of some tryptophan metabolites to cross or not the blood-brain barrier.

Tryptophan metabolism is also an essential component of gut function and homeostasis (Roager & Licht, 2018). Israelyan et al. (Israelyan et al., 2019) illustrated how a defect in 5-HT synthesis causes both brain and intestinal dysfunction by using mutated mouse for the TPH2 gene which is responsible for 5-HT biosynthesis in both enteric and central nervous system serotonergic neurons. The role of serotonin production by enterochromaffin cells in GI functions such as motility and secretion is now widely recognised (Bayrer et al., 2023; Roager & Licht, 2018). Similarly, enterochromaffin cells in the GI tract is an important source of extrapineal melatonin production, where it acts as an important signalling hormone in the GI tract and in the systemic concentration (Bubenik, 2002). However recent research has increasingly focused on microbial regulation of tryptophan metabolism, with an emphasis on microbial-specific pathways that generate bioactive tryptophan metabolites.

1. Microbial tryptophan breakdown

In the GI tract, Tryptophan is not only metabolised through the kynurenine (by immune and epithelial cells) or serotonin (by enterochromaffin cells) pathway (Benech et al., 2021) but can also be metabolised directly by the gut microbiota and generate a range of indoles with diverse biological activity, exclusively produced by microbes (Tomberlin et al., 2017). The combination of microbial and host gastrointestinal metabolism of tryptophan is thus likely an important factor in the systemic availability of tryptophan, as well as indoles, kynurenine and serotonin (5-HT) produced locally (Roager & Licht, 2018). Indole is produced from tryptophan by the microbial enzyme tryptophanase and was detected in human gut at concentrations of 250-1100 µM (J.-H. Lee et al., 2015). Indole derivatives synthetised by gut bacteria have been found in blood, peripheral tissues, urine and brain tissues which suggests an important role of those bacterial compounds (J.-H. Lee et al., 2015). Indoles have an important role in gut function and homeostasis: the most studied mechanisms being through binding to Aryl Hydrocarbon Receptor (AhR) (Agus et al., 2018), Pregnane X Receptor (PXR)

(Illés et al., 2020a), and transient receptor potential ankyrin A1 (Trpa1) receptor (Ye et al., 2021).

An increasing number of studies are focusing on the activation of the AHR as it has shown to have profound effects upon immunological status of the GI tract. However, the range of ligands responsible for AHR-activation within the gut continues to expand (Murray & Perdew, 2017). Recently, indole and some of these derivatives have been shown to be potent activators or stimulators of AHR and thus to influence the transcription of important factors of the immune system. This discovery is potentially of major importance because it highlights activation of the host's immune system through metabolites produced by the intestinal microbiota. More specifically, AhR-activation by indole metabolites have been shown to be important in AhR-mediated IL-22 production (Zelante et al., 2013) and contribute to inflammation in a colitis model (Lamas et al., 2016) and IBS (Meynier et al., 2022). Similarly, pregnane X receptor (PXR) binds indole-3-propionate which helps regulating intestinal barrier function through TLR4 signalling (Venkatesh et al., 2014).

Interestingly, it was found that some spore-forming bacteria can regulate host serotonin biosynthesis thereby affecting luminal and circulating levels of serotonin (Yano et al., 2015). A subsequent study demonstrated that Trpa1 stimulation by indole metabolites increase intestinal motility through vagal activation of enteroendocrine cells to produce of serotonin (Ye et al., 2021). Similarly, microbial metabolites SCFAs were also shown to promote *TPH1* expression in a human cell line (Reigstad et al., 2015). Similarly, a subset of gut bacteria has an enzyme to decarboxylate tryptophan int tryptamine (Williams et al., 2014), which later has been shown to accelerate GI transit through increased ionic flux across colonic epithelium (Bhattarai et al., 2018).

2. Tryptophan metabolism: Implication for brain monoamine levels

Importantly, the use of trypophan for indole molecule production also has important implications at the central nervous system level (Tennoune et al., 2022). Pre-clinical studies demonstrated that the presence or absence of microbes can influence greatly

hippocampal levels of serotonin in a sex-dependent manner (G. Clarke et al., 2013). Colonisation of GF mice by exposure to external environment was shown to influence monoamine levels in host brain and reduced anxiety-like behaviour only after 24-h of recolonisation (Nishino et al., 2013). Surprisingly, microbial metabolites from tryptophan can also activate microglia and brain TGFα and VEGF-B production directly impacting CNS inflammation through an AhR-mediated mechanism (Rothhammer et al., 2016). Lastly, some evidence suggests that Indole 3-propionic acid might be able to mitigate BBB damage due to hypoxia in neonatal rats by binding to PXR and attenuating inflammation (Q. Zhao et al., 2022). All these evidence points to an important role of gut bacteria in shifting tryptophan metabolism towards indoles compounds, thereby affecting peripheral and central immunity, and gut-brain axis signalling.

D. Gut microbiota and stress-related disorders: Implications for tryptophan metabolism

Given that changes are observed in gut microbiota composition and function in preclinical models of stress-induced depression (L. Liu et al., 2022) and in patients with anxiety disorders or MDD (Simpson et al., 2021), it is relevant to wonder to what extent microbial metabolism of tryptophan plays a role in this regard. To understand whether tryptophan supplementation will ameliorate depression and anxiety-related behaviours, Wang et al. used a tryptophan-enriched diet in a mouse model of depression. They not only demonstrated that a tryptophan enriched diet improved-related behaviour but also altered gut microbiota composition, improved gut barrier function and reduced stress-induced inflammatory responses in the colon following a chronic unpredictable mild stress (CUMS) protocol (Wang et al., 2022). Interestingly, CUMS shifted tryptophan metabolism towards an enhancement in kynurenine pathway in serum (D. Wang et al., 2022). Another preclinical study using the same model of depression hypothesised that stress-induced inflammation could be involved in microbiota dysbiosis and changes in catecholamines and systemic tryptophan metabolites following CUMS (H. long Yang et al., 2021). There is also evidence showing that acute tryptophan depletion (ATD) led to an increased

depressive-like behaviour and a stronger reduction of tryptophan, 5-HT and 5-hydroxyindoleacetic acid in the medial prefrontal cortex and hippocampus of GF than in SPF mice (Lukić et al., 2019). Interestingly, following ATD, GF mice behave more similarly to SPF mice under basal conditions. The authors concluded that the serotonergic system of GF mice, which is abnormal at baseline, is more vulnerable to ATD.

Interestingly, some indole-derivatives are characterised by neurodepressive properties - namely oxindole and isatin - and excessive production of indole by the gut microbiota may adversely affect behaviour in rats (Jaglin et al., 2018). On the other hand, administration of prebiotics (i.e. substrates that are selectively utilised by, and promotes the growth and/or activity of, beneficial host microorganisms compared to probiotics, which are live microorganisms consumed to produce a health benefit) in mice has shown antidepressant and anti-anxiolytic effects which underlines the possibility of exerting beneficial effects on the serotonergic system by therapeutic targeting of the gut microbiome, an appealing strategy, and one that can be expedited with an enhanced knowledge of the mechanisms underpinning this important emerging aspect of host-microbe dialogue (Burokas et al., 2017).

A causal link between gut microbiota composition and MDD was assessed for the first time by performing FMT from depressed patients to rats depleted of intestinal microbiota (Kelly et al., 2016). Interestingly, recipients of these FMTs display depressive-like phenotype and an increase in systemic Kynurenine/Tryptophan ratio. Overall, studies using animals lacking a microbiome or with a depleted microbiota (GF or Abx) strongly suggests that major neurotransmitters including serotonin, dopamine and noradrenaline in the brain are modulated by gut microbes in anxiety and depression models, although the mechanisms remain unclear (F. Huang & Wu, 2021).

E. Circadian rhythmicity of gut bacteria

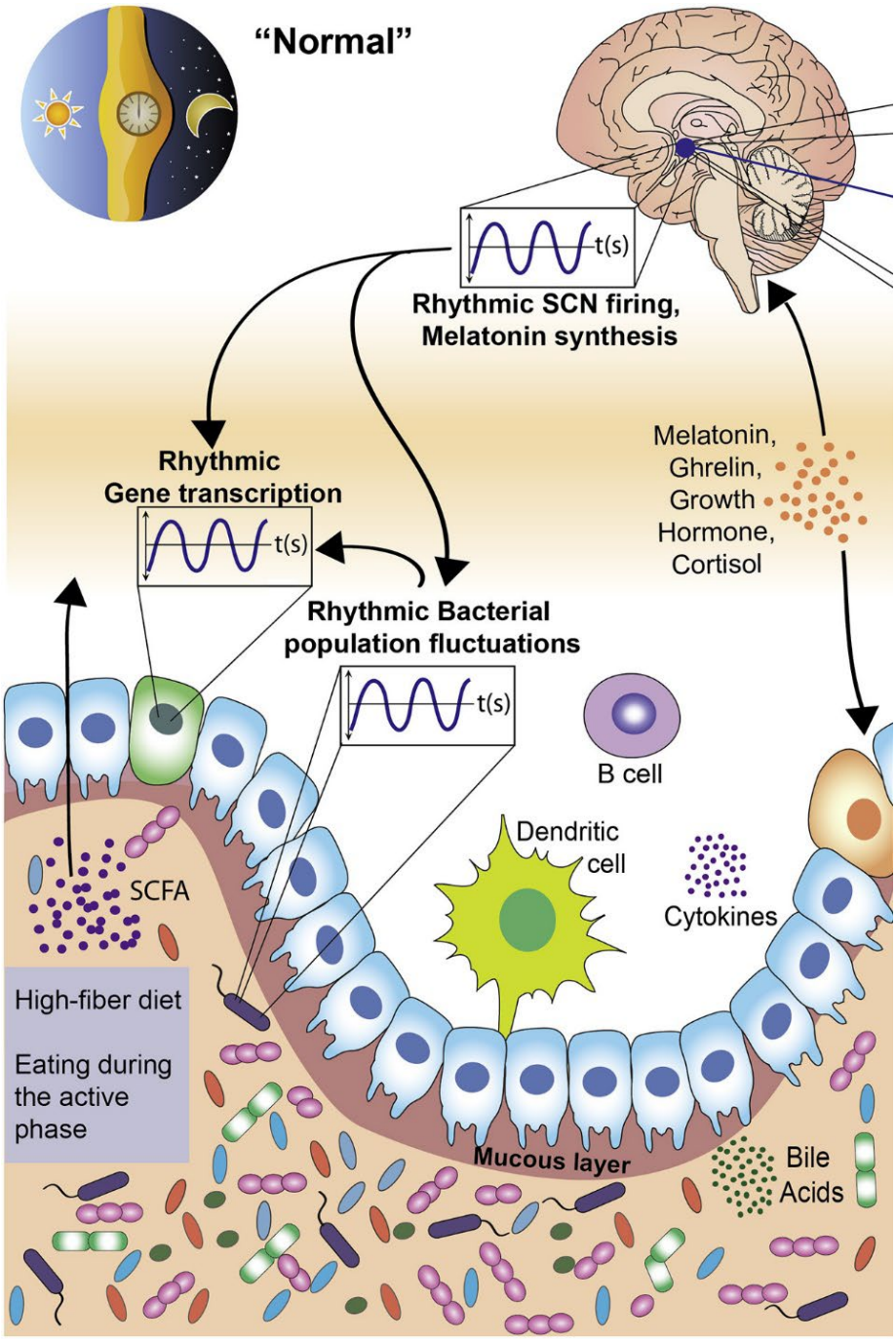

Figure 7. Circadian rhythmicity of the microbiota-gut-brain axis in healthy conditions The Suprachiasmatic nucleus (SCN) in our brain is our master clock, individual peripheral clock that can be found in all living cells, following a 24-h cycle. The majority of tissues express the same circadian genes and proteins as the SCN and possess independent biological clocks that are connected to the diurnal regulation of physiological activities. Central and peripheral clocks orchestrate circadian rhythmicity of the microbiota-gut-brain axis. Moreover, bacterial circadian rhythm is tightly regulated with our clocks, generating fluctuations in microbial metabolites and oscillation in bacteria population. Figure from (Teichman et al., 2020)

Circadian rhythms are key in maintaining overall health in almost all living organisms, including bacteria. In the past decade, our understanding on the symbiotic relationship between host and microbial circadian rhythms is expanding as well as how it influences health and disease (Teichman et al., 2020; Thaiss et al., 2014). Diurnal oscillations in gut microbiota composition and metabolite production (Butler & Gibbs, 2020; Kaczmarek et al., 2017) are highly dependent on host dietary and environmental cues (Leone et al., 2015; Thaiss et al., 2014; Zarrinpar et al., 2014; Y. Zhang et al., 2023) but microbial rhythmicity persists in the absence of host external timing cues (absence of light) (Heddes et al., 2022).

Studies using global knock-out of the core clock gene BMA1 (X. Liang et al., 2015) or specifically in intestinal epithelial cells (Heddes et al., 2022) demonstrated that host intestinal circadian controls microbiota composition and function (Heddes et al., 2022). Moreover, Heddes et al. demonstrated elegantly that host control of microbial function had implications for GI homeostasis and that arrhythmic microbiota also disturbs GI homeostasis, suggesting a bidirectional synchrony in host and microbiota rhythms in the gut (Heddes et al., 2022). Importantly, it was demonstrated that deletion of Bmal1 altered microbial composition in a sex-dependant manner (X. Liang et al., 2015). This bidirectional communication between host and microbes in a circadian fashion has important implications for gut barrier function (Mukherji et al., 2013; Thaiss, Levy, et al., 2016; Tuganbaev et al., 2020) as bacterial motility and mucus degradation undergoes diurnal fluctuations (Thaiss, Zmora, et al., 2016). However, circadian modulation of gut microbes also impacts the periphery and the liver (Leone et al., 2015; Thaiss, Levy, et al., 2016; Y. Zhang et al., 2023). GF mice exhibit altered circadian rhythmicity (Leone et al., 2015), which ultimately impacts host central and peripheral rhythms overall (Leone et al., 2015).

Some proposed mechanisms by which gut microbiota may response to host circadian signals include sensing of gut-derived melatonin (Paulose et al., 2016) or intestinal IgA secretion (Penny et al., 2022). On the other hand, microbial metabolites from tryptophan that can activate AhR could potentially impact gut barrier function in a circadian manner given that AhR also interacts with Per1 and BMAL1, key components

of the molecular clock modulating circadian activity in the liver (Xu 2010; Jaeger 2017). Research regarding AhR-related circadian modulation in the gut is at its infancy, though AhR-mediated regulation of innate lymphoid cells 3 (ILC3) (Song et al., 2020) seems to be an interesting candidate in attempts to understand whether AhR-activation will mediate beneficial effects in the gastrointestinal tract in a circadian manner (Godinho-Silva et al., 2019; S. Li et al., 2018). Overall, all these recent observations provide information of the bidirectional modulation that exists between gut microbiota and central and peripheral clocks (Teichman et al., 2020).

IV. Gut function and permeability

A. Gut physiology

1. Anatomy

The intestinal barrier is a dynamic structure which ensures absorption of nutrients and prevents pathogenic substances or microorganisms to enter the systemic circulation. The digestive tract is composed of the mouth, oesophagus, stomach, small intestine, large intestine, and anus. The small and large intestine will continue the digestive process started by the stomach and absorb nutrients. The human small intestine is the longest component of the digestive tract (~6m) is divided into the duodenum, jejunum and ileum. The large intestine consists of the cecum, colon (ascending, transverse and sigmoid), rectum and anal canal. The gastrointestinal tract is composed of several layers detailed in **Figure 8**. While absorbing nutrients, the intestinal barrier also prevents pathogens or microorganisms from the lumen of the gut to enter the systemic circulation. However, psychological stress has been shown to be an important modulator of gut barrier function and permeability across multiple levels of the gut barrier. This selective physical and immunological barrier allows a healthy bidirectional communication between the lumen and the bloodstream (Greenwood-Van Meerveld et al., 2017; Soderholm & Perdue, 2001)

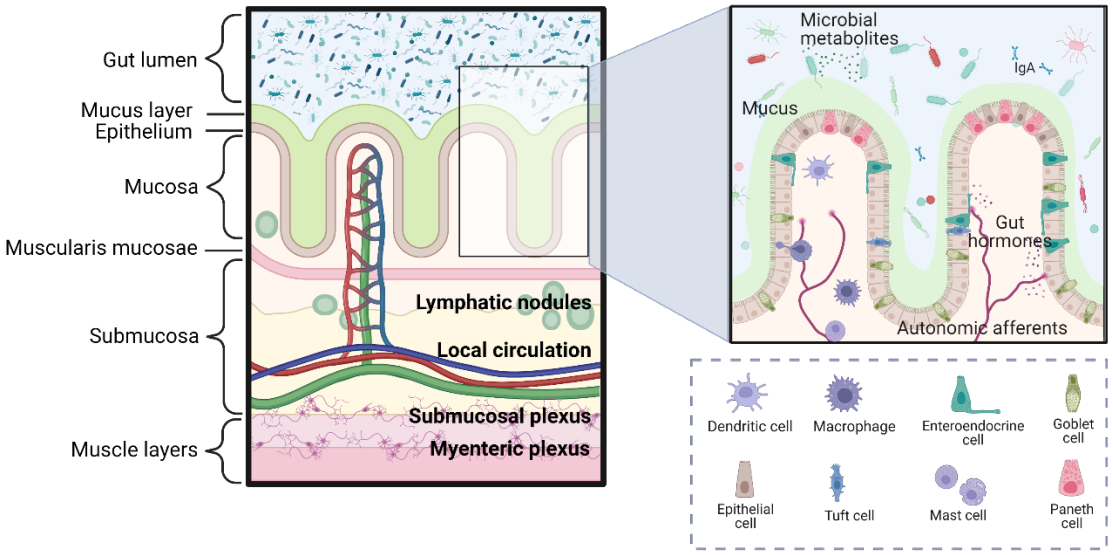

Figure 8. Anatomical organisation of the gut. The tissue of the gastrointestinal tract is organised into distinct anatomical layers. First, the inside of the intestine, called the lumen, contains all the microorganisms – referred to as gut microbiota" - as well as host and microbial metabolites from the degradation of food. Adjacent to the gastrointestinal lumen is the mucus layer, made up of mucins secreted by goblet cells found in the epithelium. The epithelium is a physical barrier made of different types of cells (goblet, enterocytes, enteroendocrine cells, goblet cells, Paneth cells, microfold cells, cup cells and tuft cells) interconnected via tight junctions' proteins. The underlying mucosa contains blood and lymphatic vessels, as well as resident immune cells (macrophages, mast cells, T helpers' cells and neutrophils), to ensure absorption of nutrients into the organism and rapid immune response to pathogens respectively. The underlying submucosa contains the blood vessels and nerves that supply the mucosa, as well as lymphoid nodules containing immune cells. The submucosal and myenteric plexi of the enteric nervous system are deep in the gut tissue, between the submucosa and muscle layers. Figure from Leigh et al. (under review). Figure created with BioRender.

B. Experimental assessment of gut permeability

Over the past decades, several methods allowed us to investigate gut permeability in-vivo, ex-vivo and in vitro as our understanding of its complexity, selectivity and dynamics has increased (Vanuytsel et al., 2021). Stress-related and GI disorders co-morbid with psychiatric disorders demonstrated impairment in gut permeability often referred to as "leaky gut". This can be defined as the entrance of food antigens, commensal and/or pathogenic bacteria, bacterial component (LPS) or bacterial metabolites into the lamina propria to further translocate into the systemic circulation (Vanuytsel et al., 2021). This translocation often provokes an increase in systemic inflammation that is characteristic of some intestinal disorders such as IBS and some

subset of psychiatric patients exhibiting increased in GI permeability (Wilmes et al., 2021).

As mentioned above, multiple layers of the intestinal tract can be impaired or altered by stress or other stress-related gut co-morbidity. The most inner part of the intestine, the mucus, is under the influence of the enteric and autonomous nervous system (Herath et al., 2020), hence its alteration by stress. Mucus barrier is different across the GI tract and mucus production is regulated in a region-specific manner. Interestingly, microbial population influence mucus properties (hydratation, viscosity, thicknesss) while also using it as a carbon and energy source (Herath et al., 2020). Differences in transport routes in the intestinal epithelium are displayed in **Figure 9** from (Vanuytsel et al., 2021). The paracellular and transcellular endocytic routes are the most relevant in the study of impairment of gut barrier permeability. Tight junctions' proteins play an important role in maintaining paracellular permeability by acting as a paracellular barrier, allowing paracellular transport of some ions and acting on ions channel and finally by maintaining cell polarity (Vanuytsel et al., 2021). Lastly, both stress (Soderholm & Perdue, 2001b) and gut microbial population (J. Y. Yoo et al., 2020) significantly alter the immune system of the GI tract ultimately altering host's innate and adaptive GI immune systems, another important component of intestinal homeostasis and gut barrier function. Table 1 Summarises the most well-characterised laboratory techniques to assess impairment in gut-barrier permeability. Adapted from (Kelly et al., 2015; Schoultz & Keita, 2020; Vanuytsel et al., 2021; Wells et al., 2017)

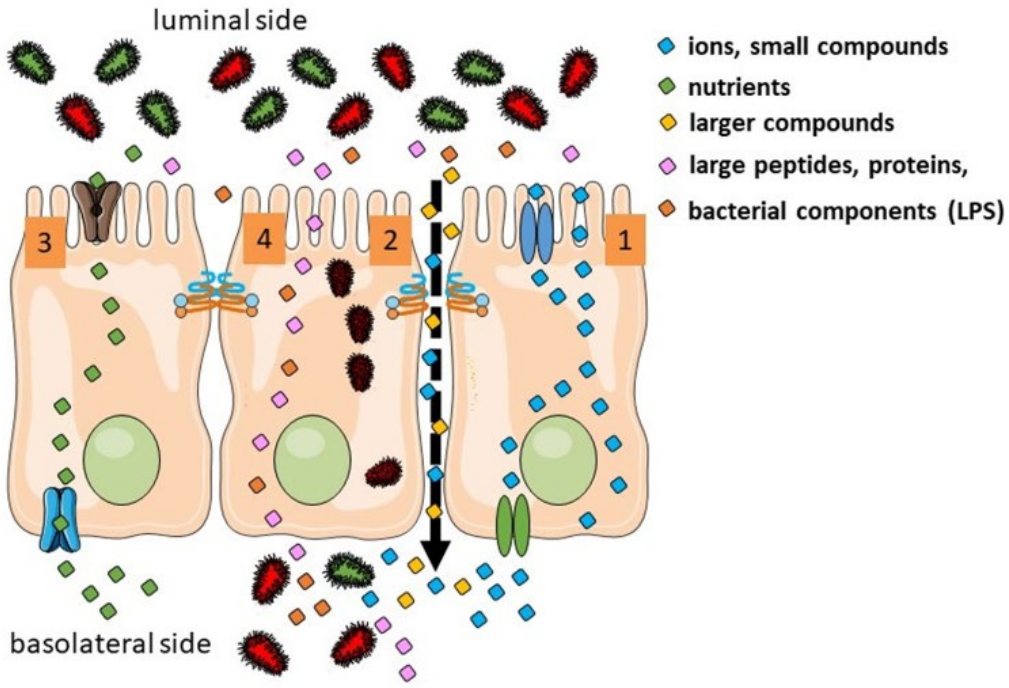

Figure 9. Transport routes in the intestinal epithelium.
When referring to intestinal permeability, several parts of the GI might be interesting to study (i.e. gastric, small and large intestines) as they have different characteristics and are differently populated by gut microbes. Across the whole physical barrier of the intestine, several routes of transport can be impaired: (1) transcellular transport of ions, (2) paracellular pathways (Ion, water, and larger hydrophilic compounds), (3) active transport (sugars, amino acids, and vitamins) and (4) endocytosis and exocytosis into vesicles (large molecules and whole bacteria). Figure from (Vanuytsel et al., 2021)

Table 1. Summary table of commonly used methods to assess intestinal permeability.

Permeability test	Sample	Measures	Advantages	Limitations
Challenge tests				
Lactulose/mannitol	Urine	Small intestine permeability	+ Non-invasive and easy to implement in clinical trials. + Biomarkers easily detectable in the blood + Low cost	- Time consuming for the participant - Sugar concentrations in urine requires HPLC or LC-MS equipment and trained researchers - Non-specific assessment of the type of permeability damage (only region-dependent) - Measures paracellular permeability only - Time and labor-intensive
Lactulose/L-rhamnose	Urine	Small intestine permeability		
Sucrose	Urine	Gastric permeability		
Sucralose	Urine	Colonic permeability		
Polyethylene glycols	Urine	Overall intestinal permeability		
^{51}Cr-EDTA	Urine	Overall intestinal permeability		
Circulating markers				
Claudin-3	Urine	Epithelial cell damage	+ Indicative of bacterial translocation and epithelial damage (LPS, LBP, sCD14, I-FABP, Zonulin, Claudin-3) + Reasonable cost + Easy to perform in large cohorts + Most detectable by ELISAs	- Validation of these biomarkers against standard permeability measurements lacking
Zonulin	Plasma/Serum			
Intestinal fatty acid binding protein (I-FABP)	Plasma/Serum	Small intestine permeability		
Citrulline	Plasma/Serum			
α-glutathione S-transferase	Plasma/Serum	Epithelial cell damage		
Lipopolysaccharide Binding protein (LBP)	Plasma/Serum	Indirect evidence of endotoxemia		
Soluble CD14 (sCD14)	Plasma/Serum	Indirect evidence of endotoxemia		
Endotoxin core antibodies (EndoCAb)	Plasma/Serum	Overall intestinal permeability		

D-lactate	Plasma/Serum	Overall intestinal permeability	
FITC-dextran	Plasma/Serum	Overall intestinal permeability	+ Different molecular weight molecules can inform on paracellular (4-20 kDa) or transcellular route (40-80 kDa) - Use only in preclinical models - Affected by other factors such as gastrointestinal motility and mucosal blood flow
Faecal markers			
Albumin	Feces	Assessment of flow from blood to intestinal lumen	+ Comparable results in intestinal permeability when comparing to orally administered FITC-Dextran (4kDa) + Non-invasive - Non-specific
Calprotectin	Feces	Intestinal inflammation	+ Marker of intestinal inflammation + Non-invasive - Non-specific
Zonulin	Feces	Intestinal permeability	+ Non-invasive - Non-specific
Ex-vivo			
Ussing chambers	Ex-vivo biopsies	Measures epithelial permeability to ions (TEER), epithelial permeability to molecules of different molecular weight to assess paracellular and transcellular pathways	+ Gold standard technique to assess epithelial integrity: different characteristics of gut permeability can be measures. + Can investigate differences between tissues from different regions of the gut + Used in preclinical (ex-vivo) and clinical (biopsies) models - Time consuming - Requires specific equipment and trained researchers - Experimental setup is limited by the number of chambers

C. Circadian regulation of gut function and permeability

The SCN transmits circadian information to peripheral organs via neuronal and endocrine signals (glucocorticoids or humoral signals) but also direct (sympathetic nervous system) and indirect cues (i.e feeding-fasting cycles, body temperature, rest-activity cycles) (Dibner et al., 2009). Peripheral clocks are regulated independently or directly synchronised with the master clock in our brain (S.-H. Yoo et al., 2003). In the GI tract, clock genes change their phase of expression depending on feeding cycle without shifting clock gene expression in the central clock (Hoogerwerf et al., 2007; Pan & Hussain, 2009). Interestingly, the expression of clock genes in the GI tract increases from duodenum to colon and from mucosal cells to epithelial cells (Oster et al., 2017), suggesting differences in the GI peripheral clock itself.

Circadian rhythmicity is essential for core GI functions such as motility, maintenance and restoration of the gut barrier, digestive enzyme production, nutrient transport in small intestine and finally for adequate immune system in the GI tract (Konturek et al., 2011). Even though circadian modulation of gut permeability and function has been reported for a long time, the precise mechanisms remain to be elucidated. In the colon, expression of clock genes exhibited similar rhythms than in the liver but displayed a shift in rhythms when compared to the brain master clock (Sládek et al., 2007). Another study found that 3.7% of distal colonic genes display a circadian pattern of expression, suggesting implication for colonic physiology (Hoogerwerf et al., 2008). Following a circadian disruption such as constant light, a significant increase in gut permeability in ileum and colon was observed and linked to alteration in ß-catenin, that acts potentially by regulating tight junction protein gene expression (Eum et al., 2022). Colonic motility has also been shown to be modulated by *Period* genes, two important clock genes (Hoogerwerf et al., 2010).

As detailed earlier, intestinal microbiota exhibits endogenous circadian rhythmicity that is impacted by host circadian rhythms and feeding pattern (Leone et al., 2015; Thaiss et al., 2014). Circadian modulation of mucus barrier is shaping host-microbiota spatial segregation over the day characterised by: changes in mucus thickness, mucus degradation by microbes, mucus production by goblet cells and secretion of IgA and antimicrobial peptides (Gombert et al., 2019). All these tightly regulated mechanisms help limit inflammation by maintaining a healthy

spatial relationship between host barrier and gut microbes with implications in IBD (Gombert et al., 2019).

D. HPA axis modulation of gut permeability: impact on MGBA communication

1. Acute stress

Little is known about the effect of a short acute stress (<1hour) on gastrointestinal permeability, possibly leading to adaptive response to stress in the gut. Human studies indicate that acute psychological stress can increase gastrointestinal permeability (Alonso et al., 2008; Gerdin et al., 2022). By comparing healthy women experiencing either low or moderate background stress, Alonso 2008 et al. demonstrated that an acute stressor elicited a stronger response in participants with moderate background stress (Alonso et al., 2008). This stronger response was combined with a diminished secretory ability in the jejunum characterised by an increased permeability to macromolecules (Alonso et al., 2008). In healthy volunteers, acute psychological stress was found to increase paracellular, but not transcellular, permeability and modulated immune cell activity in the rectal mucosa (Gerdin et al., 2022). Vanuytsel et al. found that changes in small intestinal permeability following acute stress was only observed in participants with a significant cortisol elevation (Vanuytsel et al., 2014). Interestingly, CRH administration increased intestinal permeability which was prevented by blocking mast cell degranulation (Vanuytsel et al., 2014). Mast cells are resident innate immune cells with a variety of receptors allowing them to respond to different stimuli (including immune, metabolic, neural and microbial triggers) by releasing mediators contained in their cytoplasm (Albert-Bayo et al., 2019).

In animal models, one hour of acute stress is sufficient to alter histology in the small intestine and increase paracellular and mucosal permeability (Y. Zhang et al., 2021). These changes are accompanied by an increase in intestinal inflammation, a reduced number of goblet cells and a reduction in occludin protein expression (Y. Zhang et al., 2021). The role of CRF R1 in mast cell degranulation in the context of short (1h) or extended (3h) acute stress has been investigated in mast cell deficient mice, demonstrating a modulation of mast cell degranulation by CRF R1 receptors on mast cells (Ayyadurai et al., 2017). Interestingly,

histamine release following 1h of restraint stress is significantly increased only in presence of mast cells with a CRF R1 receptor (Ayyadurai et al., 2017). Further research is needed in this area of research as a better understanding how our intestine reacts to a short-term acute stressor, will lead to potential avenue for a better understanding of the adaptive mechanisms in place that are necessary to overcome a maladaptive stress response.

2. Chronic stress

Human and animal studies indicate that psychological stress can increase intestinal permeability (X. Li et al., 2013; Vanuytsel et al., 2014). Increased intestinal permeability is the result of changes of tight junction protein expression, decreased epithelial resistance and (generally) increased short circuit current as determinants of macromolecular permeability, increased sensitivity to stimulation and ion transport. Indeed, 24 hours of social isolation and chronic social stress impacts electrical resistance and tight junction protein expression (Lauffer et al., 2016). Stress or CRF treatment has been shown to influence the colonic gut barrier through disruption of tight junction proteins (Machorro-Rojas et al., 2019), increased paracellular permeability (Machorro-Rojas et al., 2019), increased pro-inflammatory cytokines (Machorro-Rojas et al., 2019; Nozu et al., 2017), increased mast cell (Lauffer et al., 2016) and mononuclear cell infiltration (Vicario et al., 2012), and increased TLR4 expression (Yu et al., 2013). Overall, chronic psychological stress appears to induce overexpression of TLR4 on intestinal cells, mediated by CRFR1 activation, leading to increased pro-inflammatory cytokine secretion, disrupted tight junctions and increased permeability in the jejunum, ileum and colon. In both, mice and humans, these changes are associated with local and systemic inflammation (X. Li et al., 2013) and altered microbiota composition (Philip Karl et al., 2017) suggesting that inflammatory and bacterial mediators might play a causative role. Indeed, stress-induced hyperpermeability in humans in the small (Vanuytsel et al., 2014) and large (Wallon et al., 2008) intestine appears to be, at least in part, mediated by mast cells.

A well-characterised mechanism by which psychological stress in mice (for several days) impacts gut barrier permeability is through CRFR1 activation (Nozu et al., 2014; Vicario et al., 2012). Indeed, intraperitoneal CRF injection increases gut permeability. This effect can be blocked by a non-selective CRF receptor antagonist but not by a specific CRFR2 antagonist (Nozu et al., 2018). CRF induces changes indirectly via TLR4 activation that can lead to cytokine

production (Nozu et al., 2018), thereby modifying the immune function of the gut barrier. In contrast, CRFR2 does not seem to mediate most of stress-induced changes in gut permeability (Nozu et al., 2017; Vicario et al., 2012) although it can abolish CRF-induced TLR4 expression in vitro (Yu et al., 2013). Inhibition of TLR4 and NFkB has been shown to improve the microstructure of intestinal mucosa (X. He et al., 2017) providing a direct link between TLR4 and innate immune system activation in intestinal barrier structure. Interestingly, in porcine ileum it has been shown that ex vivo CRF treatment is able to increase paracellular flux with no effect on electrical conductance (Overman et al., 2012). This CRF-induced increase in paracellular permeability was inhibited in the presence of a non-selective CRF receptor antagonist or a mast cell stabiliser, suggesting again an important role of mast cells in ileal stress-induced hyperpermeability (Overman et al., 2012). Importantly, this study suggests that neuronal activity is required for CRF-induced gut barrier dysfunction and essential for triggering mast cell degranulation (Overman et al., 2012). Another relevant regional difference in the small intestine that is relatively unexplored is how epithelium that overlies Peyer's lymphoid follicles responds to stress: Zhang et al. demonstrated that follicle-associated epithelium was more sensitive to both chronic stress and ex vivo CRF in rat ileum and colon and exhibited increased paracellular permeability through the same mast cell-dependent mechanism (L. Zhang et al., 2017). Additionally, glucocorticoid secretion in response to HPA axis activation could also be one of the main player in stress-induced gut permeability changes as reviewed recently (Tena-Garitaonaindia et al., 2022) although more research is required to determine their relative contribution.

In addition to intestinal permeability, chronic stress has been shown to modulate mucous secretion. In contrast to permeability which has been studied throughout the intestine, effects on mucous secretion are best documented for the colon usually by examining goblet cells, specialised epithelial cells that secrete mucins. These studies show enlarged goblet cells (Machorro-Rojas et al., 2019), inconsistent effects on goblet cell number (Arciniega-Martínez et al., 2022; Machorro-Rojas et al., 2019) and a reduced colonic mucus layer thickness (J. M. Allen et al., 2022; Da Silva et al., 2014; Jaggers et al., 2022). Dysregulation of transcellular junctions associated with goblet cells may underlie some of the effects of stress-induced gut barrier dysfunction. Rengarajan and colleagues showed that colonic goblet cell-associated passages, that are usually inhibited by goblet cell-intrinsic sensing of gut microbes (Knoop et

al., 2016), increased with chronic stress exposure in the proximal colon, thereby increasing bacterial translocation and host-produced antibacterial IgA (Rengarajan et al., 2020).

Secretion of IgA, an important antibody at mucosal sites, is another important mechanism to consider in stress-induced disruptions in gut permeability. For example, duodenal but not ileal IgA was increased in mice exposed to repeated forced swims (Lara-Padilla et al., 2015). IgA secretion in response to acute, subacute and chronic stress has been reviewed by Campos-Rodriguez et al. which highlights the role of this immunoglobulin in modulating inflammation and permeability in response to stress, depending on the intensity and duration of exposure to stress (Campos-Rodríguez et al., 2013).

E. Stress, gut microbiota and gut barrier dysfunction

Both acute and chronic stress can alter gut permeability and function through several routes since HPA axis activation impact different layers of the intestine. Several routes of communication between the microbiota and the brain can be altered in response to stress such as systemic inflammation (Delaroque et al., 2021; Tian et al., 2021), microbial metabolite production (i.e. SCFAs (Tian et al., 2021)) all impacting different layers of gut permeability (i.e. changes in mucus level, intestinal inflammation, tight junction, colon length). We know now that the gut bacteria influence greatly stress-responsiveness (Foster et al., 2017) across different routes of communication along the gut-brain axis: systematic (Delaroque et al., 2021) and intestinal (Rao et al., 2021) inflammation, intestinal mucosa disruptions (J. M. Allen et al., 2022; Da Silva et al., 2014), paracellular permeability (Da Silva et al., 2014), colonic intestinal epithelial cell (J. M. Allen et al., 2022), alteration in intestinal permeability and physiology (Jaggers et al., 2022). These alterations in systemic inflammation or intestinal permeability can be alleviated by antibiotic depletion (Delaroque et al., 2021; Jaggers et al., 2022) or recapitulated by faecal microbiota transplantation highlighting an important role of gut microbes in subsequent effect of stress (Delaroque et al., 2021; Rao et al., 2021). Stress-induced chances in intestinal mucus barrier can be prevented by probiotic treatment in rats (Da Silva et al., 2014).

Interestingly, chronic stress triggered pro-inflammatory, pro-oxidative and antimicrobial RNA production in intestinal epithelial colonic cells in response to stress in conventional mice while

also decreasing mucus layer. Interestingly, no changes in RNA production in intestinal epithelial colonic cells were detectable in animal lacking a microbiome suggesting that the presence of microbiota helps adaptive response to stress in the gut (J. M. Allen et al., 2022).

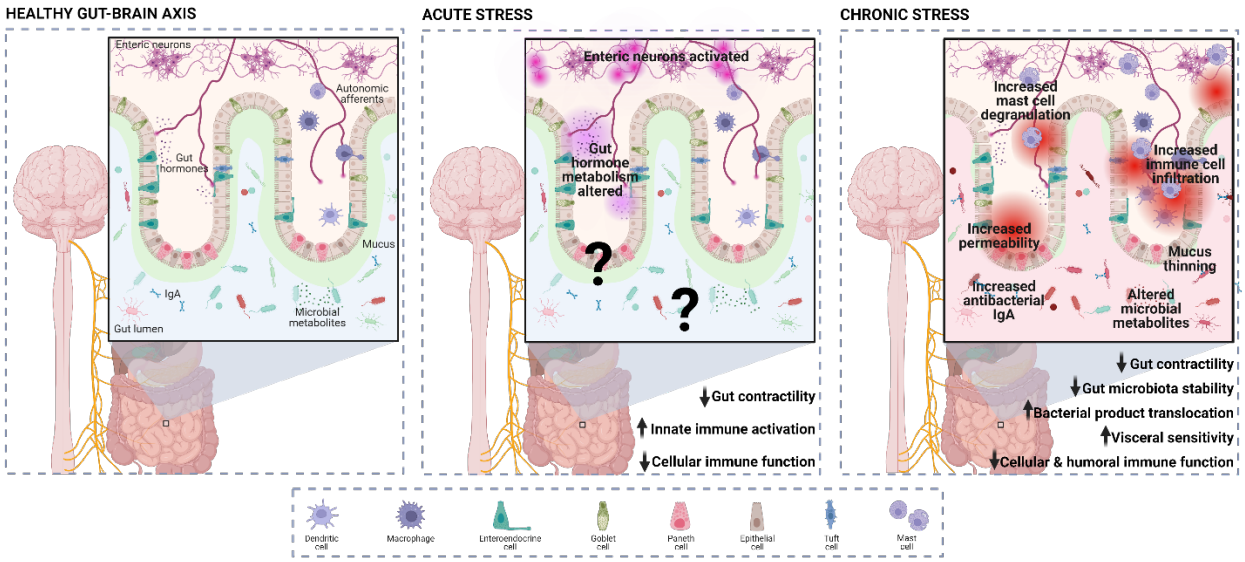

Figure 10. Acute and chronic stress exert wide-ranging effects across the gut-brain axis.
In the case of acute stress, following release of stress hormones, the enteric nervous system is activated and gut contractility is reduced. This is associated with altered serotonin metabolism in the gastrointestinal tract as well as activation of the innate immune system and immunosuppression of the adaptive immune system. Little is known about how acute stress impacts gastrointestinal physiology and the gut microbiome. Chronic stress impairs vagal signalling and enteric function, impairing gut motility. Visceral hypersensitivity is induced. Both cellular and humoral immunity are suppressed, and the gut barrier exhibits reduced mucus layer thickness, increased paracellular permeability and infiltration of circulating immune cells as well as increased mast cell degranulation. The gut microbiome exhibits reduced stability and altered composition and function. Specifically, the relative abundance of pathogenic bacteria is increased while beneficial bacteria are relatively reduced. Figure from Leigh et al. (under review)

Investigation of the potential of microbial gut-brain signalling to modulate the effect of stress through different routes of communication, including gut permeability and function, could lead to innovative therapeutic targeting of the microbiome and prevent gut permeability alterations in response to stress or in stress-related gastrointestinal disorders with psychiatric co-morbidity such as IBS/IBD.

Prebiotic supplementation in mice subject to chronic stress led to a reduced increase in circulating cytokines and ameliorated immunoreactivity to an inflammatory challenge (J. M. Allen et al., 2019). Administration of short-chain fatty acids, to mice undergoing chronic stress alleviates stress-induced increase in intestinal permeability (Tian et al., 2021; van de Wouw et al., 2018). Probiotic administration to prevent stress-induced barrier dysfunction was able to

reduce stress-induced intestinal ion secretion changes in the ileum with no change in gut permeability (Zareie et al., 2006). Another study testing another probiotic strain demonstrated a prevention in stress-induced endotoxemia (Ait-belgnaoui et al., 2012). Probiotic treatment also prevented stress-induced bacterial translocation to mesenteric lymph nodes in a rat model (Zareie et al., 2006).

V. Exercise as a modulator of HPA axis, tryptophan metabolism, gut microbiota and gut barrier function: clinical perspective

A. Exercise as a modulator of CNS: implications for stress-resilience and MDD

1. Evidence of beneficial effects of exercise in MDD

The beneficial effects of exercise on anxiety, depressive symptoms, and cognition in MDD patients are widely established (Brondino et al., 2017; Tasci et al., 2019; X. Wang et al., 2022). Beneficial effects of exercise on MDD of mild to moderate severity were reported especially for aerobic exercise (A. L. Dunn et al., 2005; Shimura et al., 2023). Although exercise demonstrated to have favourable effects on mental health, a certain threshold of exercise confers beneficial effects whilst over-exercising can lead to unfavourable effects (Shimura et al., 2023). In athletes, overtraining symptoms are associated with changes in mood, cognition and behaviour, similar to symptoms observed in MDD patients (Smith, 2000). This might be caused by an overproduction of cytokines (IL-6, IL1-β and TNF-α) evolving into chronic inflammation. Chronic systemic inflammation can significantly impact the stress-axes (HPA axis and ANS) potentially leading to those changes in behaviour, mood and cognition (Smith, 2000).

The optimal time range and duration associated with exercise leading to favourable mental health benefits for depression and anxiety range between 21-31h/week (Shimura et al., 2023). Importantly, several brain regions showing consistent volumetric reductions in MDD patients (hippocampus, anterior cingulate cortex and prefronal cortex) display structural plasticity in response to exercise (Gujral et al., 2017). A systematic review reported that exercise exerts therapeutic activity on MDD patients as a single, adjuvant or combination therapy at the moderate or vigorous intensities (Xie et al., 2021). Exercise act as at a psychological and physiological level: it enhances self-worth and self-esteem while also having antioxidant, anti-inflammatory, neuroplasticity, and neurogenesis effect (Kandola et al., 2019; Xie et al., 2021). A recent systematic review and meta-analysis identified that exercise-mediated improvement of depressive symptoms are shown to be driven by a decrease in circulating levels of

kynurenine, BDNF and IL-6 (da Cunha et al., 2023). Overall, exercise triggers the two main axes of stress and adaptation to exercise could positively modulate them (Kandola et al., 2019; Sohail et al., 2019), although the direct mechanisms have not yet been fully discovered.

2. Exercise as a regulator of HPA axis reactivity

During exercise, to adapt to a higher energy demand, HPA axis and the autonomic nervous system activate to increase carbohydrate and fat availability (Ball, 2015; Sohail et al., 2019). HPA axis activation following exercise depends on both intensity and duration, with the minimum intensity exercise to produce cortisol response appears to be 60% of VO_{2max} (Duclos & Tabarin, 2016). At a lower intensity training (i.e. 40% of VO_{2max}), the duration of exercise influence the rise in cortisol (Duclos & Tabarin, 2016). Interestingly, endurance-trained individuals have comparable markers of HPA axis at resting state but during an exercise challenge they have a decreased tissular sensitivity to glucocorticoids (Duclos et al., 1997, 2003; Duclos & Tabarin, 2016). This suggests a chronic adaptation of HPA axis activation in trained individuals of repeated acute increases in glucocorticoid secretion (Duclos et al., 2003). An important physiological adaptation to training could be a better inactivation of cortisol into cortisone to cope with the regular increase in cortisol due to training (Atlaoui et al., 2004; Gouarné et al., 2005). This agrees with another study demonstrating that cortisol levels in saliva and blood are significantly lower in the morning following an evening exercise session (T. Anderson et al., 2023), although there is some inter-individual variability (Minetto et al., 2008). This could potentially be a long-term adaptation in trained individuals to increase stress resilience by reducing glucocorticoid sensitivity and inactivating cortisol.

3. Exercise-induced cytokines production in skeletal muscle

To response to high metabolic demand, skeletal muscle – considered the largest organ in the body – has an important endocrine activity (M. A. W. Petersen & Pedersen, 2005). Muscle responds to exercise or physical activity by producing important signalling molecules, referred to as "myokines". Over time, their role as immune mediators but also as a driver of metabolic changes has become more evident (M. A. W. Petersen & Pedersen, 2005). Most of the myokines secreted by skeletal muscles in response to exercise are cytokines, intracellular signalling molecules of the immune system, that can act as pro- and anti-inflammatories

(Docherty et al., 2022). Amongst cytokines modulated by exercise, Interleukin-6 (IL-6), IL-10, TNF-α, IL-1ra, IL-15, IL-8 and IL1-β are the best-known to rise in response to an acute bout of exercise (Docherty et al., 2022; M. A. W. Petersen & Pedersen, 2005). Their immunoregulatory properties following exercise mediates HPA and ANS axes activation, triggering the release of cortisol (Path et al., 1997; Smith, 2000) and catecholamines (Smith, 2000). Although acute cytokine changes are well characterised, data are scarce regarding chronic adaptations in the immune profile in response to exercise (Docherty et al., 2022). Some studies suggests that a low level or lack of physical activity leads to increase low-grade inflammation (Forti et al., 2017; Panagiotakos et al., 2005; M. A. W. Petersen & Pedersen, 2005).

4. Myokines relevant for stress-related disorders

Exercise exerts multiple beneficial effects across the body and is now well-recognised to be an important modulator of the CNS, which has important implication for mood, cognition, and stress. Indeed, skeletal muscles act as endocrine organs, myokines produced and release by skeletal muscle in response to exercise exert either autocrine, paracrine or endocrine effects (Severinsen & Pedersen, 2020). Circulating myokines mediate the communication between skeletal muscles and other organs such as the brain, the gut, adrenal glands, and adipose tissues (Severinsen & Pedersen, 2020); to meet the energetic demands required by physical activity.

The effect of exercise on cognition (especially complex attention, executive function and memory) in healthy individuals are recognised and its magnitude dependent on age, sex and exercise intervention (Ludyga et al., 2020). Aside from cytokines, other myokines were demonstrated to be important mediators of brain-muscle crosstalk. BDNF has been the mostly widely studied and characterised in relation to exercise, for its effects on hippocampus (P. Z. Liu & Nusslock, 2018). BDNF induces improvement in cognitive functions and increase in hippocampal neurogenesis and neuroplasticity in response to exercise (Wrann et al., 2013). Some of the beneficial effects of exercise on hippocampal functioning might be mediated by a signalling cascade of neurotrophic factors (including BDNF) resulting in the production of VGF, peptides having strong antidepressant efficacy in a preclinical model (Hunsberger et al., 2007). Preclinical work demonstrated that exercise-induced skeletal muscle contraction could promote the release of other myokines (e.g., irisin and cathepsin B) and the expression of

kynurenine aminotransferase; ultimately conferring benefits at the CNS level (Lourenco et al., n.d.; Moon et al., 2016). In this work, we are interested in exercise-mediated regulation of pathways involved in CNS changes and whether those changes could be mediated by gut microbial shifts in function and composition following exercise.

5. Exercise-related changes in gut function and permeability: implication of gut microbes

Exercise may exert an hormetic effect on immune and gastrointestinal changes: initially increasing gut permeability due to hypoxia and ischemia (reduced oxygen and blood supply, respectively) or hyperthermia, thereby increasing endotoxemia but changes in inflammation following exercise appear to be transient (Dokladny et al., 2016; Sohail et al., 2019). The study of the impact of acute and chronic exercise on gut permeability has led to conflicting results. They are, in part, due to differences in study protocol, exercise regimen, intensity and duration. A meta-analysis study in healthy participant demonstrated a large effect sise for markers of endothelial cell damage in the gut (I-FABP) and moderate effect sise for gut permeability changes following a single bout of exercise (Chantler et al., 2021). Sympathetic nervous system activation following an exercise bout causes a redistribution of blood and oxygen supply, reducing supplies to the gut. Exercised-induced changes in gut permeability can lead to mucosal damage, loss of barrier integrity, increased intestinal permeability, endotoxemia and intestinal inflammation (Bosenberg et al., 1988; Costa et al., 2017; Gill et al., 2015; Karhu et al., 2017; van Wijck et al., 2012). Some evidence demonstrated that the effect of exercise on those different parameters are intensity- and duration- dependent (Costa et al., 2017). Chronically, the effect of exercise on gastrointestinal distress have been well documented in runners – with evidence showing 30-90% of them experience GI symptoms (Stuempfle & Hoffman, 2015). Interestingly, athletes or active individuals experiencing subjective feelings of stress and anxiety may be more prone to GI symptoms following exercise (Wilson, 2018, 2020). This is not surprising given that exercise act as a physiological stressor by activating stress-related pathways: the ANS and the HPA axis, thereby potentially exacerbating the impact of exercise on GI functions (Wilson, 2020).

Exercise-induced changes in gut microbiota composition and function are likely to drive some of the adaptation of exercise on gut permeability and function. Both skeletal muscle (Sohail et al., 2019) and the gut microbiome (G. Clarke et al., 2014) are important endocrine organ modulating metabolic and immune functions. Recent evidence observed that cardiorespiratory fitness is correlated with increased in microbial diversity in healthy participants (Estaki et al., 2016), with increase abundance of butyrate-producing taxa. To understand whether exercise-remodelled microbiota conferred benefits to the host, Allen et al. (2018) colonised GF animals with microbiota from exercised or control mice. They observed differences in the two groups in terms of β-diversity, metabolite profile and colon inflammation (J. Allen et al., 2018). Furthermore, they showed that mice harbouring the exercised-remodelled microbiota had an attenuated response to chemical colitis (attenuated mucus depletion and increase expression of cytokines involved in tissue regeneration) (J. Allen et al., 2018). However, this area of research is still in its infancy, and we do not yet fully understand whether the exercised-remodelled gut microbiota play a beneficial role for gut barrier permeability and function.

B. Exercise: modulator of tryptophan metabolism

Exercise has been shown to have multiple beneficial effect on mental health (Gubert & Hannan, 2021) and has been shown to modulate several factors of the microbiota-gut-brain axis (Clark & Mach, 2016; Shahar et al., 2020). Amongst those beneficial effects immediately following exercise (acute) or physiological adaptations to exercise (chronic), modulation of tryptophan metabolism is a key mechanism investigated to understand further mental health benefits of exercise. Very preliminary preclinical studies demonstrated that acutely, exercise modulated tryptophan and serotonin pathways in the brain (Blomstrand et al., 1989; Chaouloff et al., 1986). Recent evidence summarised in **Table 2** suggests that physical exercise impact tryptophan differently depending on the type, frequency, and intensity of exercise. Physical exercise has an important impact on the kynurenine pathway in response to both single (acute) and multiple (chronic) exercise, with more evidence demonstrating changes immediately post exercise. Interestingly, chronic exercise seems to impact more KAT enzymes (converting kynurenine into kynurenic acid) in skeletal muscle rather than long-term adaptive physiological changes in circulating levels of kynurenines. The increase of KAT enzymes in skeletal muscles

as an adaptation to exercise has been linked to a PGC-1α1-dependent mechanism to increase energy efficiency and fatigue-resistance (Agudelo et al., 2019). Tryptophan conversion towards the kynurenine pathway following exercise might impact energy homeostasis through de novo synthesis of NAD+ (Joisten, Walzik, et al., 2020). Preclinical studies demonstrated that the increase of circulating kynurenic acid following exercise activates Gpr35 in adipose tissue to increase energy expenditure (Agudelo et al., 2018). More importantly, PGC-1α1 modulation of kynurenine metabolism has been hypothesised to mediate resilience to stress-induced depression in mice (Agudelo et al., 2014). Overall, recent research explores the possibility that exercise-induced modulation of the kynurenine pathway may impact the levels of tryptophan metabolites in the CNS, with a direct impact on central inflammation and potentially on stress-related disorders (Cervenka et al., 2017; Joisten, Walzik, et al., 2020).

Table 2. Clinical studies interested in acute or chronic effect of exercise on tryptophan metabolism: focus on healthy participants.

Acute effect of exercise on tryptophan metabolism		
Methods	Main results:	Refs
Healthy participants (n=24) underwent acute endurance or resistance training. Blood samples were collected before, immediately after or one hour after exercise session.	**Endurance training:** ↑ KA and KA/KYN ratio immediately after and recovery after 1 hour ↑ QA **Resistance training:** ↑ Il-6 and cortisol immediately after exercise and recovery after 1 hour (can impact TRP met)	(Joisten, Kummerhoff, et al., 2020)
Patients with multiple sclerosis (n=57) either relapsing remitting or secondary progressive underwent acute (sample immediately post) endurance exercise and training (3-weeks)	↑ Kynurenine, Kyn/Trp ↓ Tryptophan	(Bansi et al., 2018)
Trained athletes (n=33) underwent a maximal exhaustion test (aerobic exercise). Blood was taken before exercise and 5min post-exercise	↑ Tryptophan, Kyn/Trp, Kynurenine ↑ Tyrosine ↓ Phe/Tyr ↑ Neopterin (marker of inflammation)	(Strasser et al., 2016)
Boston marathon runners (n=25) blood sampling before and within 10 mins of completion of the Boston marathon	↑ Kynurenate, quinolinate, anthranilate	(Lewis et al., 2010)
Skeletal muscle and plasma from healthy men (n=12) or type 2 diabetes patients (n=12) was studied at rest, after acute exercise (immediately post) and during recovery (3h- post)	**Throughout the whole exercise intervention:** ↑ KYNA/KYN ratio ↓ Tryptophan, Kynurenine (plasma) Entire intervention: ↑ mRNA expression of PGC-1α (skeletal muscle) ↓ mRNA expression of KAT4 (skeletal muscle) ↓ Tryptophan, Kynurenine (plasma) ↑ Kynurenic acid (plasma)	(Mudry et al., 2016)

	↑ Kynurenic acid (plasma)	
Pre and post single exercise bout (moderate-intense) in non-professional athletes (males, n=40). Blood samples were collected before and within 15 min of finishing the exercise bout.	Tryptophan significantly changed in faecal metabolomic when comparing pre/post exercise	(Tabone et al., 2021)
Rugby players (n=7) provided blood, urine and saliva samples daily over a competitive week	Acute changes post-match in tryptophan metabolism in urine: ↓ Kynurenate, quinolinate, xanthurenate (urine) ↑ Kynurenine (urine)	(Hudson et al., 2021)
Skeletal muscle biopsies from either control (n=8) or endurance trained active individuals (n=9). Blood samples were collected from both group at rest and from the trained group after completion of 150-km cycling exercise. Blood was also collected on a group of active participants (n=11) before and after running a half-marathon and another group before/after performing eccentric exercise (n=9)	**1h-post 150 km road cycling exercise:** ↑ KYNA, QUIN (plasma) ↓ QUIN/KYNA after exercise and returned to baseline within 24h **Before/after half-marathon race:** ↑ KYNA (plasma) 30 min-post race **Before/after eccentric exercise:** - Unchanged KYNA, QUIN and QUIN/KYNA	(Schlittler et al., 2016)
Amateurs runners were either in the placebo (n=19) or vitamin-D supplemented (n=16) group. Blood was collected 24h before, immediately after or 24h post-ultramarathon run.	↑ Kynurenic, xanthurenic, quinolinic and picolinic acids in placebo immediately post-run compared to participants supplemented with vitamin D. ↑ 3-hydroxy-L-kynurenine levels post-run in both groups ↓ tryptophan, tyrosine and phenylalanine levels immediately post-run	(Mieszkowski et al., 2022)
Skeletal muscle biopsies from either control (n=8) or endurance trained active individuals (n=9) at rest and after completion of 150-km cycling exercise.	↑ mRNA expression of KAT1 and 3 in skeletal muscle	(Agudelo et al., 2019)
Chronic effect of exercise on tryptophan metabolism		
Healthy volunteers (n=13 (unclear)) had muscle biopsies before and 48-h after the last training	↑ mRNA expression of KAT1-4 in skeletal muscle	(Agudelo et al., 2014)

session (3-weeks intense endurance training in sedentary individual)		
Breast cancer patients (n=96) and healthy women (n=24) participated in a 12-week exercise resistance training.	**Exercise response in healthy participant:** ↓ QUIN/KYNA ratio (plasma or urine → unclear) ↓ QUIN (plasma or urine → unclear)	(Zimmer et al., 2019)
Patients with mild-to-moderate depression (n=117) underwent 12-week of exercise training at 3 different intensities	- No changes in plasma levels of kynurenine or KYNA or their ratios	(Millischer et al., 2017)
Skeletal muscle biopsies from either control (n=8) or endurance trained active individuals (n=9). Blood samples were collected from both group at rest and from the trained group after completion of 150-km cycling exercise.	Muscle biopsies: ↑ mRNA expression and protein of KAT1-4 enzymes in skeletal muscle of trained individuals compared to controls - PGC-1α1 and PPARγ genes directly correlated with KAT4 gene expression	(Schlittler et al., 2016)
Healthy men over 65yo (n=49) exercise for 12-weeks. Blood samples and muscle biopsies were collected at baseline and 12-week post-exercise	↑ mRNA expression PGC-1α1, PPARα, PPARγ and KAT1-4 - No changes in circulating kynurenines	(D. J. Allison et al., 2019)
International elite athletes (n=11) at competition or preparation stage.	- No changes in KYN between preparation and competition	(Bizjak et al., 2021)

C. Exercise shifts microbial composition

1. Changes in microbial composition and function in responsive to exercise in humans

Exercise, whether it is endurance or resistance training, requires physiological and biochemical adaptations to cope with exercised-induced stress. The main adaptation to exercise requires fast energy production and release (mainly from mitochondria, adipose tissue, liver), changes in immune activation (mainly peripheral, bone marrow and muscular) and sympathetic nervous system activation (stimulating brain, heart, lungs, periphery) (Mach & Fuster-Botella, 2017). Interestingly, relatively new research demonstrates changes in gut-brain axis signalling and gut microbiota composition in response to an exercise challenge or when comparing athletes to sedentary population. **Table 3** summarised changes in microbial composition and functions following exercise in humans and is divided into several subsections: comparison of microbial species according to fitness level, study of athlete microbiome or comparison to sedentary populations, comparison of different type of exercise (endurance or resistance) on microbial community, pre- and post- measures following an exercise intervention of several weeks, pre- and post- measures in athletes competing in a highly demanding exercise task (i.e. marathon) and finally changes in microbial population in response to changes in training intensities.

Table 3. Summary table of reported changes in microbial composition and functions following exercise in humans.

Population Exercise type	Bacterial strains responsive to exercise	Advantages for the host / Hypothesis proposed	Refs
Comparing different fitness level			
Healthy participants (age= 18-35) with varying level of cardiorespiratory fitness level: low (BMI= 25.5), average (BMI= 23.5) or high (BMI= 22.8)	↑ butyrate-producing taxa: *Clostridiales, Roseburia, Lachnospiraceae* and *Erysipelotrichaceae* (taxa level) in physically fit individuals Taxa with positive exhibiting a positive correlation with VO2 peak*: Coprococcus, Roseburia, Adlercreutzia,* and unknown members of *Clostridiales*, *Lachnospiraceae*, and *Erysipelotrichaceae*	Peak oxygen uptake (VO2 peak) can account for 20% of the variation in taxonomic richness	(Estaki et al., 2016)
Middle-aged active and sedentary women (n=40) 18-40 years old BMI: 20–25kg/m^2	Active women: (species level) ↑ *Faecalibacterium prausnitzii,* ↑ *Roseburia hominis* ↑ *Akkermansia muciniphila.*	The activity of cysteine aminopeptidase in feces was significantly higher in the active group than in the sedentary group, suggesting a potential role of microbial enzymatic activity.	(Bressa et al., 2017)
Women (age=19-49) with low (n=24, age~40, BMI~31.7), moderate (n=23, age~39.7, BMI~27.9) or high (n=24, age~30.6, BMI~24.6) cardiovascular fitness	High cardiovascular fitness associated with: ↑ *Bacteroides* ↓*Eubacterium rectale – Clostridium coccoides*	Changes in gut microbiota composition are confounded by adiposity	(Y. Yang et al., 2017)

Cyclists (professional vs amateurs) Age= 19-49 11 females, 22 males	3 different taxonomic clusters, either: • ↑ *Prevotella* • ↑ *Bacteroides* ↑ abundance of *Bacteroides, Prevotella, Eubacterium, Ruminococcus,* and *Akkermansia* (genus level)	Increased abundance of Prevotella was correlated with several amino acid and carbohydrate metabolism pathways, including BCAAs.	(L. M. Petersen et al., 2017a)
University student (n=82, age~18.4, BMI~24.4) : categorised by physical activity level	Greater physical activity associated with: (taxa level) ↑ *Paraprevotellaceae* ↑ *Lachnospiraceae* ↑ *Lachnospira* Lower physical activity associated with: ↑*Enterobacteriaceae* ↑*Enterobacteriales*	• No differences observed between body mass index and gut microbiome abundance and diversity. Habitual fiber consumption and physical activity contribute to differential abundance of specific microbial taxa	(Whisner et al., 2018)
Martial art athletes comparing low (n=16, age~20, BMI~21.7) and high-level (n=12, age~20, BMI~22.2)	High level athletes: (genus level) ↑ *Parabacteroides* ↑ *Phascolarctobacterium* ↑ *Oscillibacter* ↑ *Bilophila* Low-level athletes: (genus level) ↑ *Megasphaera*	• Histidine metabolism and carbohydrate metabolism pathways were markedly over-represented in the gut microbiota of the higher-level athletes	(R. Liang et al., 2019)
Comparing athletes and non-athletes population			
Professional male rugby players (n=40, age~29, BMI~29) and controls (age~29) including high BMI≥28 (n=23) and low BMI ≤25	Athletes compared to controls: ↑ α-diversity ↑ *Akkermansia* (genus level) in athletes compared to high BMI controls	• Protein consumption positively correlates with microbial diversity.	(S. F. Clarke et al., 2014)

(n=23) matched for age and gender			
Professional rugby players (n=40) and controls including high BMI ≥26.5 (n=24) and low BMI ≤25.2 (n=22) matched for age and gender	Athletes compared to controls: ↑ α-diversity	• Microbial-derived SCFAs (propionate, acetate, butyrate and valerate) are enhanced in athletes. • Concentrations of propionate strongly correlated to protein intake, while butyrate was shown to have a strong association with intakeof dietary fibre.	(Barton et al., 2018a)
Marathon runners (n=15, age~27.4, BMI~22.4) and sedentary controls (n=10, age~29.5, BMI~22.7), samples collected one week before and after the marathon race	↑ *Veillonella Atypica* when comparing runners to sedentary individuals (genus level)	• Propionate production from systemic lactate to enhance performance	(Scheiman et al., 2019)
21 male athletes (20-35 yo), 3-w training camp with different diet: high in carbohydrate availability (HCHO), a periodised carbohydrate availability (PCHO) and ketogenic low carbohydrate-high fat diet (LCHF)	PCHO diet compared to baseline: ↑Ruminococcaceae, ↑*Coprococcus spp.* ↑*Akkermansia muciniphila* HCHO diet compared to baseline: ↑*Clostridiaceae* ↑*Lachnospiraceae* ↑*Ruminococcaceae*		(Murtaza et al., 2019)

	LCHF: stronger impact on gut microbiota composition ↑ *Dorea spp.* ↓ *Faecalibacterium spp.*		
Elite athletes (n=37)	Species level: ↑ *Eubacterium rectale* ↑ *Polynucleobacter necessarius* ↑ *Faecalibacterium prausnitzii* ↑ *Bacteroides vulgatus* ↑ *Gordonibacter massiliensis*		(O'Donovan et al., 2020)
Comparison between runners' athletes (n=48, age 18-22) and non-athletes controls (n=10, age = 22-24)	*Athletes compared to nonatheletes : (species level)* ↑ *α-diversity* ↑ *Bacteroides uniformis* ↑ *Bacteroides caccae* ↑ *Phocaeicola dorei* ↑ *Bacteroides eggerthii* ↑ *Bacteroides thetaiotaomicron* ↑ *Phocaeicola vulgatus*	• *B. uniformis* exhibited a significant negative correlation between abundance and 3000-m race time, suggesting it could be associated with endurance	(H. Morita et al., 2023)
Comparing different exercise type (endurance vs resistance)			
Comparison of sedentary control (n=15, age~26.3, BMI~25.9), bodybuilders (resistance training, n=15, age~24.9, BMI~28.1) and distance runners (endurance training, n=15, age~19.8, BMI~20.5)	No changes in β diversity Runners compared to control: (genus and species level) ↑ *Bacteroides caccae* ↑ *Sutterella* ↑ *Enterobacter cloacaegroup*	• High-protein diets might have a negative impact on gut microbiota diversity, leading to a decrease in SCFAs-producing bacteria.	(Jang et al., 2019)

	↓ *Clostridium innocuumgroup* ↓ *Weissella confusagroup* ↓ *Blautia wexlerae* ↓ *Eubacterium hallii* Bodybuilders compared to control: (genus and species level) ↑ *Faecalibacterium* ↑ *Ruminococcus callidus* ↑ *Sutterella* ↓ *Blautia wexlerae* ↓ *Eubacterium halli* ↓ *Bacteroides stercoris* ↓ *B longum group* ↓ *Escherichia coli group* ↓ *Bifidobacterium* ↓ *Lactobacillus sakei group*		
Pre- and post-exercise 24-w cardiovascular and resistance training intervention in sedentary adults (n=22, age=50-75, BMI~27.4)	24-w post-exercise: (genus level) ↑ *Bifidobacterium* ↑ *Oscillospira* ↑ *Anaerostipes* ↓ *Prevotella* ↓ *Oribacterium*	• Stool butyrate (but not acetate or propionate) increase with exercise	(Erlandson et al., 2021)
Exercise intervention study			

Intervention: 6-w of aerobic exercise (moderate-high intensity in lean (n=18, age~25, BMI~22.2) and obese (n=14, age~31, BMI~35.7) sedentary participants	Genera positively associated with exercise-induced increase in butyrate concentration*:* ↑ Faecalibacterium spp. ↑ Roseburia spp. ↑ *Lachnospira spp.* ↑ *Clostridiales spp.*	• Aerobic exercise increased faecal concentrations of SCFAs which was strongly dependent on BMI • Exercise induced changes in microbiota are reversed once returned into a sedentary lifestyle	(J. M. Allen et al., 2018)
Intervention: 6w of aerobic exercise comparing at baseline, before and after exercise in sedentary women (n=17, age~36.8, BMI~31)	Increase following exercise intervention (genus level): ↑ *Verrucomicrobiaceae* ↑ *Bifidobacteriaceae* ↑ *Dorea* ↑ *Anaerofilum* ↑ *Akkermansia* ↓ Porphyromonadaceae ↓ Odoribacter ↓ unidentified Desulfovibrionaceae ↓ unidentified Enterobacteriaceae	• Modest changes in microbial composition and function Modest changes in systemic metabolites	(Munukka et al., 2018)
Before and after 12-w intervention (resistance or endurance) study in elderly women (n=29, age<65, ~70, BMI~21.4):	Endurance training: ↑ *Baceroides* ↓*Clostridium* cluster IX Resistance training: ↑ *Clostridium* cluster IX	• Different type of exercise (resistance or endurance) affects microbiota composition differently (in elderly women).	(E. Morita et al., 2019)

3-w intervention of HIIT training in lean (n=14, age~29, Fat mass~21%) or overweight individuals (n=15, age~31, Fat mass~33%)	No differences in α and β diversity	• Short-term HIIT did not alter significantly overall microbiome composition	(Rettedal et al., 2020)
Before/after an exercising competition			
Before and after a half-marathon (21 km) (n=20, age~31.3, BMI~22.6)	No differences in α diversity At Genus level: ↑ *Pseudobutyrivibrio,* ↑ *Coprococcus_2,* ↑ *Collinsella* ↑ *Mitsuokella,*	Potential microbial function altered by exercise: ↑cell motility, ↓energy production and conversion	(X. Zhao et al., 2018)
Microbiome analysis before, during and after a 33-day 5000km race (n=4, male, age~26.5, BMI~24.4)	↑ Dorea longicatena ↑ Roseburia hominis ↑ Unclassified members of the genus *Subdoligranulum* ↓ Bacteroides finegoldii	• ↑ functional potential of microbial amino and fatty acid synthesis • Taxonomic variation included changes in butyrate-producting species and species associated with improved metabolic health	(Keohane et al., 2019)

Changes in microbiome composition in an ultramarathon runner before (21 weeks and 2 weeks) and after (2 hours and 10 days) the race (males, n=1, age=32)	Increase 2-hours post-race: (genera level) ↑ *Veillonella* ↑ *Streptococccus* ↓ *Alloprevotella* ↓ *Subdolingranulum*	• Rapid and pronounced changes in gut microbiome composition after acute exercise in humans	(Grosicki et al., 2019)
Pre and post single exercise bout (moderate-intense) in non-professional athletes (males, n=40, age~35.8, BMI~22.8)	No differences in α and β diversity Bacterial taxa modified after an acute exercise session: ↑ Romboutsia ↑ Escherichia coli TOP498 ↑ Ruminococcaceae UCG-005 ↑ Blautia genus ↓ Ruminiclostridium 9 ↓ Clostridium phoceensis	• "Functional analysis revealed that alanine, aspartate and glutamate metabolism and the arginine and aminoacyl-tRNA biosynthesis pathways were the most relevant modified pathways in serum, whereas the phenylalanine, tyrosine and tryptophan biosynthesis pathway was the most relevant pathway modified in feces" • Decrease in serum tryptophan and an increase in kynurenic acid, increasing the kynurenic/tryptophan ratio	(Tabone et al., 2021)
Changes in gut microbiome composition in ultramarathon runners before and after the race (n=9, age~46.9, BMI~21.6)	No differences in α and β diversity Pre- and post-race: (species level) ↑ *Collinsella aerofaciens* ↑ *Catenibacillus scindens* ↑ *Clostridium sp E2* ↑ *Clostridium sp E2* ↑ *Alistipes putredinis* ↑ *Drancourtella massiliensis*		(Sato & Suzuki, 2022)

	↑ *Massiliomicrobiota timonensis* ↑ *Clostridium sp Culture Jar 17* ↑ *Romboutsia timonensis* ↑ *Streptococcus sanguinis* ↓ *Faecalibacterium prausnitzii* ↓ *Blautia luti* ↓ *Eubacterium rectale* ↓ *Eubacterium rectale*		
Changes in training volumes			
Longitudinal study to analyse microbiome dynamic during changes in training volumes in swimmers (n=13, age=18-24)	Microbial genera with shift in relative abundance correlated to change in exercise training: Coprococcus Faecalibacterium	• Changes in training volume were significantly associated with shifts in the microbial community structure. The average ratio of Firmicutes:Bacteroidetes (microbial metric of energy utilization) was approximately 2:1 at the peak of volume and 1:1 as training volume reached zero suggestive of reduced energy harvesting by Firmicutes-derived microbes	(Hampton-Marcell et al., 2020)
Comparing changes in training volume in runners (males, n=8, age~20.7, Body mass~71; females, n=6, age~22, Body mass~55.1): normal training, high-intensity and one-week taper	High-intensity training: (species level) ↑ *R. callidus* ↓ *S. parasanguinis* ↓ *H. parainfluenzae*	No significant changes in nutritional intake, alpha-diversity, or global microbial composition following high intensity or taper training compared to normal training.	(Craven et al., 2022)

2. Beneficial effects of an exercised-remodelled microbiota

Since gut bacterial species have been shown to be important in energy metabolism, inflammation, and oxidative stress (Mach & Fuster-Botella, 2017; Marttinen et al., 2020), among other things, it is not surprising to see microbial adaptation and relevant function in trained individuals. Pre-clinical studies provide a deeper understanding of the mechanisms underpinning these changes. Interestingly, animals lacking a gut microbiota, GF (Dohnalová et al., 2022; Hsu et al., 2015; W. C. Huang et al., 2019) or antibiotic-treated (Dohnalová et al., 2022; W. C. Huang et al., 2019; Nay et al., 2019), display a reduce exercise performance compared to conventional animals. However, these changes in exercise performance can be reversed by cessation of antibiotic treatment or conventionalisation of germ-free animals (Dohnalová et al., 2022). Interestingly, continuous acetate infusion in antibiotic-treated mice can restore their exercise capacity suggesting an important role for systemic acetate as energy substrate during exercise (Okamoto et al., 2019).

The most well-known mechanism by which gut microbes are contributing to exercise-remodel adaptions is through the production of SCFAs (namely acetate, propionate and butyrate) in the colon as an energy fuel that can be used by colonocyte, liver, muscle, or adipose tissue (Mach & Fuster-Botella, 2017). Recently, Scheiman et al. demonstrated elegantly that systemic lactate produced during anaerobic exercise can cross gut lumen and be metabolised by *Veillonella atypica* into propionate in the colon, which can then serve to promote exercise performance (Scheiman et al., 2019). Administration of a SCFA-producing bacteria in SPF mice, *B. uniformis*, not only ameliorate exercise performance but also increased SCFA concentration in the cecum and hepatic glycogen concentration (H. Morita et al., 2023) suggesting a role for SCFA in facilitating glucose supply from the liver during exercise. Huang et al. demonstrated that acetate and butyrate, but not propionate, stimulate mitochondrial biogenesis and promote endurance performance (L. Huang et al., 2022). An interesting hypothesis suggests that exercise training could trigger changes in gut microbial composition by inducing a state of hypoxia, defined as a low oxygen level in body tissues (L. Huang et al., 2022). Indeed, several SCFA-producing bacteria are anaerobes therefore do not require the oxygen to produce energy. Therefore, Huang et al hypothesised that exercise-induced hypoxia could remodel gut microbiota composition towards SCFA-producing microbes (L. Huang et al., 2022).

Changes in SCFAs and other metabolites including inflammatory signals in response to exercise can also affect gut permeability and function during exercise (Mach & Fuster-Botella, 2017). Exercise-remodelled microbiota is thought to be beneficial for gut permeability and function though the underlying mechanisms remain to be uncovered. Allen et al. demonstrated that colonisation of exercise-remodelled microbiota in a mouse model of colitis, characterised by inflammation in the colon, attenuated colitis-induced mucus depletion and increases cytokines involved in muscle regeneration (J. Allen et al., 2018). Clinical studies investigating the role of SCFAs in obese population demonstrated that SCFAs improved lipolysis (Canfora et al., 2017). Furthermore, exercise-induced alterations in gut microbial population conferred improvement in glucose metabolism and insulin sensitivity in prediabetic participants (Y. Liu et al., 2020). Beneficial changes could also be due to antioxidant activity of gut microbes: both aerobic and anaerobic exercise lead to oxidative stress, which is characterised by an overproduction of reactive oxygen species than the antioxidant system can handle (F. He et al., 2016). Interestingly, Hsu et al. demonstrated that different microbial status can affect differently antioxidant defence system, directly impacting exercise performance (Hsu et al., 2015).

Moreover, some preclinical evidence suggests that gut microbiota influence muscle mass and function (Lahiri et al., 2019). For example, Nay et al. found that gut microbiota depletion by antibiotic induces muscle fatigability that can be reversed by environmental recolonisation (Nay et al., 2019). They hypothesised that changes in intrinsic contractile muscle properties could be due to changes in energy metabolism since SCFAs and glucose transporters expression were decrease in the ileum and skeletal muscle of antibiotic-treated animals and normalised by recolonisation. Finally and surprisingly, a recent study demonstrated elegantly that microbiome-dependent production of endocannabinoid metabolites in the gut could stimulates dopamine levels in the striatum (Dohnalová et al., 2022) thereby potentially modulating motivational states to exercise.

Aims and Objectives

The role of the microbiota-gut-brain axis in mental health is now widely recognised. Relentless research efforts enable us to grasp the impact of chronic stress on the emergence of psychiatric disorders. A growing body of evidence suggests that chronic stress alters microbiota-gut-brain axis communication, with important consequences for brain function and behaviour. However, little is known about the impact of acute stress on the microbiota-gut-brain axis. Alterations in tryptophan metabolism are a neurobiological hallmark of stress-related psychiatric disorders. Tryptophan is also a key building block for a number of bioactives in the gut brain axis. This work aims to our understanding of the fate and metabolism of tryptophan in response to a stressor. In other words, we seek to understand the tryptophan-related response along the gut-brain axis triggered by short-term psychological or physical stress. More specifically, we focused on the complex interaction between changes in host and microbial tryptophan metabolism and gut permeability in response to an activation of the HPA axis. Our attempt to decipher the underlying beneficial mechanism of acute stress and physical training could result in wholesome ways to avoid allostatic overload or maladaptive coping to severe or repeated stressors.

Aim1: Is the serotonergic response to acute stress along the gut-brain axis dependent on the microbiome?

Since microbial status has an impact on the hippocampal serotonergic system (G. Clarke et al., 2013), we aimed at identifying the role of gut microbes in serotonergic responsivity to acute stress along stress-sensitive sites of gut-brain axis signalling. A better understanding of the role of gut microbes in shaping stress-induced changes in tryptophan metabolism along the gut-brain axis enables us to understand the influence of the gut microbiome in brain and gut serotonergic activity. To do so, we analysed conventional, microbiota-deficient, and recolonised mice response to an acute stressor (15 mins) and assessed changes in central and gastrointestinal tryptophan metabolism. Moreover, we investigated whether those changes are sex-specific (***Chapter 2***).

Aim 2: Does microbial and host metabolism of tryptophan in the gut, at baseline or following acute stress, impact gut permeability in a circadian manner?

The results from ***Chapter 2*** revealed that gut serotonergic response to acute stress is region- and microbiota- dependent. On the other hand, emerging evidence suggests that gut microbial species display circadian rhythmicity (See **Introduction - Section III - E**.). Given that the availability and metabolism of tryptophan in the periphery will dictate its supply to CNS for neuroactive synthesis and that psychiatric disorders often display circadian-related impairments, we aimed at understanding further whether gastrointestinal tryptophan metabolism at baseline and following stress is circadian-dependent. To do so, we used a mouse model of microbial depletion (germ-free or antibiotic treatment) at baseline and following stress to investigate: (1) how the gut microbiota and exposure to acute stress shapes gut barrier function and permeability over the day (*in vivo*, *ex vivo* and *in vitro*), (2) whether an acute stressor impact host and microbial tryptophan metabolites and finally (3) determine if changes in gut barrier function and tryptophan metabolism at baseline and following an acute stressor are time-of-day dependent.

Aim 3: Understand how different intensity of exercise shapes key pillars of the microbiota-gut-brain axis both acutely and chronically.

Recently, skeletal muscle appeared as a new player in determining the peripheral fate of kynurenine pathway tryptophan metabolism concentrations following exercise (See **Introduction - Section V - B**, Table 2), potentially mediating some of the beneficial effects of exercise. Over time, our understanding of the beneficial effects of exercise enables us to understand its impact on several key pathways of the microbiota-gut brain axis, with changes in gut microbes being one of the physical adaptations that potentially confer improved mental health benefits. Lastly, we aimed at understanding exercise signatures of microbiota-gut-brain signalling acutely and chronically and understand how different exercise intensities mediates those changes in healthy sedentary individuals.

Chapter 2

Gut-Brain Axis Serotonergic Responses to Acute Stress Exposure are Microbiome-Dependent

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Abstract

Background: Understanding the mechanisms underpinning the response to acute stress is critical for determining how this can be modulated in both health and disease and across sexes. Stress can markedly alter the microbiome and gut-brain axis signalling with the serotonergic system being particularly sensitive to acute stress. As the impact of acute stress on regional serotonergic dynamics in the gut-brain axis and the contribution of the microbiome to this is poorly appreciated, we used microbiota deficient mice to assess whether the serotonergic response to acute stress exposure is microbiome dependent.

Methods: Adult male and female conventional, germ-free, and colonised germ-free mice underwent a single acute stressor and samples harvested immediately or 45min following stress. Serotonin, related metabolites, and serotonergic gene expression were determined.

Key Results: Our data clearly show the microbiota influenced gastrointestinal serotonergic response to acute stress in a sex- and region-dependent manner. Male-specific post-stress increases in colonic serotonin were absent in germ-free mice but normalised following colonization. mRNA serotonergic gene expression was differentially expressed in colon and ileum of germ-free mice on a sex-dependent basis. Within the frontal cortex, absence of the microbiome altered basal serotonin, its main metabolite 5-hydroxyindoleacetic acid, and prevented stress-induced increases in serotonin turnover.

Conclusions & Inferences: The gut microbiome influences the set points of the brain and gastrointestinal serotonergic systems and affected their response to acute stress in a sex- and region-dependent manner.

Keywords: Stress; neuroendocrine; germ-free; serotonin; microbiota-gut-brain axis; gastrointestinal

Introduction

The impact of stress on host physiology involves the recruitment of a variety of systems including the microbiota-gut-brain axis [1] and neuroendocrine pathways have for decades been recognised as important nodes of host-microbe interaction [2]. Serotonin (5-hydroxytryptamine; 5-HT) is an important monoamine neurotransmitter within both the gastrointestinal tract [3,4] and central nervous system (CNS) [5], and plays a key role in the adaptive response of the host to stress. Recent studies have implicated 5-HT produced along the microbiome-gut-brain axis in mediating physiologically and clinically important local, systemic and CNS effects [6,7].

Within the gastrointestinal tract, serotonergic signalling is involved in the local response to stress and 5-HT plays a critical role in stress-induced diarrhea as gut serotonergic signalling may affect intestinal motility [8,9] and faecal transit [10]. With ~95% of the body's supply of 5-HT derived from the gut, it is notable that the microbiota can alter the bioavailability of free luminal ileal and colonic 5-HT [11,12], the uptake of extracellular 5-HT [13], and influence local [14] as well as CNS [15] 5-HT synthesis. Gut-derived 5-HT may also modulate visceral sensitivity [16], as well as elicit pro- and anti-inflammatory responses through signalling via different 5-HT receptor subtypes [17]. The gut microbiota regulates baseline colonic enterochromaffin cell (EC) 5-HT synthesis and related gene expression (e.g. tryptophan hydroxylase (Tph)-1) [18]. Although the abundance of the microbiota increases by several logs proceeding from the ileum to the colon [19], the implications for microbial regulation of the serotonergic system at these distinct sites is currently unclear [11].

Acute stress-induced changes in serotonergic neurotransmission within the frontal cortex [20] and hippocampus [21] are implicated in behavioural disorders [22] including major depressive disorders [23]. Serotonergic signalling within the brain also affects the activation of

stress-responsive systems that are strongly affected by the microbiome, such as the hypothalamic-pituitary-adrenal (HPA)-axis. Despite these important observations, it is unknown whether gut-brain axis serotonergic responsivity to acute stress is affected by the microbiome.

Assimilating the insights from the impact of acute stress on serotonergic signalling within the gut-brain axis could thus have profound implications for mental and gastrointestinal health and identifying this novel regulatory role by the microbiome could reveal new treatment modalities. [24]. Hence, we sought to identify a novel effect of the microbiome in determining serotonergic responsivity to acute stress within known stress-sensitive sites in the gut-brain axis including CNS and gastrointestinal regions that may also be receptive to host-microbe dialogue.

Methods and Materials

Ethical Approval

All animal-related experiments were approved by the Animal Experimentation Ethics Committee of University College Cork and Health Products Regulatory Authority (HPRA) before beginning this study. All experimentation was carried out in accordance with European Directive 2010/63/EU.

Animals

C57/BL6 mice breeding pairs were acquired from Taconic Biosciences (Germantown, NY, USA) and F_1-generation male and female offspring were used in all experiments. GF, ex-GF, and conventional mice were housed 2-4 mice/cage under a 12h light/dark cycle and maintained on *ad libitum* autoclaved water and autoclaved, pelleted diet (Special Diet Services, Product code 801010). Male and female mice were kept in separate cages. All female mice were used at random stages of the estrous cycle. Housing conditions for GF, conventional, and ex-GF adhered to the same environmental conditions of temperature (21 ± 1°C) and humidity (55-60%). GF mice were housed in gnotobiotic flexible-film isolators. Ex-GF mice were born and maintained as GF mice in gnotobiotic flexible-film isolators until post-natal day 21 when they were removed from the isolators and, for the remaining duration of this study, re-located to the standard animal facility and housed in wire-top cages that contained used-bedding from age- and sex-matched conventional mice; bedding from cages that housed female conventional mice was used for colonization of GF female mice, and conventional male bedding for GF male mice. Previous studies have demonstrated natural murine coprophagic behaviour to be an effective method of microbial colonization [15,25]. The choice of colonization at post-natal day 21 was based on previous reports that bacterial colonization at this age can

rescue a variety of deficiencies related to the serotonergic system in the germ-free mouse including correction of basal serotonin levels in the gastrointestinal tract [11] and in serum [14]. Conventional mice were born and raised within the standard animal facility in the same typical wire-top cages. Adult (10-11 weeks old) male and female mice were used in all experiments.

Restraint stress

Perforated sterile polypropylene screw-cap 50mL conical tubes (Sarstedt, Nümbrecht, Germany) were used for restraint stress. Each tube was used only a single time (i.e. one mouse, one tube) after which the tube was discarded. In order to reduce handling variability, the same researcher handled all mice for all restraint stress experiments, which were conducted during the light cycle between 0800h and 1400h. Different rooms were used for where the mice were housed, subjected to restraint stress, and culled. Cages were randomly assigned to either non-stress or stress groups so that each experimental group contained an n=7-9 mice/sex/group. Animal group numbers were informed by previous studies assessing murine serotonin concentrations [5,15].

To begin the restraint stress experiment, a mouse was removed from its home-cage, placed into a new cage containing fresh bedding, and transported into the restraint stress room. Each mouse that underwent stress was placed into the 50mL tube and restrained for 15min. The restraint tube was laid horizontal and secured for the entire 15min stress session. After 15min of restraint stress, mice were removed from restraint and either transported immediately to the cull room or returned to their home-cage to recover from stress. Mice that were returned to their home-cage were left undisturbed for 45min, at which time the mice were moved to the cull room. To control for the variable of transport stress, mice from the

non-stress control group were placed into new cages containing fresh bedding and transported the same distance before entering the cull room.

Upon entering the cull room, mice were immediately decapitated, trunk blood collected, and tissues harvested. Trunk blood was collected into K_2 EDTA lavender-top vacutainer (BD Life Sciences, Franklin Lakes, NJ, USA) and centrifuged at 3500 x g for 15min at 4°C. Plasma was collected and stored at -80°C until analysis. Full thickness intestinal samples, which contained both mucosal and neuronal 5-HT, were excised and flushed with cold phosphate-buffered saline to remove contents. Distal ileum (2.0cm long sample taken 1.0cm away from the caecum) and proximal colon (2.0cm long sample taken 1.0cm away from the caecum), were collected. Intestinal samples and brain regions were manually-dissected on separate petri-dishes each containing dry-ice ,weighed, snap frozen for HPLC analysis or, for RNA isolation and qRT-PCR analysis stored in RNA-later (Sigma-Aldrich) for 48h at 4°C at which point RNA-later was removed, and stored in PCR-grade microfuge tubes (Sarstedt) at -80°C until analyses.

High performance liquid chromatography (HPLC)

5-HT and 5-hydroxyindoleacetic acid (5-HIAA) monoamine concentrations of intestinal and brain samples were determined as previously described [5,10]. Briefly, mobile phase consisted of a solution of HPLC-grade (Alkem/Reagecon, Shannon, Ireland) 0.1M citric acid, 0.1M sodium dihydrogen monohydrate, 5.6mM 1-octane sulfonic acid, 0.01mM EDTA, and 10% (v/v) methanol, which was adjusted to a final pH of 2.8 with 4N sodium hydroxide. Intestinal homogenization buffer consisted of mobile phase spiked with the internal standard HPLC-grade n-methyl 5-HT (Sigma-Aldrich) at a concentration of 20ng $20uL^{-1}$. Brain homogenization buffer consisted of mobile phase spiked with n-methyl 5-HT at a concentration of 2ng $20uL^{-1}$.

500uL of cold intestine or brain homogenization buffer was added to intestinal or brain samples, respectively. Tissues were then sonicated (Sonopuls HD2070, Bandelin, Germany), centrifuged at 14000 x g for 20min at 4°C, and supernatant was then carefully collected. Next, intestinal sample supernatant was diluted 1:10 in mobile phase before injection into the HPLC. Brain sample supernatant was not diluted. Injection volume for brain and intestinal samples was 20uL.

The HPLC system (Shimadzu, Japan) was operated at a flow rate of 0.9mL min^{-1}, oven temperature of 30°C, and consisted of a SCL-10Avp system controller, LC-10AS pump, SIL-10A autoinjector, CTO-10A oven, LECD 6A electrochemical detector, and Class VP-5 software. The column was Kinetex 2.6u C18 100a 100mm x 4.6mm (Phenomenex, UK). 5-HT and 5-HIAA in intestinal and brain samples were identified by characteristic retention times as determined using 5-HT and 5-HIAA external standards (Sigma-Aldrich) which were injected at regular intervals during sample analyses. Analyte:Internal standard peak height ratios were measured and compared with external standards, and results were expressed as ug of neurotransmitter per gram of tissue.

mRNA isolation and qRT-PCR

Total RNA from colonic and ileal samples was isolated using the mirVana™ miRNA isolation kit (Thermofisher Scientific, Waltham, MA, USA) and molecular biology-grade acid-phenol chloroform (Thermofisher Scientific) according to kit manufacturer instructions. All pipet tips, tubes, and plates were PCR-grade certified (Sarstedt). A Nanodrop™ spectrophotometer (Thermofisher Scientific) was used to quantify total RNA concentration and determine 260/280 ratio; only samples at or above a 260/280 ratio of 2.0 were used. Complementary DNA (cDNA) was reverse-transcribed from RNA using the High Capacity

Reverse Transcription cDNA kit (Thermofisher Scientific). cDNA synthesis was performed on a GeneAmp Thermocycler 9700 (Perkin Elmer, Applied Biosystems). Gene expression analysis was performed using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in duplicate on 384-well plates on a LightCycler 480 system (Roche Life Sciences, Penzberg, Germany) using primers designed by Integrated DNA Technologies (**Table 1**) and TaqMan™ Universal Master Mix II, no UNG (Thermofisher Scientific). qRT-PCR data was normalised using beta-Actin (*Actb*) and transformed using the $2^{-\Delta\Delta Ct}$ method as previously described [26].

Enzyme-linked immunosorbent assay (ELISA)

Corticosterone concentration (ng mL^{-1}) in plasma were determined using an ELISA kit (Enzo life sciences, Farmingdale, NY, USA) according to manufacturer instructions. Plasma was diluted in kit-provided dilution buffer 1:40 for stressed mice or 1:20 for control non-stress mice, assayed in duplicate and absorbance read at 450nm using a Biotek Synergy H1 plate reader with Gen5 software (Biotek, Winooski, VT, USA).

Statistics

All data were reported as mean ± SEM. Datasets were analysed with outliers removed using the ROUT test [27] followed by two-way ANOVA to analyse significant differences between groups, followed by Fisher's LSD post-hoc test. Stress and germ-free status were used as factors in the two-way ANOVA. Main effect values are contained in Supplemental Tables 1 and 2. Statistical significance was set at $p<0.05$. The Shapiro-Wilk normality test was used to assess the normal distribution of data. Male and female data sets were analysed separately.

Results

Absence of the microbiota affects corticosterone response to acute stress in a sex-dependent manner

The HPA-axis response to acute stress, as indicated by plasma corticosterone, (**Figure 2**) was significantly affected by GF status in a sex-dependent manner. Male and female GF exhibited greater ($p<0.05$) baseline corticosterone compared to conventional and colonised groups. Acute restraint stress caused a significant ($p<0.05$) elevation in corticosterone in all groups regardless of sex immediately following stress. At 45min-post stressor, corticosterone of each group except for male GF mice remained elevated ($p<0.05$) relative to baseline concentrations.

Figure 1. Time-course of restraint stress and post-stressor recovery.

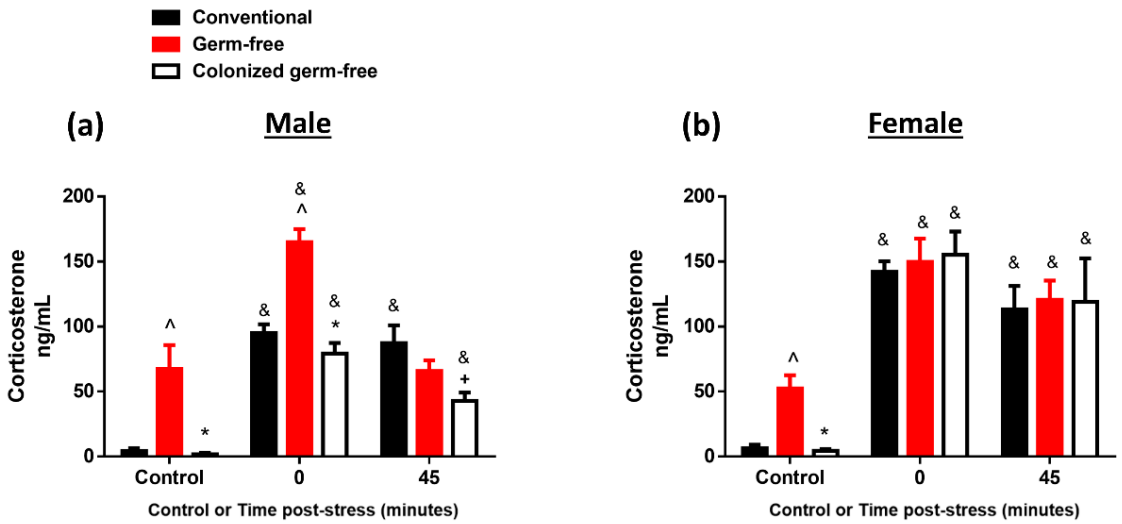

Figure 2. Stress affects corticosterone (ng mL^{-1}) response to acute stress in male (a) and female (b) mice in a sex- and microbiome-dependent manner. Corticosterone was determined in murine plasma using ELISA as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=7-9/mice/sex/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Region-dependent gut serotonergic response to acute stress depends on presence of the microbiota

Colonic 5-HT system

Changes in the male and female colonic (**Figure 3A**) serotonergic system were influenced by sex and GF-status. Acute restraint stress caused a significant ($p<0.05$) increase in colonic 5-HT of conventional and ex-GF but not GF male mice. Female colonic 5-HT did not significantly ($p>0.05$) change following acute restraint stress. Baseline serotonin was significantly ($p<0.05$) lower in female GF but not male GF mice compared to same-sex conventional mice. Male GF mice exhibited lower ($p<0.05$) 5-HIAA compared to conventional mice at baseline and following stress. Conversely, female GF mice was not significantly

different ($p>0.05$) from conventional mice. The 5-HIAA/5-HT ratio, a measure of 5-HT turnover, was significantly ($p<0.05$) altered only in male conventional mice immediately following stress. Acute restraint stress did not significantly ($p>0.05$) alter the 5-HIAA/5-HT ratio in GF or ex-GF mice.

Table 1. qRT-PCR primer assay probes †

Gene	Probe catalog #
Beta actin (*Actb*)	Mm.PT.39a.22214843.g
Tryptophan hydroxylase (*Tph*-1)	Mm.PT.58.8815029
Indoleamine 2,3-dioxygenase (*Ido*-1)	Mm.PT.58.29540170

†Probes purchased from Integrated DNA Technologies, Inc.

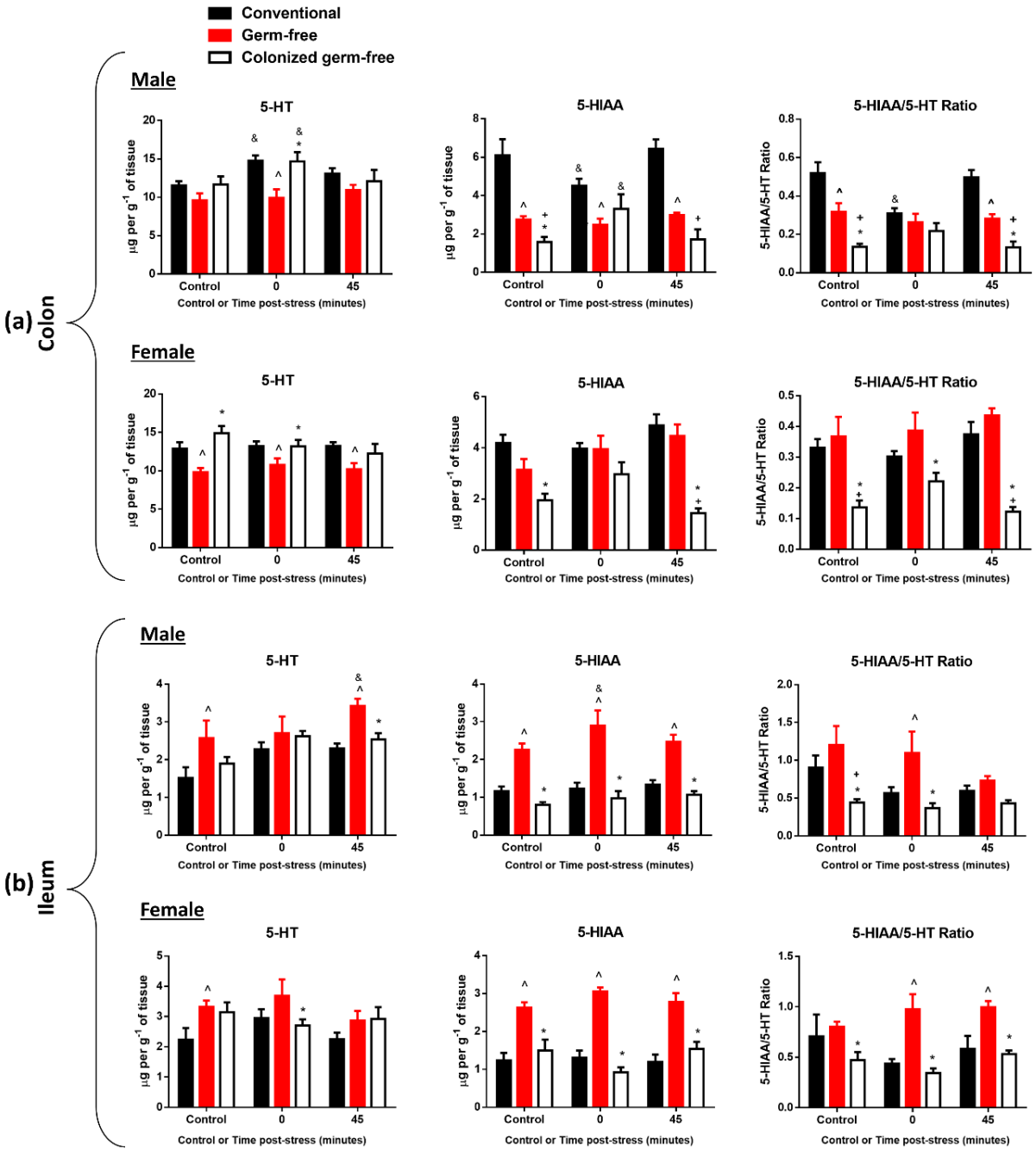

Figure 3. Stress influences male and female **(a)** colonic and **(b)** ileal 5-HT, and its main metabolite 5-HIAA. 5-HT and 5-HIAA concentrations were determined using high performance liquid chromatography (HPLC) as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=7-9/mice/sex/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Ileal 5-HT system

5-HT was significantly elevated ($p<0.05$) in male and female GF mice compared to conventional mice. Acute restraint-stress caused a significant increase ($p<0.05$) in male but not female ileal 5-HT at 45min-post stressor. 5-HIAA concentrations were greater ($p<0.05$) in the ileum of male and female GF mice compared to conventional and ex-GF groups. Acute restraint stress caused a significant increase ($p<0.05$) of 5-HIAA only in GF male ileum. Baseline 5-HIAA/5-HT ratio did not significantly differ ($p>0.05$) between GF and conventional groups. Following stress, male and female GF mice exhibited greater 5-HIAA/5-HT ratios compared to conventional and ex-GF mice ($p<0.05$).

Colonic and Ileal 5-HT gene expression

Colon

The effect of GF-status on *Tph*-1 (**Figure 4A**) and indoleamine 2,3-dioxygenase (*Ido*)-1 (**Figure 4C**) colonic mRNA expression was sex-dependent. Male GF *Tph*-1 was significantly ($p<0.05$) downregulated at baseline and following stress compared to conventional. Female GF *Tph*-1 was only downregulated ($p<0.05$) in comparison to conventional mice following stress. Compared to conventional mice, colonization corrected *Tph*-1 expression to conventional-like levels in male ex-GF ($p>0.05$) but not female ex-GF mice ($p<0.05$). *Ido*-1 mRNA expression was significantly elevated ($p<0.05$) in male GF colon compared to conventional mice. GF male *Ido*-1 expression was decreased ($p<0.05$) following stress whereas conventional male *Ido*-1 remained unaffected ($p>0.05$). Conversely, female conventional mice exhibited a stress-induced increase ($p<0.05$) in *Ido*-1 expression that was absent in GF ($p>0.05$).

Ileum

Tph-1 (**Figure 4B**) mRNA expression was downregulated ($p<0.05$) in male but not female GF mice ($p>0.05$) compared to conventional mice. *Ido*-1(**Figure 4D**) mRNA expression was downregulated ($p<0.05$) in male and female GF mice compared to conventional mice. Female conventional mice showed a stress-induced increase ($p<0.05$) in ileal *Tph*-1 mRNA expression, an effect that was absent in GF mice and not rescued by colonization ($p>0.05$). Ileal *Ido*-1 downregulation in GF mice was only partially corrected by colonization in both sexes.

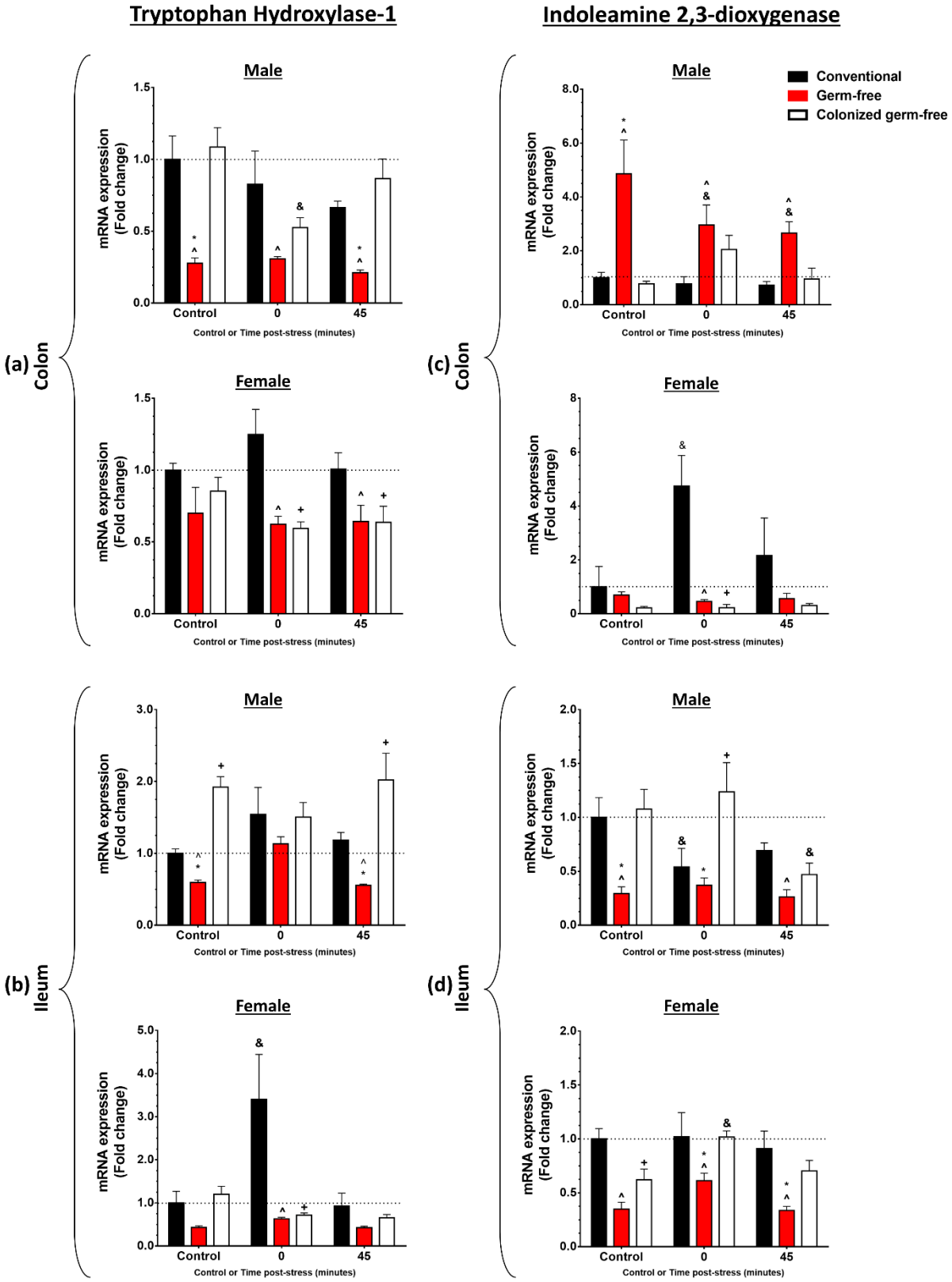
Tryptophan Hydroxylase-1
Indoleamine 2,3-dioxygenase
Conventional
Germ-free
Colonized germ-free
(a) Colon
(b) Ileum
(c) Colon
(d) Ileum
Male
Female
mRNA expression (Fold change)
Control
0
45
Control or Time post-stress (minutes)

Figure 4. Stress alters male and female colonic and ileal tryptophan hydroxylase (*Tph*)-1 **(a;b)** and indoleamine 2,3-dioxygenase (*Ido*)-1 **(c;d)** in a sex- and microbiome-dependent manner. mRNA expression was assessed using qRT-PCR as described in Methods. Bar graphs depict mean ± SEM of each group of mice and represent an n=6-9/mice/sex/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Region- and sex-dependent brain serotonergic response to acute stress are reliant on the presence of the microbiota

Hypothalamic 5-HT system

5-HT and 5-HIAA (**Figure 5A**) was not significantly different ($p>0.05$) between conventional and GF mice regardless of sex. Stress caused a sex-specific increase in 5-HT at immediately following stress in female GF mice ($p=0.0204$), an effect that was absent in female conventional and ex-GF female mice ($p>0.05$). Stress-induced changes ($p<0.05$) in conventional and GF male 5-HIAA at 45min-post stressor were absent in ex-GF male mice ($p>0.05$). Compared to baseline, acute restraint stress caused 5-HIAA to increase in conventional and GF female mice but decrease in ex-GF mice ($p<0.05$). Baseline 5-HIAA/5-HT ratio did not differ ($p>0.05$) between any group. A stress-induced change in 5-HIAA/5-HT was observed in GF female mice at 45min post stressor.

Hippocampal 5-HT system

5-HT was decreased ($p<0.05$) in female GF mice compared to conventional and ex-GF groups (**Figure 5B**) but was not significantly different between male groups ($p>0.05$). Stress did not ($p>0.05$) cause alteration in 5-HT in any group. Baseline 5-HIAA was only reduced in ex-GF mice compared to conventional mice. Stress caused a significant ($p<0.05$) elevation in 5-HIAA of all male and female groups at 45min-post stressor. Male but not female 5-HIAA/5-HT ratio was significantly ($p<0.05$) altered by stress at 45min-post stressor.

Frontal cortex 5-HT system

5-HT was significantly elevated ($p<0.05$) in male and female GF mice compared to conventional mice (**Figure 5C**)**.** Colonization restored 5-HT to conventional-like levels in ex-GF male but not female mice. In all groups and regardless of sex, conventional mice had greater ($p<0.05$) 5-HIAA compared to GF mice. Baseline 5-HIAA levels of ex-GF male mice ($p>0.05$) but not female ($p<0.05$) were similar to those of conventional mice. Stress caused a significant increase ($p<0.05$) in 5-HIAA/5-HT ratio only in conventional male and female. The 5-HIAA/5-HT ratio was greater ($p<0.05$) at all time points in conventional mice regardless of sex compared to GF mice.

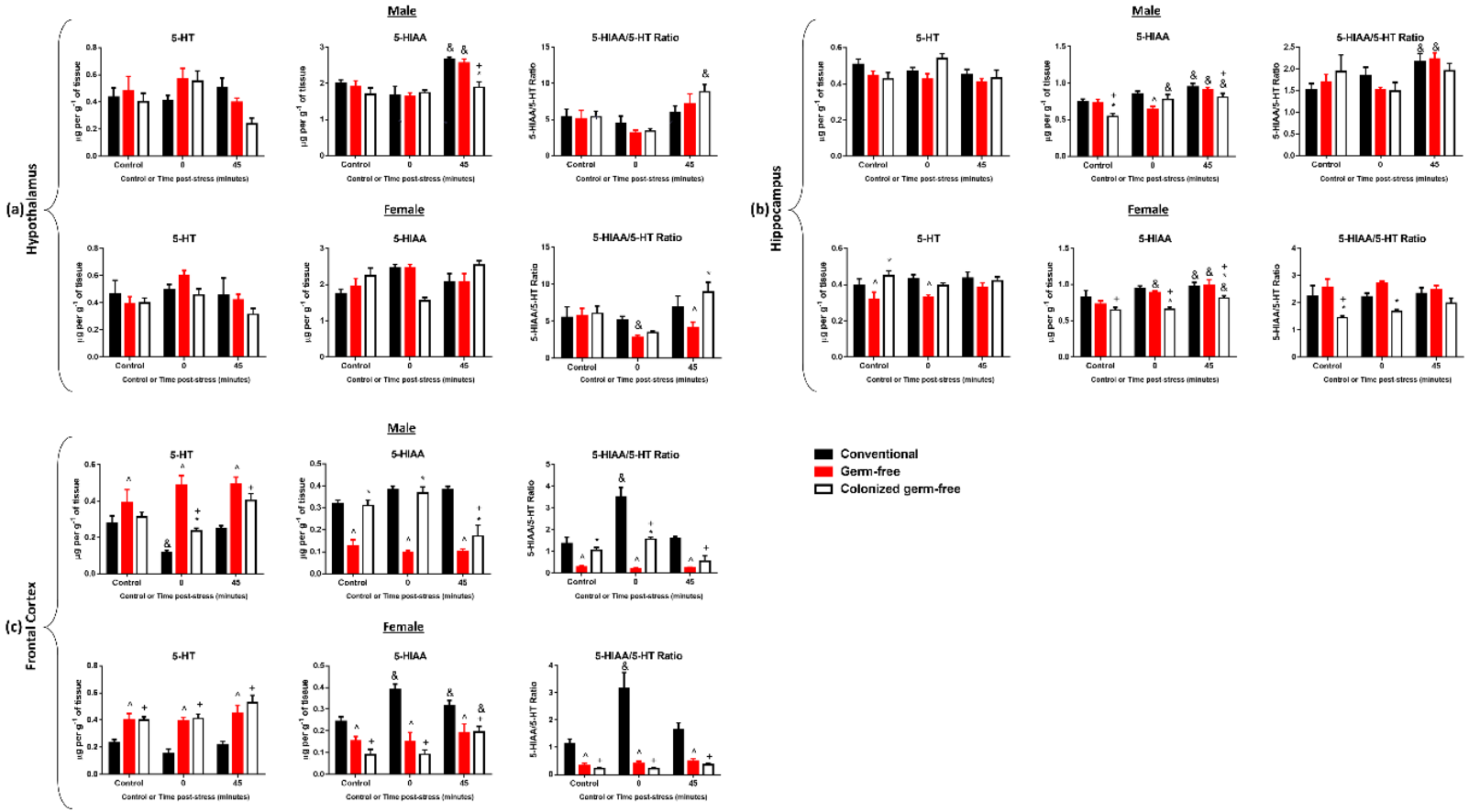

Figure 5. Stress alters male and female CNS hypothalamic **(a)**, hippocampal **(b)**, and frontal cortical **(c)** serotonergic systems in a sex- and microbiome-dependent manner. 5-HT and 5-HIAA concentrations were determined using high performance liquid chromatography (HPLC) as described in Methods. Hypothalamus and hippocampus bar graphs depict mean ± SEM of each group of mice and represent an n=7-9/mice/sex/group. Frontal cortex bar graphs depict mean ± SEM of each group of mice and represent an n=7-8/mice/male/group and an n=4-9/mice/female/group. Symbols indicating significant differences at $p<0.05$ level: &=difference between timepoint X and respective control; ^ = between same-sex GF and conventional; * = between same-sex GF and colonised GF; + = between same-sex colonised GF and conventional.

Table 2. Gastrointestinal and central responses to acute stress relative to non-stressed C, GF, and ex-GF mice †

Analyte	*Male 0 min-post stressor*			*Female 0 min-post stressor*			*Male 45 min-post stressor*			*Female 45 min-post stressor*		
	C	GF	Ex-GF	C	GF	Ex-GF	C	GF	Ex-GF	C	GF	Ex-GF
Corticosterone	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑	↑
Colon												
5-HT	↕	=	↑	=	=	=	=	=	=	=	=	=
5-HIAA	↕	=	↑	=	=	=	=	=	=	=	=	=
5-HIAA/5-HT	↕	=	=	=	=	=	=	=	=	=	=	=
Tph-1 mRNA	=	=	↓	=	=	=	=	=	=	=	=	=
Ido-1 mRNA	=	↓	=	↑	=	=	=	↓	=	=	=	=
Ileum												
5-HT	=	=	=	=	=	=	=	↑	=	=	=	=
5-HIAA	=	↑	=	=	=	=	=	=	=	=	=	=
5-HIAA/5-HT	=	=	=	=	=	=	=	=	=	=	↑	=
Tph-1 mRNA	=	=	=	↑	=	=	=	=	=	=	=	=
Ido-1 mRNA	↓	=	=	=	=	↑	=	=	↓	=	=	=
Hypothalamus												
5-HT	=	=	=	=	↕	=	=	=	=	=	=	=
5-HIAA	=	=	=	↑	↕	↓	↑	↑	=	=	=	=
5-HIAA/5-HT	=	=	=	=	↕	=	=	=	↑	=	=	=
Hippocampus												
5-HT	=	=	=	=	=	=	=	=	=	=	=	=
5-HIAA	=	=	↑	=	↑	=	↑	↑	↑	↑	↑	↑
5-HIAA/5-HT	=	=	=	=	=	=	↑	↑	=	=	=	=
Frontal cortex												
5-HT	↓	=	=	=	=	=	=	=	=	=	=	=
5-HIAA	=	=	=	↑	=	=	=	=	=	↑	=	↑
5-HIAA/5-HT	=	=	=	=	=	=	↑	=	=	↑	=	=

†An increase (↑), decrease (↓), or no change (=) as compared to control non-stressed mice. Change was defined as $p<0.05$; no change as $p>0.05$. Acute stress paradigm, tissue analyses, and statistics described in Methods. C=conventional; GF= germ-free; ex-GF= colonised GF mice.

Discussion

Serotonin is a key stress-related neurotransmitter in the gut-brain axis. Despite an increased appreciation that the microbiota influences host central and peripheral serotonergic systems at baseline, little is understood of how the microbiota can modulate the host serotonergic response following stress exposure. This is the first study, to our knowledge, to explore the impact of an acute stress challenge on serotonergic signalling at both terminals of this bidirectional communication system. We identified novel region- and sex-dependent effects of the microbiota in modulating serotonergic responses to acute stress in the gut-brain axis (**Table 2**) and provide definitive evidence that the gut microbiome is a key regulator of the gastrointestinal and CNS serotonergic system stress response. Importantly, we demonstrated that upon colonization of GF mice, the response to stress of the gastrointestinal and central serotonergic systems was restored to that observed in conventional mice thereby indicating that microbiota manipulation can be exploited to control host serotonergic responsivity to acute stress. These results enhance our understanding of microbial regulation of central and peripheral serotonergic response to stress and can help inform next-generation approaches to optimizing host-microbiota interaction performance under stress and ultimately in managing the serotonergic component of stress-related gut-brain axis disorders.

Our results indicate that the gastrointestinal serotonergic response to acute stress requires the presence of the microbiome and occurs in a region- and sex-dependent manner. Notably, the stress-induced increase in colonic serotonin of male conventional mice was absent in male GF mice, an effect that was restored following colonization. Interestingly, this effect of stress on the serotonergic system was not observed in the colon of female mice. Randomly cycling females were used in this study and an effect of estrous cycle stage was not

observed to influence serotonin concentrations (data not shown). This novel sex-specific impact of acute stress on the colonic serotonergic system underscores the value of including male and female animals to fully appreciate the consequences of acute stress interventions, particularly in relation to gut-brain axis disorders which frequently manifest differentially in males and females [28,29]. For example, irritable bowel syndrome (IBS), now viewed as a prototypical stress-related gut-brain axis disorder [30,31], has greater incidence in women compared to men with key symptoms frequently emerging following acute stress exposures [32]. Although baseline levels of tryptophan metabolism are altered in male [33] and female [34] patients, it was previously unknown whether the microbiome regulates gastrointestinal serotonergic response to stress in a sex-dependent manner. Considering the multiple roles 5-HT plays within the gut, alterations in gastrointestinal 5-HT as a consequence of stress exposure may have implications for inflammatory signalling, intestinal motility, and secretory functions [35] [17]. The role of male and female microbiome differences in these sex-specific alterations warrant assessment and microbial metabolites are likely to play a role in this regard. Metabolomics coupled with shotgun metagenomic sequencing to identify functional differences between male and female microbiota could prove fruitful in unraveling these mechanisms while cross colonization of male with female microbiome could be a useful validation strategy.

The microbiota influence both the serotonergic system and HPA-axis is important as interactions between these systems play several roles in the onset and regulation of the stress response[36]. In agreement with previous findings[37], compared to conventional and colonised mice, GF mice had an exaggerated corticosterone response to acute stress. We did find that, relative to conventional mice, GF mice had elevated baseline corticosterone. While this may

suggest GF mice are more stressed under basal conditions compared to conventional counterparts, the biological relevance of greater corticosterone concentrations in the absence of an external stressor requires further study considering GF mice have been reported to exhibit reduced anxiety-like behaviour compared to conventional mice[38]. Another important factor when considering corticosterone following acute stress is circadian rhythm, which is well known to affect basal corticosterone concentrations. Gong et al. [39] examined corticosterone in mice from 0300 to 2200 and found that corticosterone concentrations at 0800 did not differ from those at 1500, but did significantly increase at 2200. Few reports exist comparing basal corticosterone between germ free and conventional mice. Of the literature reporting basal corticosterone comparisons, Sudo et al. found no difference[37], while Neufeld et al. did identify increased concentrations in GF compared to conventional mice[38]. While Sudo et al. used the Balb/c mouse strain, Neufeld et al. utilised Swiss Webster strain, suggesting mouse strain may also play a role.

The lower baseline concentrations of serotonin observed here in the colon of female but not male GF mice as compared to conventional mice is in partial agreement with previous findings indicating the microbiota in influencing host colonic 5-HT synthesis [11,14]. Moreover, compared to conventional mice, male and female GF colon showed lower mRNA expression of *Tph*-1, a key enzyme in the synthesis of 5-HT. Conversely, the ileum of male and female GF mice exhibited increased levels of 5-HT despite the fact that ileal *Tph*-1 mRNA expression in GF mice remained lower compared to conventional mice. This may indicate a microbial influence on gut 5-HT concentration that is not exclusively reliant on transcriptional regulation of *Tph*-1, such as the microbial metabolism of serotonin. Indeed, bacteria that are part of the microbiome respond to and uptake serotonin[7,13], and that enzymes that degrade

neurotransmitters, including monoamine oxidases are found in the membranes of many bacterial taxa[40]. This is an important consideration as the role of 5-HT as an interkingdom signalling molecule, and the 5-HT system as a bi-directional pathway of host-microbe interaction is only beginning to be understood [13]. Indeed, the present study confirms the importance of the often neglected small intestine microbiota for host 5-HT responses to stress [41].

Ido-1, a key enzyme in the conversion of tryptophan into kynurenine instead of serotonin, has been implicated as a biomarker in gastrointestinal disease [42]. Whereas *Ido*-1 mRNA expression was downregulated in the ileum of male and female GF mice, microbial regulation of colonic *Ido*-1 diverged according to sex. The stress-induced increase in *Ido*-1 mRNA expression only within the female colon is notable and suggests sex differentially regulates stress-induced changes in enzymes important for tryptophan metabolism at the transcriptional level within the colon with implications for the conversion of tryptophan into various bioactive metabolites capable of modifying gastrointestinal function.

Given that the 5-HT system of the frontal cortex is a major therapeutic target for behavioural disorders [22], and the frontal cortex 5-HT and 5-HIAA levels in GF rats were previously reported to be affected by acute stress [43], we investigated the impact of stress-microbiome interactions in the frontal cortex and other important brain regions in the microbiota-gut-brain axis. We also report here a previously unidentified role for the microbiota in the regulation of the central serotonergic response to acute stress in these important brain regions in a sex- and region-dependent manner.

Acute stress caused a significant reduction in conventional male, but not female, frontal cortical 5-HT, an effect contingent on the presence of the microbiome. Colonization did not

fully restore stress responsivity of the frontal cortex serotonergic system. Reduced 5-HT turnover has been strongly associated with the consequences of psychosocial stress [44]. Both male and female conventional mice displayed a stress-induced increase 5-HT turnover, an effect that was absent in GF mice. Several antidepressants have specific effects on 5-HT turnover in the frontal cortex [45] and our results suggest that microbiome-based interventions should be assessed for their capacity to similarly influence 5-HT in this brain region.

Although the hypothalamic 5-HT system is involved in modulating neuroendocrine responsivity to stress [24], little is known regarding the involvement of the microbiota. Such a consideration is important as acute stress-induced activation of the HPA-axis was shown to be greater in females, impacted by the microbiota [37], and under serotonergic influence [46]. Consistent with these sex-dependent observations, we found that hypothalamic 5-HT of GF mice increased following acute stress in female but not male mice. Female conventional and ex-GF did not exhibit such a stress-induced increase in 5-HT thereby indicating the microbiota may blunt hypothalamic serotonergic response to acute stress in females. Although male and female exhibited differences in the timing of their serotonergic responses to acute stress exposure, it is interesting to note that this stress-induced effect was observed in conventional and GF mice of both sexes but not ex-GF. This indicates hypothalamic response to acute stress may not be primed by the microbiome but is still modifiable by colonization and thus open to modification via microbiota-directed interventions.

As GF-status was previously demonstrated to alter hippocampal 5-HT and 5-HIAA concentrations in a sex-dependent manner [15], we sought to investigate if the microbiome also influenced hippocampal 5-HT and 5-HIAA concentrations following acute stress. GF female, but not male mice, displayed significantly lower hippocampal 5-HT in non-stress and post-

stress groups compared to conventional mice. Colonization of female GF mice appeared to correct this effect as in ex-GF female mice, hippocampal 5-HT was not significantly different compared to conventional mice. These results contrast with a previous study [15] where baseline differences in hippocampal 5-HT and 5-HIAA concentrations were reported only in male GF mice. However, it is important to note that outbred Swiss Webster mice were employed in that study whereas the present study used C57/BL6 mice, and stress-induced differences in brain 5-HT gene expression between mouse strains have been previously demonstrated [5]. It is worth noting that regardless of sex and group, acute stress elicited an increase in hippocampal 5-HIAA, indicating that the more robust aspects of the hippocampus 5-HT response to stress is not specifically microbiome dependent per se. Stress-induced changes in hippocampal 5-HIAA in both conventional and GF rodents were also reported in rats that underwent behavioural testing in an open field apparatus [43]. Altered functionality of 5-HT transmission in the hippocampus has been reported to associate with depression [23], which is known to be more prevalent in women than men [47]. Taken together, these results indicate an exquisite specificity in terms of brain regions which are influenced by the microbiota and vulnerable to the impact of acute stress on the gut-brain axis.

As the physiologically and clinically relevant roles of central and peripheral serotonergic systems in stress-related disorders are increasingly understood within the context of the microbiota-gut-brain axis, it is necessary to understand how the microbiota affects the function of these systems following acute stress. Moreover, as the incidence of stress-related psychopathologies unequally affects men and women, any examination of the microbial influence on serotonergic systems therefore warrants the use of both male and female animals. Baseline gastrointestinal tract and brain serotonergic profiles were altered in GF mice a region-

and sex-dependent manner. The present study is to our knowledge the first demonstration that the microbiota also regulates gastrointestinal and central serotonergic responses to acute stress and does so with a high degree of temporal and regional specificity, an effect which is rescued in a sex-dependent manner following microbial colonization. Future studies are needed to understand the mechanisms underpinning these stress-related alterations in host-microbe dialogue and to investigate the potential functional and translational implications of these important new findings.

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Supplementary Tables

Table S1. Main effect† values of stress and germ-free status

Analyte	Tissue	Main effect	
Corticosterone	**Plasma**	**Male**	**Female**
	Stress	F(2,56) = 60.92, p<0.0001	F(2,59)=47.52, p<0.0001
	Germ-free status	F(2,56) = 26.34, p<0.0001	F(2,59)=1.255, p=0.2927
Serotonin	**Colon**		
	Stress	F(2,56)=3.873, p<0.0266	F(2,59)=0.4972, p=0.6108
	Germ-free status	F(2,56)=8.576, p<0.0006	F(2,59)=14.510, p<0.0001
	Ileum		
	Stress	F(2,56)=4.742, p=0.0125	F(2,59)=1.172, p=0.3170
	Germ-free status	F(2,56)=8.046, p=0.0008	F(2,59)=4.206, p=0.0196
	Hypothalamus		
	Stress	F(2,56)=2.901, p=0.0633	F(2,59)=2.872, p=0.0645
	Germ-free status	F(2,56)=1.231, p=0.2997	F(2,59)=1.491, p=0.2334
	Hippocampus		
	Stress	F(2,56)=1.957, p=0.1508	F(2,59)=0.9312, p=0.3998
	Germ-free status	F(2,56)=2.348, p=0.1049	F(2,59)=7.7130,p=0.0011
	Frontal cortex		
	Stress	F(2,56)=5.242, p=0.0082	F(2,58)=3.389, p=0.0406
	Germ-free status	F(2,56)=30.60, p<0.0001	F(2,58)=36.53, p<0.0001
Tph-1	**Colon**		
	Stress	F(2,57)=3.379, p=0.0410	F(2,58)=0.4954, p=0.6119
	Germ-free status	F(2,57)=21.75, p<0.0001	F(2,58)=12.930, p<0.0001
	Ileum		
	Stress	F(2,54)=0.8006, p=0.4543	F(2,57)=6.791, p=0.0023
	Germ-free status	F(2,54)=17.760, p<0.0001	F(2,57)=12.89, p<0.0001
Ido-1	**Colon**		
	Stress	F(2,54)=1.205, p=0.3076	F(2,51)=2.885, p=0.0650
	Germ-free status	F(2,54)=16.32, p<0.0001	F(2,51)=12.64, p<0.0001
	Ileum		
	Stress	F(2,55)=3.576, p=0.0347	F(2,56)=4.736, p=0.0126
	Germ-free status	F(2,55)=12.80, p<0.0001	F(2,56)=21.50, p<0.0001

†Main effect values calculated from two-way ANOVA as described in Methods.

Table S2. Main effect† values of stress and germ-free status

Analyte	Tissue	Main effect	
5-HIAA	**Colon**	**Male**	**Female**
	Stress	F(2,56)=0.2983, p=0.7432	F(2,59)=1.647, p=0.2014
	Germ-free status	F(2,56)=46.080, p<0.0001	F(2,59)=24.08, p<0.0001
	Ileum		
	Stress	F(2,56)=1.941, p=0.1531	F(2,59)=0.1267, p=0.8813
	Germ-free status	F(2,56)=59.88, p<0.0001	F(2,59)=70.180, p<0.0001
	Hypothalamus		
	Stress	F(2,56)=18.54, p<0.0001	F(2,57)=1.570, p=0.2170
	Germ-free status	F(2,56)=4.659, p=0.0134	F(2,57)=0.1345, p=0.8744
	Hippocampus		
	Stress	F(2,56)=15.80, p<0.0001	F(2,59)=10.56, p=0.0001
	Germ-free status	F(2,56)=6.408, p=0.0031	F(2,59)=13.20, p<0.0001
	Frontal cortex		
	Stress	F(2,55)=5.218, p=0.0084	F(2,50)=4.863, p=0.0118
	Germ-free status	F(2,55)=81.61, p<0.0001	F(2,50)=42.680, p<0.0001
5-HIAA/5-HT	**Colon**		
	Stress	F(2,56)=2.037, p=0.1399	F(2,59)=0.5411, p<0.5849
	Germ-free status	F(2,56)=42.79, p<0.0001	F(2,59)=28.000, p<0.0001
	Ileum		
	Stress	F(2,56)=2.669, p=0.0782	F(2,59)=0.9049, p=0.4101
	Germ-free status	F(2,56)=13.05, p<0.0001	F(2,59)=15.76, p<0.0001
	Hypothalamus		
	Stress	F(2,56)=11.80, p<0.0001	F(2,59)=6.528, p=0.0027
	Germ-free status	F(2,56)=0.4614, p=0.6328	F(2,59)=3.398, p=0.0401
	Hippocampus		
	Stress	F(2,56)=5.335, p=0.0076	F(2,59)=0.6290, p=0.5367
	Germ-free status	F(2,56)=0.0424, p=0.9585	F(2,59)=14.28, p<0.0001
	Frontal cortex		
	Stress	F(2,56)=18.98, p<0.0001	F(2,50)=6.077, p=0.0043
	Germ-free status	F(2,56)=62.07, p<0.0001	F(2,50)=48.90, p<0.0001

†Main effect values calculated from two-way ANOVA as described in Methods.

Chapter 3

The Microbiota Drives Diurnal Rhythms of Tryptophan Metabolism in the Stressed gut

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Abstract

Tryptophan metabolism is a key signalling pathway of gut-brain axis communication involved in stress-related disorders although its modulation across the circadian cycle remains unknown. We find that acute stress alters microbial and host tryptophan metabolism, and increases gut permeability *in vivo*. Tryptophan-metabolizing bacteria and their predicted metabolites exhibit circadian rhythmicity at baseline. Gut microbiota depletion disrupts circadian rhythmicity of host genes related to gut barrier function and for tryptophan enzymes, altering the balance of kynurenine and serotonin metabolism in the gut. We show for the first time that gastrointestinal response to acute stress is circadian-, microbial- and region-dependent with a signature of stress induced functional alterations in the ileum and altered tryptophan metabolism in the colon. Our findings highlight a novel role for gut microbes, both at baseline and following acute stress, in regulating circadian rhythmicity of tryptophan metabolism and barrier function in the gut.

Key words: Tryptophan metabolism, microbiota-gut-brain axis, acute stress, gut function, gut permeability, circadian rhythms

Introduction

Chronic stress impairs both mental and physical health, which can promote or exacerbate the development and progression of disease[1]. The physiological effects of stress often manifest in the gut, with a substantial body of work showing that chronic stress exposure alters gut barrier function[2], tryptophan metabolism[3] and gut microbiota composition[4] as well as other components of the gut-brain axis[5]. Altered gut tryptophan metabolism is of particular interest as this may lead to altered concentrations of tryptophan and its metabolites in the circulation, and thus availability to the brain where they play a key role in neuropsychiatric health and functioning[6-8]. However, very little is known about the effects of acute stress, the "building block" of the maladaptive sequelae of chronic stress, on the interface between gut homeostasis and tryptophan metabolism.

Circadian rhythms are disrupted in many stress-related disorders[9-11] and thus a key consideration in the study of acute stress[12]. Shift-workers, first responders, and deployed personnel often experience both stress and circadian rhythm disruption that can affect health and performance[13,14]. Furthermore, gut homeostasis[15], and gut microbiome composition and function[16,17] exhibit rhythmicity. Tryptophan availability and metabolism is microbially regulated and dependent on feeding patterns, and therefore its metabolism may depend on time-of-day. Tryptophan can be catabolised by the host towards the kynurenine or serotonin pathways both in the gut and in the brain. In contrast, gut bacteria degrade tryptophan into tryptamine or indole compounds, which can alter gut physiology, barrier function and homeostasis by interacting with host receptors in the gut epithelium, underlying tissue and liver, including the Aryl Hydrocarbon Receptor (AhR)[18,19], Transient Receptor Potential cation channel, subfamily A, member 1 (TRPA1)[20] and Pregnane X Receptor (PXR)[21,22]. Gut bacteria also shape host tryptophan metabolism by altering its availability[23-25], and affecting gut barrier function and nutrient absorption[26,27]. We have recently shown that acute stress is sufficient to disrupt the serotonergic system in the terminal ileum and proximal colon in mice, and that these stress-induced changes are dependent on the gut microbiota[28].

Here, we assess whether tryptophan-metabolizing bacteria respond to acute stress, determine circadian rhythmicity of their inferred metabolites, and examine microbial modulation of gut

tissue rhythmicity and expression of genes involved in host tryptophan metabolism. We utilised mouse models of microbial depletion (germ-free and antibiotic treatment) to investigate how an intact gut microbiota shapes host tryptophan metabolism and gut barrier function over the day. We evaluated the impact of a 15 minutes acute stressor on microbial and host tryptophan metabolites as well as microbiota-dependent mechanisms of paracellular permeability of the gut *in vivo*. Finally, we determine the interaction between time-of-day and microbial depletion on the gut response to acute stress in a region-dependent manner. Understanding the mechanisms contributing to health and performance under the burden of stress and circadian rhythm disruption will inform interventions to counteract their impact.

Methods

Resource Availability

Lead Contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Gerard Clarke at G.Clarke@ucc.ie.

Materials Availability: This study id not generate new unique reagents.

Data and Code Availability:

Metagenomics data have been deposited at the European Nucleotide Archive and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. PCR and ex vivo experimental data will be shared by the lead contact upon request.

All original code has been deposited at Github and is publicly available. The url is listed in the key resources table.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Studies in Animals

All animal work carried was approved by the Animal Experimentation Ethics Committee of University College Cork and Health Products Regulatory Authority (HPRA) before beginning this study. All experimentation was carried out in accordance with European Directive 2010/63/EU and was approved by both the Animal Experimentation Ethics Committee of University College Cork (Project Authorization AE19130/P160) and United States Air Force Surgeon General's Office of Research Oversight and Compliance. For studies containing germ-free mice, C57/BL6 mice breeding pairs were acquired from Taconic Biosciences, and F1-generation male and female offspring were used in all experiments. Germ-free, ex-germ-free, and conventional mice were housed 2-4 mice/cage under a 12-hour light/dark cycle and maintained on ad libitum autoclaved water and autoclaved, pelleted diet (Special Diet Services). Housing conditions for germ-free, conventional, and colonised germ-free adhered to the same environmental conditions of temperature (21 ± 1°C) and humidity (55%-60%).

Germ-free mice were housed in gnotobiotic flexible-film isolators. Colonised germ-free mice were born and maintained as germ-free mice in gnotobiotic flexible-film isolators until postnatal day 21 when they were removed from the isolators and, for the remaining duration of this study, re-located to the standard animal facility and housed in wire-top cages that contained used-bedding from age- and sex-matched conventional mice. For all other studies, adult male C57/BL6 (20–29 g) mice purchased from Envigo were group-housed 2–3 per cage and maintained on a 12/12 h dark–light cycle with a room temperature of 22 ± 1 °C on ad libitum water and autoclaved, pelleted diet (Envigo). ZT 23 and ZT11 were determined on the dark-light cycle in the animal unit.

Studies in Cell Lines

The AhR activity of animal caecal supernatants was measured using Human HT29 colon adenocarcinoma Lucia™ AhR reporter cells (InvivoGen).

Gut microbiota depletion by antibiotic cocktail

Mice were subjected to antibiotic treatment for 14 consecutive days in drinking water, replaced every 2 days for the duration of the treatment. The full antibiotic cocktail consisted of ampicillin (1g/L), gentamicin (1g/L), vancomycin (0.5 g/L) and imipenem (0.25 g/L) while Gram-positive-depleted mice received vancomycin (0.5 g/L) and Gram-negative-depleted mice received gentamicin (1 g/L). All antibiotics were purchased at Discovery Fine Chemicals. Antibiotic-containing water was replaced every 2 days for the duration of the treatment. Mice had access to water *ad libitum*. Body weight and water intake were monitored every day the first week of antibiotic treatment.

Acute restraint stress

The acute restraint stress procedure was performed using a clean perforated polypropylene screw-cap 50 mL conical tubes as previously described [28]. For the FITC *in vivo* and metabolomics studies acute restraint stress was administered during the light phase between 8:00am and 2:00pm (ZTs1-7). For the circadian-acute stress experiments, acute restraint stress was administered either at ZT23 (6:30am +/- 15 mins) or ZT11 (6:30pm +/- 15 mins). Cages were randomly assigned to either non-stress or stress groups. Each mouse that underwent stress was placed into the 50 mL tube and restrained for 15 minutes. After 15 minutes of

restraint stress, mice were removed from the restrainer and either transported immediately to the cull room or returned to their home cage and left undisturbed for 45 minutes. To control for the variable of transport stress, mice from the non-stress control group were placed into new cages containing fresh bedding and transported the same distance before entering the cull room.

FITC in vivo

Gastrointestinal permeability was assessed using the FITC-dextran intestinal permeability test, which was conducted as previously described [59]. Mice were fasted overnight and then gavaged with Fluorescein-5-isothiocyanate (FITC; Sigma-Aldrich) 4kDa orally at a concentration of 600mg/kg in sterile PBS 30mins prior to acute restraint stress. Following a pre-stress tail bleed to assess baseline levels of FITC, mice were subjected to acute restraint stress as described above. Following the stress procedure, mice were allowed to recover in their cage except for tail bleeds at different timepoints following stress (T0, T45, T90 and T240). Whole blood was collected in an EDTA-containing capillary. Blood was then transferred to a tube, centrifuged for 15 min at 3500 *g* at 4°C, and plasma was collected and stored at −80°C for later analysis. Plasma FITC-dextran concentrations were assessed with a Victor 2 plate reader (PerkinElmer) with an excitation of 490 nm and emission of 520 nm.

Tissue Collection

Upon entering the cull room, mice were immediately decapitated, trunk blood collected, and tissues harvested. Trunk blood was collected into K2 EDTA lavender-top vacutainer (BD Life Sciences) and centrifuged at 3500 *g* for 15 minutes at 4°C. Plasma was collected and stored at −80°C until further analysis. Full-thickness intestinal samples were excised and flushed with cold phosphate-buffered saline to remove contents or 10mM glucose-containing krebs buffer (1.2mM NaH_2PO_4, 116mM NaCl, 4.8mM KCl, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 2.5mM $CaCl_{2)}$ for Ussing Chambers experiments. Intestinal samples were manually dissected and stored in PCR-grade microfuge tubes at −80°C until analyses.

Ussing chambers

Distal ileum (1.5cm segment taken 1cm proximal to caecum) and proximal colon (1.5cm segment taken 1cm distal to caecum) samples were mounted into the Ussing chambers with

4mm round ap[e]rture (0.126cm2 exposed tissue area) following seromuscular stripping. Krebs buffer was added to both the mucosal and serosal chambers: Krebs buffer contained 10mM D-glucose in the serosal chamber and 10mM mannitol in the mucosal chamber to avoid activation of sodium/glucose co-transporters in the epithelial cells of the small intestine, which is known to increase tight junction permeability [60]. Chambers were maintained at 37°C and continuously supplied with carbogen (9_5% O2 and 5% CO2). Tissues were voltage clamped to zero using an automatic voltage clamp (DVC-1000/EVC-4000, World Precision Instruments). Short-ci*rcu*it current (Isc) was recorded in a zero voltage clamp mode; transepithelial electrical resistance (TEER) was measured by discharging a 2 mV pulse. All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using Labscribe software (World Precision Instruments). Transepithelial permeability was investigated by measuring mucosal-to-serosal flux of FITC. FITC was added to the mucosal chamber to a final concentration of 2.5mg/ml and 200µl samples were taken from the serosal chamber every 30 minutes and replaced by fresh Krebs for the following 120 minutes. Samples taken were measured at 485nm excitation/535nm emission wavelengths.

Quantitative RT–PCR

Total RNA from proximal colon and distal ileum samples were isolated using the mirVana™ miRNA isolation kit (Thermo Fisher Scientific) according to kit manufacturer instructions using zirconia/silica beads (BioSpec) in a bead beater (MP Biomedical). RNA concentration was determined using the Nanodrop Spectrophotometer (Nanodrop Technologies).

For TaqMan assays (Applied Biosystems) RNA was retro-transcribed using a HighCapacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time PCR. HPRT was used as housekeeping gene. Genes of interest were normalised using the mean of the housekeeping genes for gut tissues (HPRT). Analysis of relative gene expression was performed using the ΔΔCT method normalised to an independent calibrator [61].

Measurement of AhR activity

The AhR activity of animal caecal supernatants was measured using Human HT29 colon adenocarcinoma Lucia™ AhR reporter cells (InvivoGen) as recommended by the supplier.

Caecal supernatants were resuspended in 1xHBSS solution at a concentration of 100 mg/mL. The mixture was vortexed thoroughly then centrifuged 10 mins at 4000xg, 4°C. The supernatant obtained was filtered through 0.45 um filter, centrifuge again and filtered at 0.2 um. Caecal supernatants were stored at -80°C until immediately prior to AhR reporter cell treatment. HT29-AhR reporter cells were seeded into clear 96-well standard cell-culture plates . Following 24hr from seeding, cells were stimulated with processed caecal supernatant, 50uM kynurenine (known AhR ligand, Sigma-Aldrich) or the complete antibiotic cocktail for 24 hours in a total culture volume of 200uL per well. Experiments were performed in triplicate and each experiment contained at least two biological replicates. Luciferase activity was quantified as relative luminescence units (RLU) using a Victor 2 plate reader (PerkinElmer) and Quanti-Luc reagent (InvivoGen) according to the manufacturer's instructions. The results were normalised on the basis of the negative luciferase activity of the control and corrected for cell viability determined using the MTT assay as previously described [62], in brief below.

MTT cell viability assay

Immediately following media collection for the luciferase assay, the remaining media was removed from the cells and 50µL of 0.5% MTT (Sigma-Aldrich) was added to each well. The plate was covered and placed in the 37°C incubator for 2-3hrs to allow crystals to form. The MTT was then removed from the wells and 100µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was placed on a plate shaker for 15-20min to allow the crystals to dissolve. The plate was then read at absorbance 570nm. Percent viability compared to the untreated wells was calculated by subtracting the average reading of wells containing dead cells (24hr 50% DMSO) and then dividing this number by the average reading of the untreated wells minus wells containing dead cells and then multiplied by 100 for percentage.

Shotgun sequencing library preparation

DNA was extracted from caecal contents using the QIAamp Fast DNA Stool mini kit (Qiagen) according to kit instructions. Whole genome shotgun sequencing library was performed the Nextera XT DNA Library Preparation kit (Illumina) and the library was prepared according to the Nextera XT DNA Library Preparation Guide. Briefly, DNA concentrations were diluted to 0.2 ng/µL and fragmented by incubating at 55°C for 7 minutes. Paired-end Nextera XT indexes

were added and 12 cycles of amplification process were completed. Samples were purified with AMPure XP beads (Beckman Coulter Life Sciences) according to the manufacturer's instructions. In order to calculate molarity, DNA concentration was quantified using the Qubit dsDNA High Sensitivity Assay (Thermofisher Scientific) and amplicon size was measured by Agilent Bioanalyser 2100. Lastly, 1 mM of libraries were pooled before loading on the Illumina NextSeq platform for 150 bp paired-end sequencing.

Taxonomic and functional analysis

We performed quality checks on raw sequences from all faecal samples using the FastQC program, using the default quality score of 30 threshold. Shotgun metagenomic sequencing data were then cleaned and host genome sequences were filtered using Bowtie2 [63] via the Kneaddata wrapper program with following parameters: ILLUMINACLIP:/NexteraPE-PE.fa:2:30:10, SLIDINGWINDOW:5:25, MINLEN:60, LEADING:3, TRAILING:3. Reads were aligned to the Web of Life database[64] using Bowtie2 and taxonomic and functional profiling of the microbial community was performed using woltka[65]. Next, the uniref90-based gene abundance matrix was further collapsed by KEGG Orthology (KO) term mapping via the "woltka tools collapse" function provided within woltka. Woltka SOP is available online (https://github.com/qiyunzhu/woltka/blob/master/doc/wolsop.sh). Gut-Brain Modules (GBMs) and Gut-Metabolic Modules (GMMs) were calculated using the R version of the Gomixer tool [33].

Metabolomics

Caecal contents and colonic mucosa metabolomics were conducted by Metabolon Inc. using ultrahigh performance liquid chromatography-tandem mass spectrometry, described below. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with

positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

All methods utilised a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analysed using acidic positive ion conditions, chromatographically optimised for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analysed using acidic positive ion conditions, however it was chromatographically optimised for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analysed using basic negative ion optimised conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analysed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate, pH 10.8.

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities based on three criteria: retention index within a narrow RI window of the proposed identification, accurate

mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. Metabolites that were found to be altered with stress were mapped to their corresponding Human Metabolome Database identifier and subjected to enrichment analysis using the MetaboAnalyst online pipeline[66], choosing the murine KEGG library as a reference.

Bioinformatics and statistical analysis

Further data-handling was undertaken in R (version 4.2.0) using the Rstudio GUI (version 2022.2.2.485). In all microbiome analysis with the exception of alpha diversity, taxa with a prevalence of < 10% of samples at the species level were excluded from analysis as ratios are invariant to subsetting and this study employs compositional data analysis techniques [67,68]. Principal component analysis was performed on centred log-ratio transformed (clr) values. Zeroes were replaced using the 'const' approach described by Lubbe and colleagues [69]. Beta diversity was computed in terms of Aitchison distance, or Euclidean distance between clr-transformed data, and assessed by PERMANOVA using the vegan package. Alpha diversity was computed using the iNEXT library [70]. In order to assess circadian rhythmicity, we fitted linear models for each clr-transformed feature to a fourier-decomposed sine and cosine element, similar to the method described by Singer and Hughey [32]. Code used to fit these models can be found online at https://github.com/thomazbastiaanssen/kronos. Variables were selected based on the Benjamini-Hochberg procedure with a q-value of 0.1 as a threshold. This procedure controls the False Discovery Rate (FDR) using sequential modified Bonferroni correction for multiple testing. Custom R scripts and functions are available online at https://github.com/thomazbastiaanssen/Tjazi [71]. Remaining studies were analysed using general linear models with the rstatix package or using functions available through https://github.com/thomazbastiaanssen/Tjazi. Where appropriate post-hoc pairwise comparisons were performed using Tukey's procedure. Heatmaps were produced using r package pheatmap. In order to assess associations between host gene expression and microbial functional potential in the context of tryptophan metabolism, we converted all relevant genes and functions to KEGG orthologues and fitted linear mixed-effects models between those pairs of features that shared membership in a KEGG-pathway. This method was

implemented previously here[36]. Custom R scripts and functions are available online at https://github.com/thomazbastiaanssen/anansi.

In silico analysis of tryptophan metabolism

Raw counts from our metagenomic analysis from conventional mice were cross-referenced with the TrpNet database (https://www.trpnet.ca/home.xhtml)[34]. This allowed us to estimate the potential of tryptophan metabolites production for each bacterial strain characterised in the database. We then summed the predicted abundance of each metabolite for all bacterial strains present in an individual mouse, to estimate the predicted abundance for each metabolite in the TrpNet database. The TrpNet database was used to classify bacteria as tryptophan-metabolising or other for the metagenomic analysis.

Results

Acute stress increases gut permeability *in vivo* and alters host and microbial tryptophan metabolite concentration in the gut

Chronic and severe stressors increase gut paracellular permeability which is associated with and may underlie stress-induced changes in immune and gut-brain axis signalling[2,5]. In contrast, little is known about the effects of acute stress on paracellular permeability. Here, we examined if 15 minutes of acute restraint stress was sufficient to impact gut permeability in conventional or antibiotic-treated animals by orally administering 4kDa Fluorescein-5-isothiocyanate-dextran (FITC) (**Figure 1A**). We show for the first time that a 15-min acute stressor transiently increased paracellular permeability *in vivo* immediately following stress independent of microbial status (**Figure 1B**; all statistical results reported in Table S1). Since this rapid response is likely to alter interactions between the gut microbiota and host tissue, we examined whether this stressor exposure could alter caecal and colonic mucosa metabolites (**Figure 1C**). When comparing conventional, germ-free, and colonised germ-free mice subjected to an acute stressor, we observed that caecal metabolites were significantly altered by stress and microbial status (**Figure 1D**) while metabolites from the colonic mucosa were only affected by microbial status (**Figure S1**). Pathway enrichment analysis of caecal metabolites identified tryptophan metabolism as the pathway most affected by acute stress exposure in conventional mice (**Figure 1E**). This result suggests that stress-induced tryptophan metabolite changes are dependent on an intact microbiome.

Our metabolomics approach revealed that stress only exerted effects on tryptophan metabolites in microbially-colonised mice. Microbially-derived metabolites tryptamine (**Figure 1F**) and indole acetate (**Figure 1G**) were altered by stress in conventional animals, while 3-formylindole (**Figure 1H**), N-acetyltryptophan (Figure 1I), indole lactate (**Figure 1J**) and indole propionate (**Figure 1K**) were altered by stress in colonised germ-free mice. Interestingly, host-derived tryptophan metabolites in caecal contents were also altered by stress only in microbially-colonised mice (**Figures 1L-N**). Additional tryptophan metabolites measured in the caecal contents are available in **Figure S2**. While a clear difference was observed between colonised and germ-free mice in terms of tryptophan metabolism following stress, this is less

evident between conventional and colonised germ-free mice. While both conventional and colonised germ-free mice exhibited stress-induced changes in caecal tryptophan metabolite expression, different sets of metabolites were altered. These results suggest that stress-induced tryptophan metabolite production in the caecum may depend on both the gut microbiome in adulthood and its effects on gut homeostasis in development. In summary, it appears that both gut barrier permeability and tryptophan metabolism are dynamically and transiently altered by acute restraint stress, and that the latter effect depends on the gut microbiome. Specifically, acute stress appears to increase microbial tryptophan metabolite production while reducing host tryptophan metabolite availability in the gut lumen. Although we know that the response to psychological stress varies depending on time-of-day[29] and that gut microbial abundance can oscillate over the day[30], to our knowledge little is known about microbial tryptophan metabolism over the day.

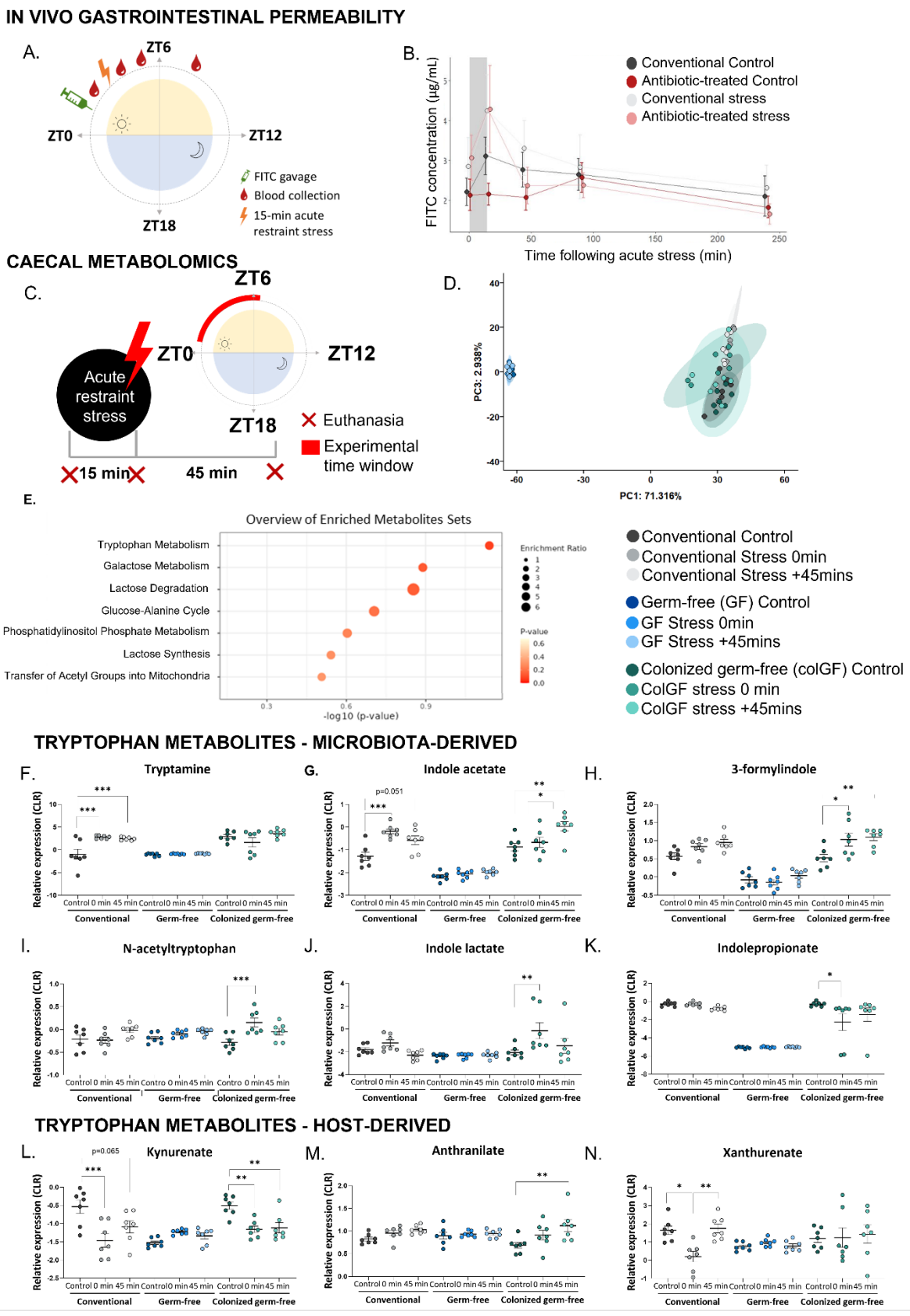
IN VIVO GASTROINTESTINAL PERMEABILITY
A.
ZT6
ZT0
ZT12
ZT18
FITC gavage
Blood collection
15-min acute restraint stress
B.
FITC concentration (µg/mL)
Time following acute stress (min)
Conventional Control
Antibiotic-treated Control
Conventional stress
Antibiotic-treated stress
CAECAL METABOLOMICS
C.
ZT6
ZT0
ZT12
ZT18
Acute restraint stress
15 min
45 min
Euthanasia
Experimental time window
D.
PC3: 2.938%
PC1: 71.316%
E.
Overview of Enriched Metabolites Sets
Tryptophan Metabolism
Galactose Metabolism
Lactose Degradation
Glucose-Alanine Cycle
Phosphatidylinositol Phosphate Metabolism
Lactose Synthesis
Transfer of Acetyl Groups into Mitochondria
-log10 (p-value)
Enrichment Ratio
P-value
Conventional Control
Conventional Stress 0min
Conventional Stress +45mins
Germ-free (GF) Control
GF Stress 0min
GF Stress +45mins
Colonized germ-free (colGF) Control
ColGF stress 0 min
ColGF stress +45mins
TRYPTOPHAN METABOLITES - MICROBIOTA-DERIVED
F.
Tryptamine
G.
Indole acetate
H.
3-formylindole
I.
N-acetyltryptophan
J.
Indole lactate
K.
Indolepropionate
Relative expression (CLR)
Control 0 min 45 min
Conventional
Germ-free
Colonized germ-free
TRYPTOPHAN METABOLITES - HOST-DERIVED
L.
Kynurenate
M.
Anthranilate
N.
Xanthurenate

Figure 1. Acute stress increases gut permeability in vivo and alters host and microbial tryptophan metabolites concentration in the gut. (A) Experimental design schematic and legend for panel B: mice were treated orally with FITC (4kDa) 30 minutes prior to the 15-min acute restraint stress exposure between ZT1/ZT2 (ZT= zeitgeber). Blood was taken from the tail to assess baseline levels of FITC and following stress (B) FITC concentration (µg/mL) in blood at baseline and following stress (T0, T45min, T90min, T240min) to measure FITC translocation from gut lumen to the circulation (n=8 mice/group). Data expressed as mean ± SEM; data analyzed by three-way mixed general linear modelling; grey area represents stress exposure. (C) Experimental design schematic and legend for panels D-N: conventional, germ-free and colonized germ-free mice were subjected to the same 15-min acute restraint stress. All mice were euthanized between ZT1 and ZT7 and were either naïve (control), euthanised immediately after stress, or allowed to recover for 45mins post-stress (n = 7 mice/group/timepoint) (D) Principal component analysis showing the effects of acute stress on caecal metabolome in conventional, germ-free, and colonized germ-free mice in terms of β-diversity as measured in Aitchison distance. Ellipses indicate 95% confidence intervals per group. (E) Enrichment analysis of caecal metabolites altered by stress in conventional mice. The enrichment ratio is represented by the size of the circle. (F-K) Relative expression of selected caecal tryptophan-related metabolites from microbial degradation. (L-N) Relative expression of selected caecal tryptophan-related metabolites from host degradation. Data are expressed as mean ± SEM with individual values overlaid; data analyzed by 2-way general linear modelling followed by Tukey-adjusted post-hoc comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Tryptophan-metabolizing bacteria and predicted metabolites display circadian rhythmicity in conventional mice

The composition and function of gut bacteria oscillate during the day due to environmental changes such as the sleep/wake cycle and nutrient availability[30] as well as internal clocks in gut epithelial tissue[31]. However, there is no data for these circadian oscillations at the strain level in conventional animals (**Figure 2A**). We assessed circadian rhythmicity by mathematically estimating the acrophase (peak), nadir (trough) and amplitude of our variables of interest[32]. From our metagenomic analysis we found that microbial species richness exhibited circadian effects (**Figure 2B**) and microbiome composition significantly changed according to time-of-day when assessed by PERMANOVA (**Figure 2C**; PCA plot). We found that approximately 3% of the strains identified (104/3637) exhibited oscillating relative abundances in conventional mice. Of interest, 61% of bacterial strains exhibiting rhythmicity can metabolise tryptophan (**Figure 2D**), implying that microbially-derived tryptophan metabolites exhibit circadian rhythmicity. Figure 2E represents a heatmap of tryptophan-metabolizing bacteria highlighting that they display oscillations in abundance over time. We then inferred the functional capacity of the microbiome over the day involved in gut-brain communication[33]. This analysis revealed that predicted bacterial tryptophan degradation was significantly different over the day (**Figure 2F**) - with a significant reduction from ZT11 to ZT17 - while bacterial kynurenine degradation into 3-hydroxykynurenine exhibited circadian rhythmicity (**Figure 2G**). To assess whether the oscillation of tryptophan-metabolizing species led to differences in microbially-derived tryptophan metabolites, we cross-referenced our shotgun sequencing data with the TrpNet database[34] to predict their potential abundance. From this analysis, we saw that all of tryptophan metabolites in the TrpNet database displayed a circadian rhythm following the same pattern: our linear models predicted peaks at ZT5 and nadir at ZT17 for selected microbial tryptophan metabolites. It has already been established that microbial tryptophan metabolites are important for modulating gut function and immunity. Our novel results indicate that the effects of tryptophan on gut homeostasis and function may be further regulated by circadian rhythms in microbial degradation of tryptophan and its metabolites. We then aimed to investigate the effects of those changes on host tryptophan metabolism.

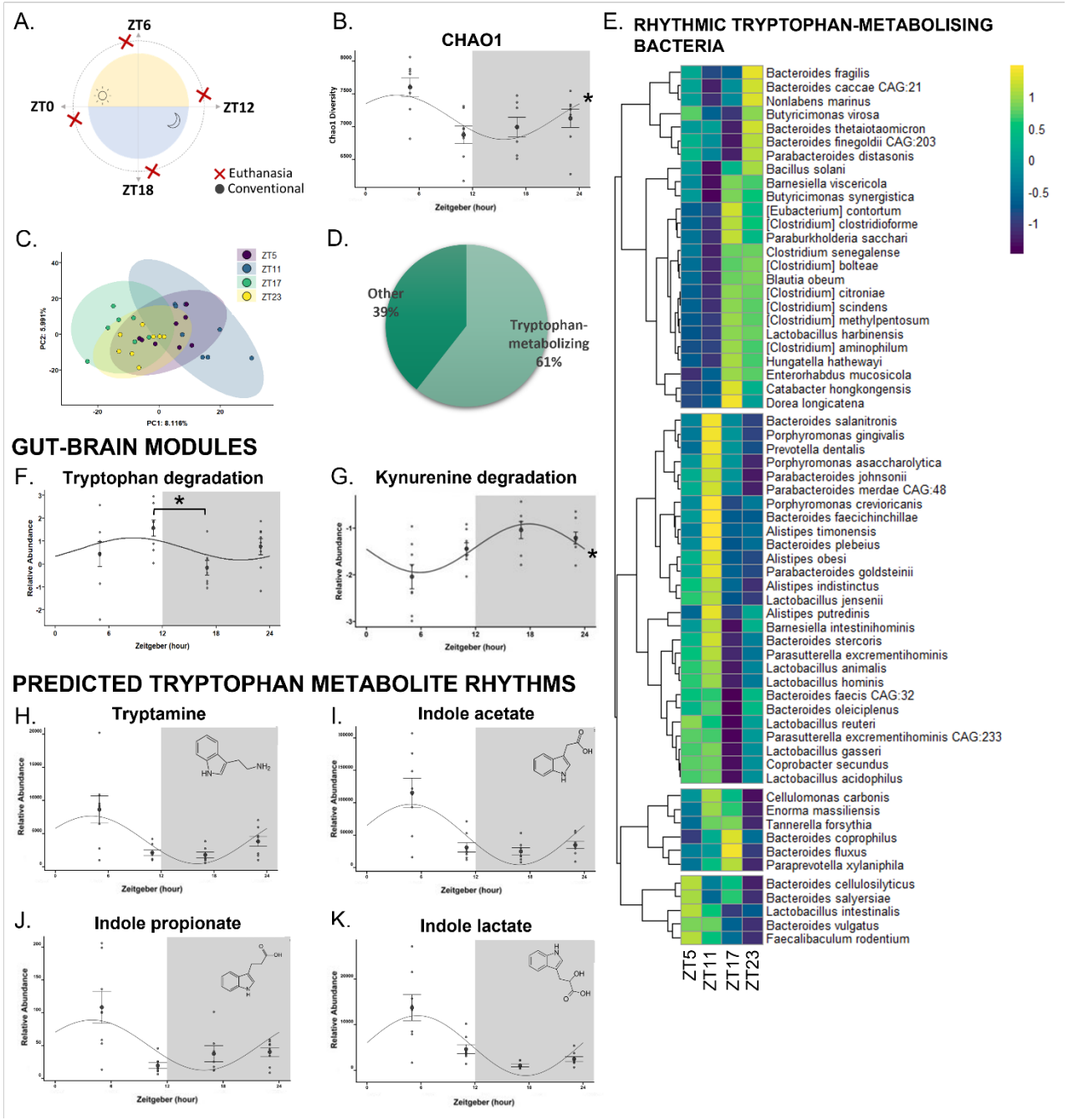

Figure 2. Tryptophan-metabolizing bacteria and predicted metabolites display circadian rhythmicity in conventional mice. (A) Experimental design schematic: conventional mice were euthanised at different times of the day and night: ZT5, ZT11, ZT17 and ZT23 (ZT= zeitgeber) (n=8 mice/timepoint). (B) Rhythmicity of bacterial species richness represented by Chao1 index. Data are expressed as mean ± SEM; data analyzed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity. (C) Principal component analysis showing differences in microbial composition at different time of the day and night (ZT5, ZT11, ZT17, ZT23) in conventional mice in terms of β-diversity as measured in Aitchison distance. Ellipses indicate 95% confidence intervals per group. (D) Pie chart indicating percentages rhythmic bacteria that are tryptophan-metabolizing in conventional mice. (E) Heatmap representing relative abundance of significantly rhythmic tryptophan-metabolizing bacteria (determined by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity; with row-scaling to highlight rhythmicity). (F-G) Tryptophan-related gut-brain modules (F; data analysed by one-way general linear modelling as arrhythmic). (H-K) Predicted relative abundance of selected microbial tryptophan metabolites analysed with TrpNet database. Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity.

Microbial status modulates rhythmicity of genes implicated in tryptophan metabolism in a region-dependent manner

To understand microbial and circadian influence on genes related to host tryptophan metabolism (*TDO*, *IDO*, *TPH*) and genes implicated in downstream signalling of tryptophan metabolites (*AhR*, *AhRR*, *PXR*), we analysed ileum, colon and liver tissues of conventional, germ-free and antibiotic-treated mice over the day (**Figure 3A**). The liver receives absorbed metabolites from the gut via the hepatic portal vein where tryptophan is mainly degraded through host kynurenine pathway. While it is known that circadian rhythms are a critical modulator of liver metabolism [35], the role of the gut microbiota in this interaction is not well-characterised. In the liver, *TDO2* relative expression was rhythmic in all groups, with a significantly different rhythm induced by germ-free status (**Figure 3B**). Interestingly, *AhR* and *AhRR* expression in the liver were rhythmic only in conventional animals, with no statistically significant rhythms detected in mice lacking microbes (**Figure 3C, D**).

In the gut, tryptophan is catabolised by the host either through the kynurenine pathway (by indoleamine 2,3-dioxygenase (*IDO1*)) or through the serotonin pathway (by Tryptophan hydroxylase (*TPH1*)). In conventional mice, *IDO1* and *TPH1* gene expression and rhythmicity appear to be region dependent. In the ileum of conventional mice, *TPH1* (**Figure 3E**) - but not *IDO1* (**Figure 3F**) - displays circadian rhythmicity, suggesting a time-dependent effect when tryptophan is converted into serotonin in the ileum. *AhR* and *PXR* are two important receptors in the gut, highly responsive to microbial- and host-derived tryptophan metabolites. In the ileum, all experimental groups display rhythmicity in *AhR* (**Figure 3G**) and *PXR* (**Figure 3H**) expression with significant differences in amplitude and acrophase induced by microbial depletion. In the colon, both *IDO1* (**Figure 3I**) and *TPH1* (**Figure 3J**) relative gene expression follow significant and reciprocal rhythms in conventional mice. This suggests that in the colon of conventional mice, there are time-windows when the metabolism of tryptophan is directed towards either the serotonin or kynurenine pathway. Even more interestingly, microbial status in the colon seems to play a key role in host enzyme rhythmicity: both antibiotic-depletion and germ-free mice lost *TPH1* and *IDO1* rhythmicity and exhibited reduced expression for these genes overall. *AhR* (**Figure 3K**) and *PXR* (**Figure 3L**) expression in conventional mice

were significantly lower than expression levels observed in germ-free and antibiotic-depleted animals.

To further understand the relationship between host gene expression and microbial functions, we used a knowledge-based integration approach. We first converted both microbial functions and host genes to their corresponding KEGG orthologues. Then, we used linear mixed-effects models to assess differential associations between all pairs of host and microbiome features that share a KEGG-pathway group[36,37]. Several microbe-host gene pairs show significantly disjointed associations based on host tissue. In general, microbial functions are more positively associated to corresponding host gene expression in the colon compared to their counterparts in the ileum which are typically negatively associated (**Figure 3M**). Since we know that tryptophan metabolites and important receptors implicated in gut barrier function are affected by microbial status and circadian rhythm, we wanted to see if similar circadian effects were seen in gut barrier markers.

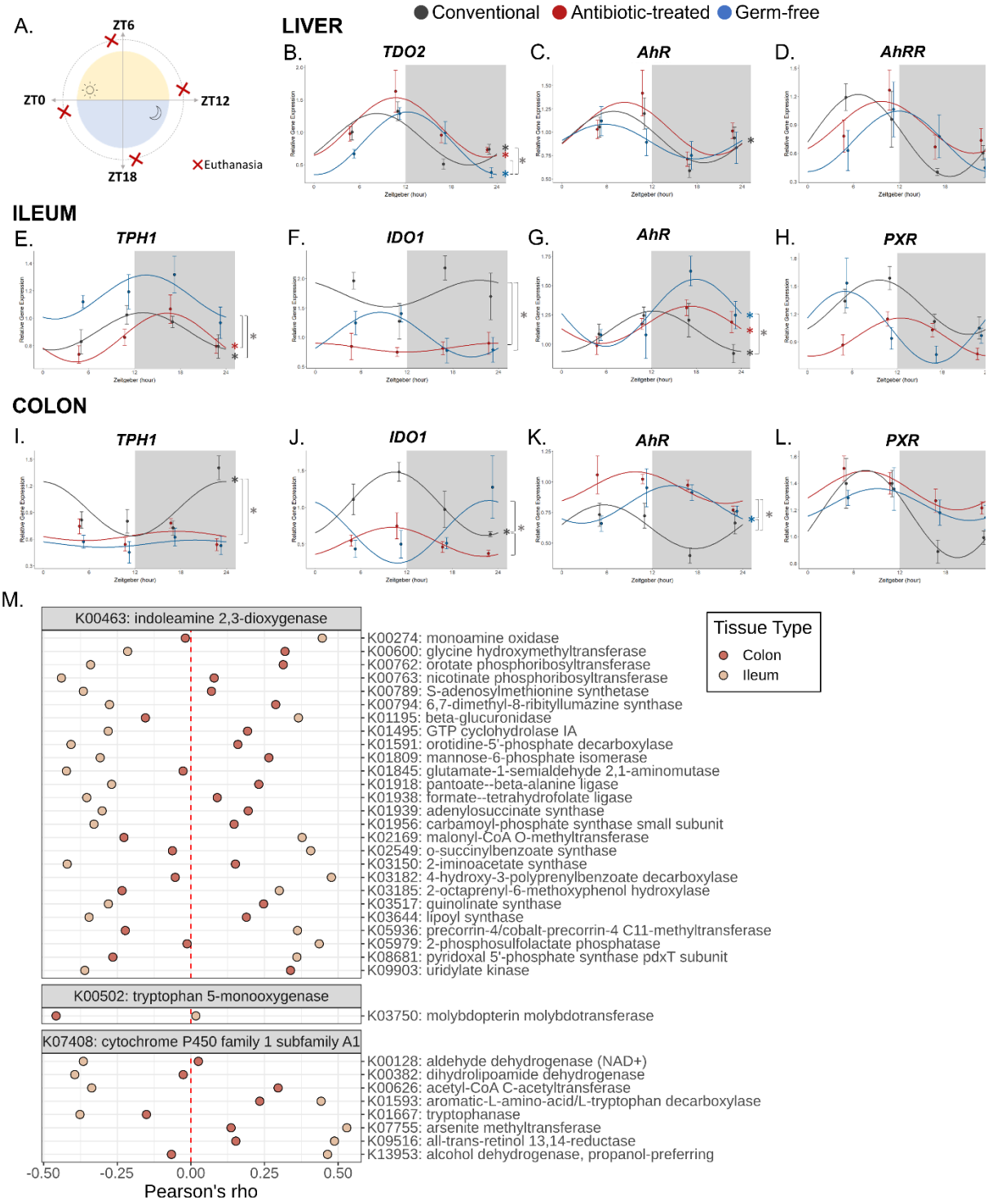
Conventional
Antibiotic-treated
Germ-free
A.
ZT6
ZT0
ZT12
ZT18
Euthanasia
LIVER
B.
TDO2
C.
AhR
D.
AhRR
ILEUM
E.
TPH1
F.
IDO1
G.
AhR
H.
PXR
COLON
I.
TPH1
J.
IDO1
K.
AhR
L.
PXR
Relative Gene Expression
Zeitgeber (hour)
M.
K00463: indoleamine 2,3-dioxygenase
K00502: tryptophan 5-monooxygenase
K07408: cytochrome P450 family 1 subfamily A1
Tissue Type
Colon
Ileum
K00274: monoamine oxidase
K00600: glycine hydroxymethyltransferase
K00762: orotate phosphoribosyltransferase
K00763: nicotinate phosphoribosyltransferase
K00789: S-adenosylmethionine synthetase
K00794: 6,7-dimethyl-8-ribityllumazine synthase
K01195: beta-glucuronidase
K01495: GTP cyclohydrolase IA
K01591: orotidine-5'-phosphate decarboxylase
K01809: mannose-6-phosphate isomerase
K01845: glutamate-1-semialdehyde 2,1-aminomutase
K01918: pantoate--beta-alanine ligase
K01938: formate--tetrahydrofolate ligase
K01939: adenylosuccinate synthase
K01956: carbamoyl-phosphate synthase small subunit
K02169: malonyl-CoA O-methyltransferase
K02549: o-succinylbenzoate synthase
K03150: 2-iminoacetate synthase
K03182: 4-hydroxy-3-polyprenylbenzoate decarboxylase
K03185: 2-octaprenyl-6-methoxyphenol hydroxylase
K03517: quinolinate synthase
K03644: lipoyl synthase
K05936: precorrin-4/cobalt-precorrin-4 C11-methyltransferase
K05979: 2-phosphosulfolactate phosphatase
K08681: pyridoxal 5'-phosphate synthase pdxT subunit
K09903: uridylate kinase
K03750: molybdopterin molybdotransferase
K00128: aldehyde dehydrogenase (NAD+)
K00382: dihydrolipoamide dehydrogenase
K00626: acetyl-CoA C-acetyltransferase
K01593: aromatic-L-amino-acid/L-tryptophan decarboxylase
K01667: tryptophanase
K07755: arsenite methyltransferase
K09516: all-trans-retinol 13,14-reductase
K13953: alcohol dehydrogenase, propanol-preferring
-0.50
-0.25
0.00
0.25
0.50
Pearson's rho

Figure 3. Microbial status impacts circadian rhythmicity of host gene expression implicated in tryptophan metabolism and signalling in a region-dependent manner. (A) Experimental design schematic: conventional, germ-free and antibiotic-treated mice were euthanised at different times of the day and night: ZT5, ZT11, ZT17 and ZT23 (ZT= zeitgeber) (n=8-12 mice/timepoint). Relative gene expression of (B-D) liver, (E-H) ileum and (I-L) colon host genes implicated in tryptophan metabolism and signalling (n=7-10 mice/group/timepoint). Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons * $p<0.05$. Colored stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences. (M) Associations between host genes and microbial functions that share a KEGG-pathway. The x-axis depicts pearson correlation coefficients between host gene expression in conventional mice and microbial associated KEGG functions. Only associations between genes and functions that share a KEGG-pathway group were considered and only those associations where an ANOVA of the entire model resulted in a BH-adjusted q-value < 0.1 as well as a differential association (slope interaction with host tissue $p < 0.05$) are shown. Yellow circles: ileum; Pink circles: colon

Microbial status modulates circadian gene expression oscillations of gut barrier markers in a region-dependent manner

While circadian disruption is known to impact gut barrier gene expression[38,39], fewer studies have examined the contribution of the gut microbiota to gut barrier circadian rhythmicity. Here we investigated the impacts of microbial status on gene expression related to both the mucus barrier and tight junctions, two key features of gut barrier integrity. *Mucin-2* (**Figure 4A**; gel-forming mucin) and *Mucin-3* (**Figure 4B**; membrane-bound mucin) did not exhibit circadian rhythmicity in the ileum of conventional mice. Antibiotic treatment but not germ-free status induced a phase-shift in ileal *Mucin-2* expression (**Figure 4A**), while *Mucin-3* expression was significantly disrupted by both antibiotic treatment and germ-free status (**Figure 4B**) relative to conventional mice. Similarly, ileal tight junction gene expression did not exhibit circadian rhythmicity overall: *Claudin-1* (**Figure 4C**), *Claudin-5* (**Figure 4D**), *Tight-Junction Protein 1* (TJP1, **Figure 4E**) and *Occludin* (**Figure 4F**) were not rhythmic in conventional mice. Claudin-1 (**Figure 4C**) and *Claudin-5* (**Figure 4D**) expression were increased by both methods of microbial depletion, while *TJP1* expression was reduced by antibiotics and increased by germ-free status respectively relative to conventional mice (**Figure 4E**). Interestingly, microbial depletion induced circadian rhythmicity in ileal *Occludin* expression (**Figure 4F**).

In contrast, both *Mucin-2* (**Figure 4G**) and *Mucin-3* (**Figure 4H**) gene expression exhibited circadian rhythmicity in colonic tissue from conventional mice, with peaks in gene expression during the inactive phase. Germ-free status reversed rhythmicity in both *Mucin-2* and *Mucin-3*, such that gene expression peaked during the active phase, while antibiotics exposure

disrupted rhythmicity of *Mucin-2* and increased expression of *Mucin-3* throughout the day. Similarly, colonic gene expression of *Claudin-1* (**Figure 4I**), *Claudin-5* (**Figure 4J**), *TJP1* (**Figure 4K**) and *Occludin* (**Figure 4L**) exhibited circadian rhythmicity in conventional mice. *Claudin-1* (**Figure 4I**) and *Claudin-5* (**Figure 4J**) gene expression in the colon was increased by both methods of microbial depletion, with reversal in rhythmicity observed for *Claudin-5* with both antibiotics exposure and germ-free status. Germ-free status also reversed the rhythm of *TJP1*, shifting the peak of gene expression into the active phase (**Figure 4K**). Overall, *TJP1* and *Occludin* (**Figure 4L**) gene expression were increased by both methods of microbial depletion.

In summary, gut barrier-related genes exhibit robust circadian rhythms in the colon but not the ileum. Germ-free status reversed the rhythmicity of several genes assessed, shifting the acrophase from the rest phase to the active phase of the animals. In contrast, microbial depletion through antibiotic exposure often increased the expression of these genes without causing dramatic shifts in rhythmicity. These results indicate a role for the gut microbiota in the development of appropriate circadian rhythms in gut barrier- related gene expression as well as in the maintenance of these rhythms once they are established.

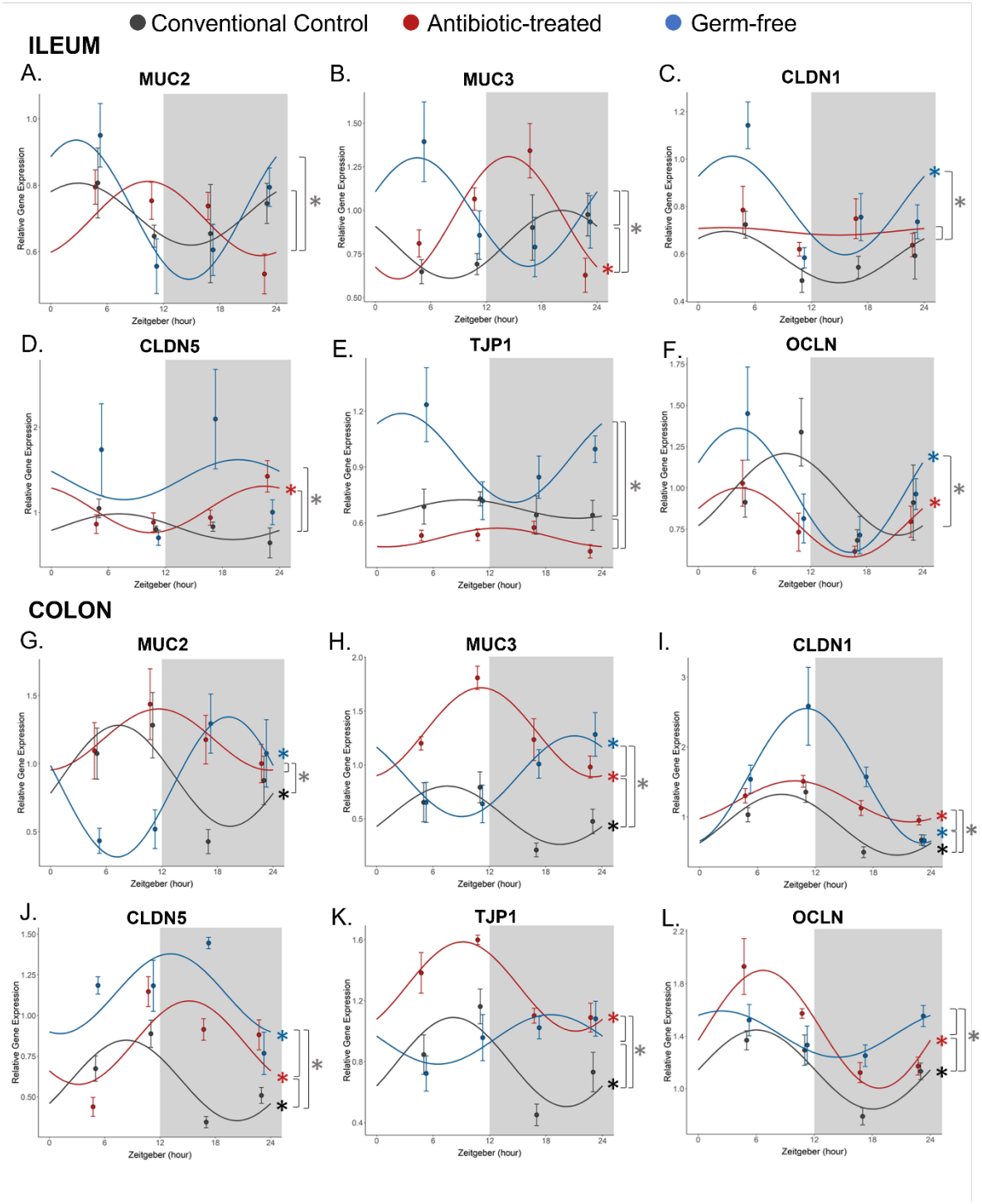

Figure 4. Microbial status modulates circadian gene expression oscillations of gut barrier markers in a region-dependent manner. Relative gene expression of host genes implicated in gut barrier function in the (A-F) ileum and (G-L) colon of conventional, germ-free or antibiotic-treated mice (n=7-10 mice/group/timepoint). Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons, * $p<0.05$. Coloured stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences.

Both untargeted and Gram-positive microbial depletion disrupt circadian rhythms in gut barrier function and tryptophan-related gene expression

The above results indicate that disruptions in gut barrier function induced by microbial depletion may depend on the time-of-day gut barrier assessment is performed. Since host circadian rhythms in tryptophan metabolism and gut barrier function were disrupted by microbial depletion, and microbial tryptophan degradation was affected by time-of-day, we next examined the effects of shifting microbial tryptophan metabolite production on gut barrier function over the day. To accomplish this, we treated mice with either vancomycin (Gram-positive depletion; known to reduce indole-3-propionate production[40], gentamicin (Gram-negative depletion) or a broad-spectrum antibiotic cocktail) and assessed *ex vivo* gut function over the day (**Figure 5A**) using an Ussing chamber apparatus to assess both electrophysiological characteristics and paracellular permeability (**Figure 5B**).

In the ileum, ion transport (**Figure 5C**) did not exhibit circadian rhythmicity in conventional mice and was not impacted by targeted microbial depletion while the antibiotic cocktail induced circadian rhythmicity in short-circuit current. In contrast, both transepithelial electrical resistance (**Figure 5D**) and paracellular permeability (**Figure 5E**) were disrupted by vancomycin treatment. Vancomycin treatment induced rhythmicity in transepithelial electrical resistance (**Figure 5D**) and increased overall paracellular permeability (**Figure 5E**). Ion transport in the colon (**Figure 5F**) exhibited circadian rhythmicity in conventional mice that was disrupted by both gentamicin and antibiotic cocktail treatment, both of which shifted the acrophase from the inactive phase into the active phase.

In contrast, colonic transepithelial electrical resistance (**Figure 5G**) did not exhibit circadian rhythmicity in conventional mice and was significantly increased by vancomycin treatment. Paracellular permeability in the colon (**Figure 5H**) was reduced by antibiotic cocktail treatment over the day. We then assessed tryptophan-related gene expression to determine if this was associated with observed functional changes. Ileal *TPH1* gene expression (**Figure 5I**) was significantly increased by all antibiotic treatments relative to conventional mice while *IDO1* gene expression (**Figure 5J**) was reduced with both antibiotic cocktail and gentamicin treatment. Of note, host receptors of tryptophan metabolites, *AhR* (**Figure 5K**), *PXR* (**Figure 5L**) and *TRPA1* (**Figure 5M**) were significantly elevated by both antibiotic cocktail and

vancomycin treatments, suggesting that the effects observed may be mediated by Gram-negative bacteria. In the colon *TPH1* gene expression (**Figure 5N**) was significantly reduced by all antibiotic treatments relative to conventional mice while *IDO1* (**Figure 5O**) was specifically reduced by antibiotic cocktail and vancomycin treatments. Colonic *AhR* expression (**Figure 5P**) was significantly reduced by antibiotic cocktail and increased by Gram-negative depletion respectively relative to conventional mice, while *PXR* gene expression (**Figure 5Q**) was not affected by any treatment. *TRPA1* gene expression (**Figure 5R**) exhibited circadian rhythmicity in the colon and was reduced overall by all antibiotic treatment.

Since all tryptophan metabolite-related receptor gene expression assessed in the ileum was increased by broad-spectrum and Gram-positive depletion - with corresponding and compelling changes in genes downstream of *AhR* (see **Figure S3**) - we examined the capacity for caecal contents from these mice to activate *AhR* (**Figure 5S**). All antibiotic treatment groups reduced luminescence evoked in this assay (**Figure 5T**) in a dose-dependent manner with the largest effects observed with the antibiotic cocktail. However, these changes did not exhibit circadian rhythmicity and could not explain the unique functional and gene expression disruptions observed with vancomycin.

In summary, we found that both targeted and broad-spectrum microbial depletion impacted gut barrier function and related gene expression over the day. Transepithelial resistance was altered by vancomycin treatment in both gut regions while vancomycin exerted effects on paracellular permeability in the ileum alone. All antibiotic exposures appear to shift serotonin synthesis more proximally, indicated by increased and decreased *TPH1* in the ileum and colon respectively, while kynurenine production may be reduced in both regions assessed. These functional and gene expression changes do not appear to be mediated by *AhR* activity at the gut epithelium in response to altered caecal *AhR* ligand availability.

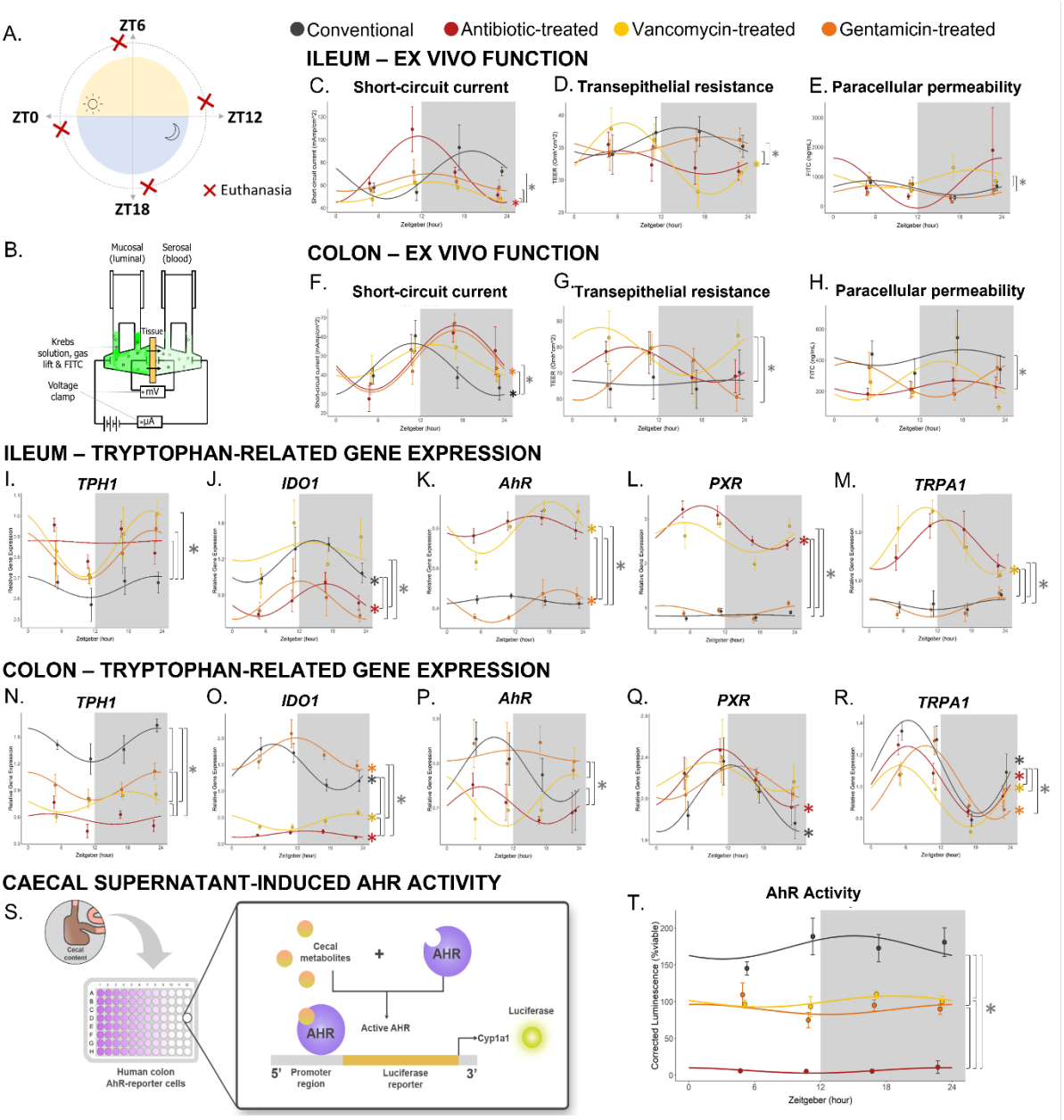

Figure 5. Targeted and antibiotic cocktail induced-depletion impact gut barrier integrity and AhR activity. (A) Experimental design schematic: mice treated with vehicle (control), gentamicin (Gram-negative depletion), vancomycin (Gram-positive depletion) and antibiotic cocktail were euthanised at different times of the day and night: ZT5, ZT11, ZT17 and ZT23 (ZT= zeitgeber) (n=7-10 mice/timepoint). (B) Ex-vivo analysis of gut tissue was done with Ussing chambers apparatus: ileum or colon tissues were stripped and then placed in the middle of two chambers to measure ion transport (short-circuit current; Isc), transepithelial electrical resistance (TEER) and paracellular permeability (Fluorescein isothiocyanate (FITC) translocation from mucosal to serosal side). (C) Isc, (D) TEER and (E) FITC permeability in the ileum. (F) Isc, (G) TEER and (H) FITC permeability in the colon. Relative gene expression of (I-M) ileum and (N-R) colon host genes implicated in tryptophan metabolism and signalling (n=7-10 mice/group/timepoint). (S) Caecal contents from mice in A were processed to remove large food particles and bacteria and then incubated with AhR Lucia cells to determine their potential to induce AhR activity. (T) Caecal supernatant-induced AhR activity. Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons * $p<0.05$. Coloured stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences.

Microbial modulation and acute stress lead to altered gut barrier function

We then investigated whether the effects of microbial depletion on circadian rhythmicity in gut barrier function and host tryptophan metabolism were important factors in the gut response to acute restraint stress. We focused on the transition between active and inactive phases (ZT11 and ZT23; **Figure 6A**), as acute stress responding to psychological stressors is known to differ between these transition phases[12].

While ileal ion transport (**Figure 6B**) was unaffected by these interventions, transepithelial electrical resistance (**Figure 6C**) was reduced by stress overall (see **Table S1**). Paracellular permeability (**Figure 6D**) was increased by stress and exhibited a significant interaction between treatment and time-of-day. While stress uniquely increased permeability in conventional mice at ZT23, it increased permeability at ZT11 in antibiotic-treated mice only. This suggests that the microbiota may interact with time-of-day to modulate the effect of acute stress on paracellular permeability. In contrast, colonic ion transport (**Figure 6E**) exhibited circadian effects only, while transepithelial electrical resistance (**Figure 6F**) was increased in the active phase and by antibiotic exposure with no effects of stress. Colonic paracellular permeability (**Figure 6G**) was affected by time-of-day, with increased permeability at ZT11.

Only a limited number of the genes we assessed in this experiment were significantly altered by stress, most likely due to the relatively short window between the acute stressor and tissue collection. In the ileum, *TPH1* (**Figure 6H**) and *IDO1* (**Figure 6I**) gene expression exhibited interactions between treatment and time-of-day: antibiotics exposure reduced the expression of these genes to a greater extent at ZT11 than ZT23. While *AhR* expression was unaffected (**Figure S4**) *CYP1a1*, a transcription factor induced by AhR activity, was significantly increased with acute stress (**Figure 6J**), indicating increased AhR activity in the ileum of stressed mice. Ileal *occludin* expression (**Figure 6K**) exhibited a significant interaction between treatment and time-of-day: antibiotics treatment increased *occludin* expression at ZT23 only. In contrast, *claudin-5* was unaffected by any intervention (**Figure 6L**).

Colonic *TPH1* gene expression (**Figure 6M**) exhibited a significant interaction between treatment and stress: *TPH1* expression was reduced by stress in conventional mice but

increased by stress in antibiotic-treated mice, although antibiotic treatment strongly reduced colonic *TPH1* expression overall. *IDO1* gene expression (**Figure 6N**) was reduced by antibiotic treatment and lower overall at ZT23. While AhR gene expression (**Figure S4**) was reduced with antibiotics treatment, *CYP1a1* in the colon exhibited a significant effect of treatment, stress and time-of-day interaction (**Figure 6O**): stress appeared to increase *CYP1a1* in conventional mice at ZT11 while increasing *CYP1a1* in antibiotic-treated mice at ZT23. In the colon, *occludin* gene expression (**Figure 6P**) was reduced by stress while *Claudin-5* gene expression (**Figure 6Q**) was reduced with antibiotics treatment.

Since we have shown that several microbial and host tryptophan metabolites that activate AhR are altered with acute stress (**Figure 1H-Q**), we assessed whether caecal supernatants collected from stressed mice would increase AhR activity *in vitro* (**Figure 6R**). While we found clear effects of antibiotic treatment on evoked AhR activity, caecal supernatants collected 45 minutes following acute restraint stress did not alter AhR activity relative to that of control mice (**Figure 6S,T**).

In summary, acute stress evokes functional changes in transepithelial resistance and interacts with time-of-day to induce impairments to paracellular permeability specifically in the ileum. These were associated with changes in *CYP1a1* expression, implying increased production of specific tryptophan metabolites in response to stress (in line with our findings in **Figure 1**). However, caecal supernatant from stressed mice did not evoke altered AhR activity *in vitro*. While colon function did not appear to be impacted by stress in these experiments, altered *TPH1* and *Occludin* gene expression indicate stress-induced changes to gut homeostasis and barrier regulation.

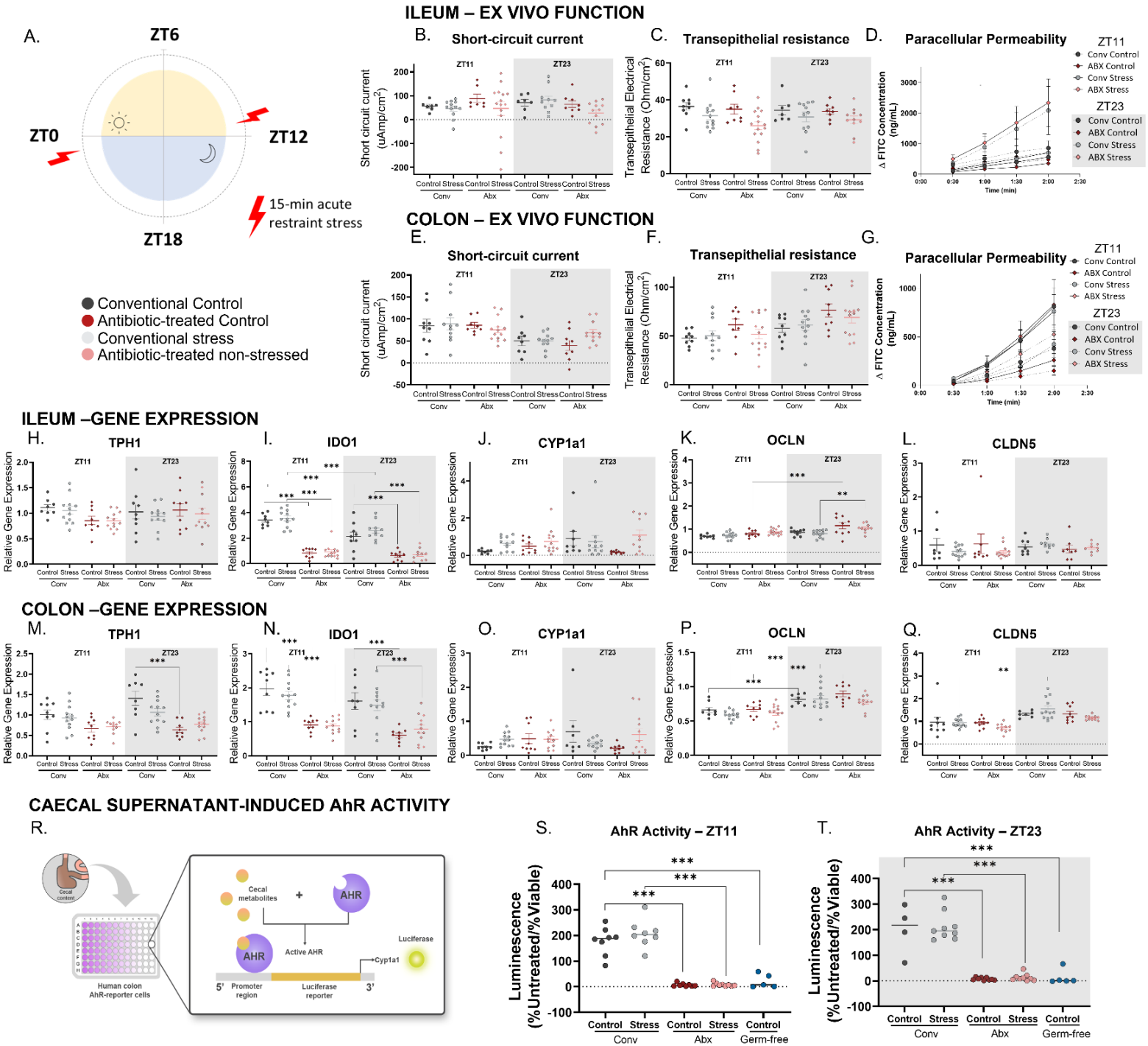
ILEUM – EX VIVO FUNCTION
A.
ZT6
ZT0
ZT12
ZT18
15-min acute restraint stress
Conventional Control
Antibiotic-treated Control
Conventional stress
Antibiotic-treated non-stressed
B. Short-circuit current
C. Transepithelial resistance
D. Paracellular Permeability
Short circuit current (uAmp/cm²)
Transepithelial Electrical Resistance (Ohm/cm²)
Δ FITC Concentration (ng/mL)
Time (min)
ZT11
ZT23
Conv Control
ABX Control
Conv Stress
ABX Stress
Control Stress Control Stress Control Stress Control Stress
Conv Abx Conv Abx
COLON – EX VIVO FUNCTION
E. Short-circuit current
F. Transepithelial resistance
G. Paracellular Permeability
ILEUM –GENE EXPRESSION
H. TPH1
I. IDO1
J. CYP1a1
K. OCLN
L. CLDN5
Relative Gene Expression
COLON –GENE EXPRESSION
M. TPH1
N. IDO1
O. CYP1a1
P. OCLN
Q. CLDN5
CAECAL SUPERNATANT-INDUCED AhR ACTIVITY
R.
Cecal content
Human colon AhR-reporter cells
Cecal metabolites
AHR
Active AHR
Luciferase
Cyp1a1
5' Promoter region
Luciferase reporter
3'
S. AhR Activity – ZT11
T. AhR Activity – ZT23
Luminescence (%Untreated/%Viable)
Control Stress Control Stress Control
Conv Abx Germ-free

Figure 6. Gut microbiota modulates the gut response to acute stress in a circadian manner with effects in tryptophan metabolism and AhR activity. (A) Experimental design schematic: mice treated with an antibiotic cocktail underwent acute restraint stress at ZT11 or ZT23 and then euthanised following 45 minutes (ZT= zeitgeber) (n=8-12 mice/timepoint). (B) Isc, (C) TEER and (D) FITC permeability in the ileum. (E) Isc, (F) TEER and (G) FITC permeability in the colon. Relative gene expression of (H-L) ileum and (M-Q) colon host genes implicated in tryptophan metabolism and signalling, and gut barrier integrity (n=7-10 mice/group/timepoint). Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons * $p<0.05$. Coloured stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences. (R) Caecal supernatant-induced AhR activity at (S) ZT11 and (T) ZT23.

Discussion

Previous work has demonstrated that microbial depletion impacts tryptophan metabolism[25,41], a key integrator of circadian homeostasis in the central clock and periphery[42]. Since then, crosstalk between gut epithelial enteroendocrine cells and microbial tryptophan metabolites in the gut has been demonstrated[20], suggesting a symbiotic relationship between host tryptophan metabolism, gut bacteria and gut barrier function. Moreover, intestinal epithelial cells clocks are critical in driving microbial rhythms, composition and host intestinal homeostasis[31]. In this study, we demonstrate that incorporating a microbial perspective can illuminate our understanding of the mechanisms underpinning the interactions between stress exposures and circadian rhythm disruption.

Our previous work has demonstrated that acute stress exposure impacts host tryptophan degradation in the gut in a region-dependent manner[28]. In the present study, we show that a 15-min acute stressor is sufficient to alter caecal tryptophan metabolism in bacterially-colonised mice and increase *in vivo* permeability independent of microbial status. To our knowledge, this is the first demonstration that an acute psychological stressor can impact the production of microbial metabolites in the gut. The effects of our acute stress protocol on gut permeability are in line with previous studies using longer stressor durations (1-4h hours)[43-47]. It is striking that an acute stressor of 15 minutes is sufficient to affect caecal metabolite production and functional readouts of intestinal permeability.

Our study reinforces the critical role of gut microbiota for normal circadian rhythms in gut barrier function and tryptophan metabolism in the host. Gut microbiota populations express circadian rhythmicity[17,30] and our study provides the first evidence of this at the strain level, showing that approximately 3% of strains identified exhibited circadian rhythmicity in conventional animals of which more than half can metabolise tryptophan. We found that inferred microbial tryptophan and kynurenine degradation exhibited differences depending on time-of-day. By cross-referencing our data with the TrpNet database[34], we demonstrated that all tryptophan metabolites displayed a circadian rhythm in their predicted abundance. We investigated whether these effects of microbial depletion were mediated through receptors known to bind to tryptophan metabolites and involved in gut barrier function and integrity,

namely AhR[48,49], TRPA1[20] and PXR[50]. Interestingly, mRNA expression in the ileum of those three receptors were upregulated following microbiota depletion by the full-antibiotic cocktail and vancomycin treatment (Gram-positive depletion) compared to conventional or gentamicin-treated mice (Gram-negative depletion). This suggests the upregulation of these receptors is likely in response to the presence of Gram-negative bacteria, or an effect of the overgrowth of antibiotic-resistant microorganisms.

Since microbial tryptophan catabolites have been shown before to act on gut barrier function[51] and immune signalling[48] through the AhR receptor, we wanted to assess whether caecal metabolites from our experimental groups would increase AhR-mediated *Cyp1a1* transcription in a circadian manner. Interestingly, AhR activity was dose-dependently lessened in cells treated with caecal supernatants from mice exposed to partial or full antibiotic treatment. However, AhR-mediated *Cyp1a1* transcription was not increased by caecal supernatants collected from stressed animals. The incongruency between our *in vivo* and *ex vivo* results here may be an issue of resolution: the differences in ileal *CYP1a1* expression may be due to changes in AhR activity in specific cell types that are not directly exposed to the caecal contents and may indicate stress-induced changes in absorption of specific AhR ligands. Future experiments should utilise a single-cell approach to determine where AhR activation is occurring in the gut.

Recent evidence indicates that mutualistic interactions between commensal microbiota and gut immunity are circadian-dependent and confers metabolic benefits to the host[52]. *TPH1* and *IDO1* are host enzymes responsible for tryptophan conversion towards the serotonin or kynurenine pathway, respectively. *IDO1* is known to be immunoregulatory[53] and to play a key role in host-microbiome symbiotic relationships at mucosal sites[48]. Here we show for the first time that *IDO1* and *TPH1* rhythmicity, at a transcriptional level, is dependent on the presence of microbes in the mouse colon. This is in line with previous work showing that bacterial metabolites can modulate *IDO1* expression[54], that *TPH1* levels are different in germ-free compared to conventional mice in the colon[55] and that specific bacteria can increase host serotonin biosynthesis[23]. In contrast, we demonstrate that any shift in microbial populations is predicted to favor disrupted host serotonin production in the colon, and this may result in

increased serotonin synthesis in the ileum, indicated by increased *TPH1* mRNA level in the ileum and decreased *TPH1* level in the colon respectively.

Additionally, future work should focus on characterizing stress-induced permeability changes in female mice since stress-related gastrointestinal disorders are more common in females[56] and microbial circadian rhythmicity is regulated also by gender[17]. Other microbial metabolites implicated in gastrointestinal homeostasis also exhibit circadian fluctuations, including branched-chain fatty acids and secondary bile acids, and should be investigated in the context of acute stress exposure.

Tryptophan metabolism and availability in the host is essential for neuropsychiatric, gastrointestinal, immune and metabolic health[7,57]. There is also substantial evidence of circadian rhythm disruption in stress-related neuropsychiatric disorders[10,11] and it is frequently experienced by shift-workers, first responders, and deployed personnel and often in the context of stress. Our study demonstrates that host tryptophan enzymes in the gut and liver exhibit circadian rhythmicity, which is microbiota-dependent. These rhythms in host tryptophan metabolism, particularly *IDO1*[58], are likely to shape immune function, host metabolism, gastrointestinal health and behaviour. To date, most studies in this area focus on single timepoints which may miss critical windows important for understanding the role of tryptophan metabolism in disease processes. Further, this work demonstrates that even brief exposures to acute stress can alter gut barrier function and caecal metabolome, which may have important implications for stress-related gastrointestinal dysfunction and disorders of gut-brain axis interactions. Importantly, our study demonstrates that gut microbiota is essential for maintaining normal circadian rhythms in gut barrier and host tryptophan metabolism. These observations may have important implications at the interface between the experience of stress and circadian rhythm disruption in health and disease.

Supplementary Figure and Table Legends

Figure S1. Acute stress and microbial status-related changes in tryptophan metabolites in colonic mucosa

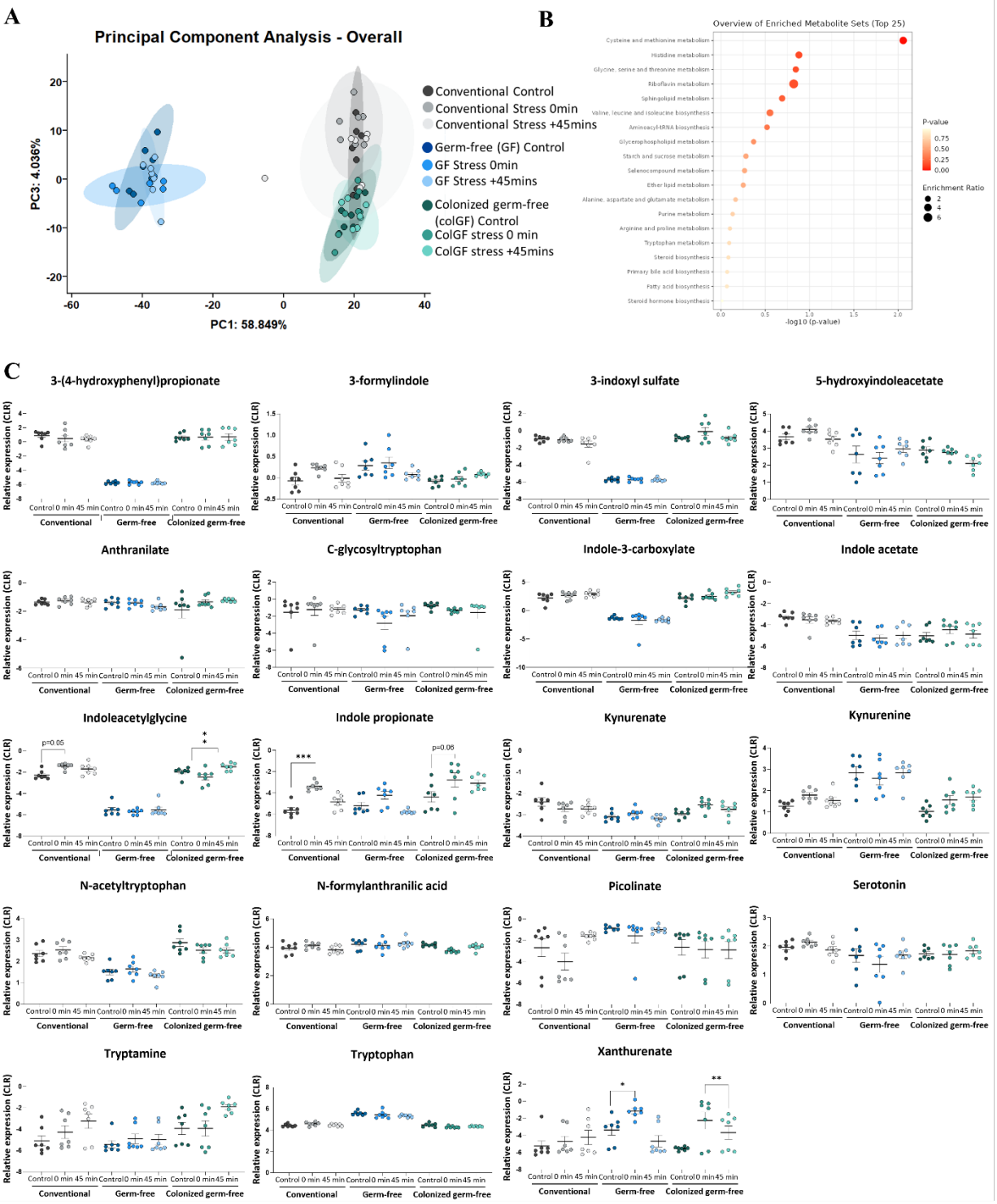

Fig S1. Acute stress and microbial status-related changes of tryptophan metabolites in colonic mucosa (A) Principal component analysis showing the effects of acute stress on colonic mucosa metabolome in conventional, germ-free, and colonised germ-free mice in terms of β-diversity as measured in Aitchison distance. Ellipses indicate 95% confidence intervals per group. (B) Enrichment analysis of colonic mucosa metabolites altered by stress in conventional mice. The enrichment ratio is represented by the size of the circle. (C) Relative expression of colonic mucosa tryptophan-related metabolites from host and microbial degradation. Data are expressed as mean ± SEM with individual values overlaid; data analysed by 2-way general linear modelling followed by Tukey-adjusted post-hoc comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure S2 . Alteration of caecal host and microbial tryptophan metabolites in response to acute stress are microbiota-dependent

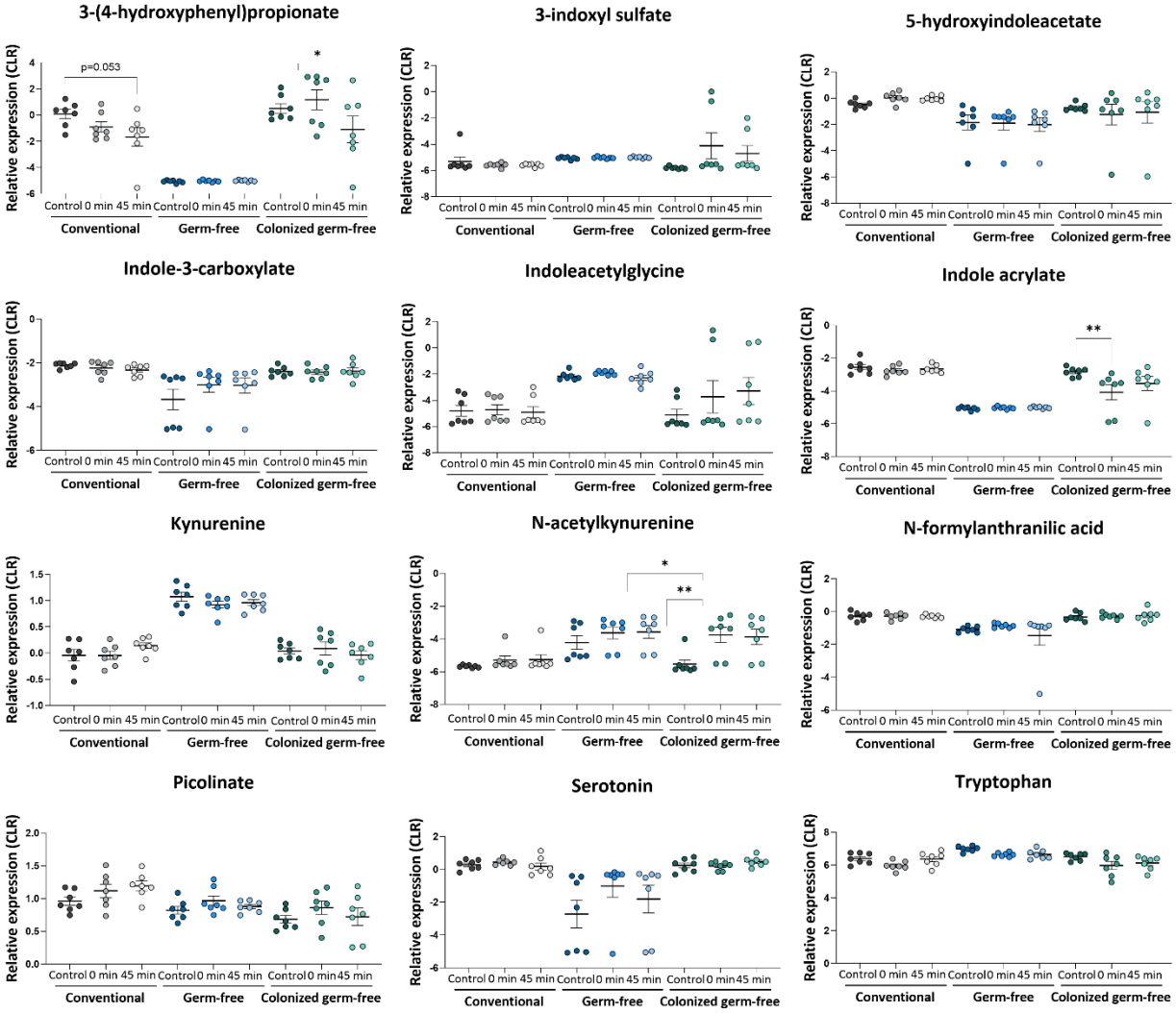

Fig S2. Alteration of caecal host and microbial tryptophan metabolites in response to acute stress are microbial-dependent
Relative expression of caecal tryptophan-related metabolites from host and microbial degradation in conventional, germ-free, and colonised germ-free mice. Data are expressed as mean ± SEM with individual values overlaid; data analysed by 2-way general linear modelling followed by Tukey-adjusted post-hoc comparisons. * p<0.05, ** p<0.01, *** p<0.001

Figure S3. AhR signaling and rhythmicity are impacted by microbial status in ileum and colon tissues

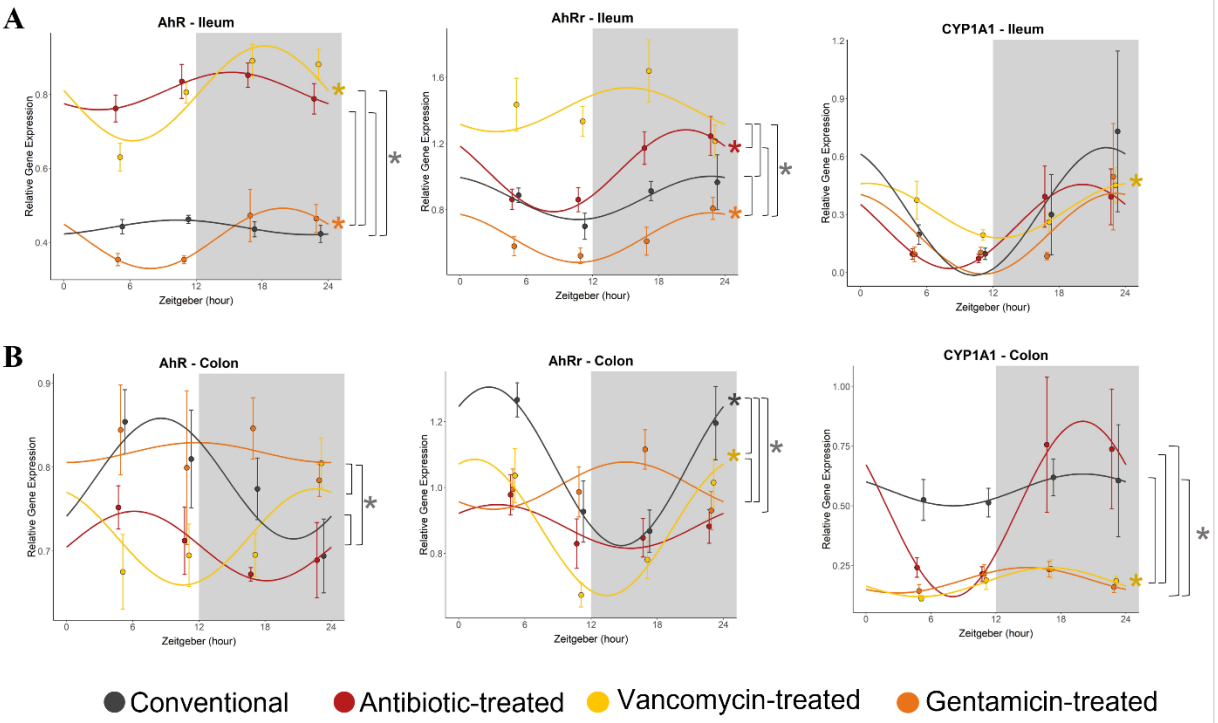

Fig S3. AhR signalling and rhythmicity are impacted by microbial status in ileum and colon tissues. (A) Ileum and (B) colon host genes implicated in Aryl Hydrocarbon Receptor (AhR)-related signalling in mice treated with gentamicin (Gram-negative depletion), vancomycin (Gram-positive depletion) and antibiotic cocktail euthanised at different times of the day and night: ZT5, ZT11, ZT17 and ZT23 (ZT= zeitgeber) (n=7-10 mice/timepoint). Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons * $p<0.05$. Coloured stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences.

Figure S4. AhR signaling in response to an acute stressor are impacted by microbial status in ileum and colon tissues

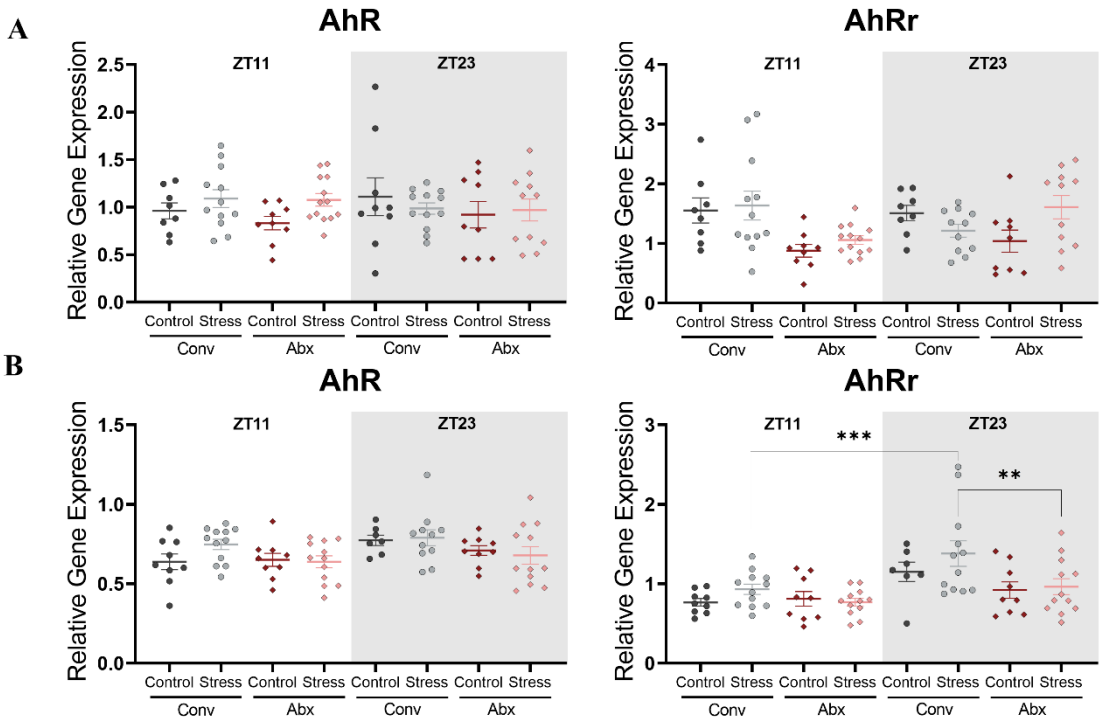

Fig S4. AhR signalling in response to an acute stressor are impacted by microbial status in ileum and colon tissues. Relative gene expression of (A) ileum and (B) colon host genes implicated in Aryl Hydrocarbon Receptor (AhR)-related signalling in mice treated with an antibiotic cocktail underwent acute restraint stress at ZT11 or ZT23 and then euthanised following 45 minutes (ZT= zeitgeber) (n=8-12 mice/timepoint). Data are expressed as mean ± SEM; data analysed by linear modelling to a Fourier-decomposed sine and cosine element for circadian rhythmicity followed by Tukey-adjusted post-hoc comparisons * $p<0.05$. Coloured stars on the right of the graphs represented individual rhythmicity for the associated experimental group; grey brackets on the far right indicate between-group differences.

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Chapter 4

Optimising Exercise-Training for Enhancing the Microbiota-Gut-Brain axis.

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Abstract

Exercise is often recommended to improve mental health as it impacts several important pathways known to be altered in stress-related disorders that are also important pillars of microbiota-gut-brain axis communication. Recently, exercise has been shown to mediate some of its beneficial effects through shifting peripheral tryptophan metabolism via transcriptional regulation of skeletal muscle enzymes. Evidence suggests that adaptation to exercise in athletes leads to functional differences in gut microbes, which could also lead to changes in microbial tryptophan metabolism. However, exercise is a physical stressor and the intensity and duration necessary to achieve these beneficial effects for mental health are still unknown and often focused on individual biomarkers. Furthermore, transient acute changes following this physical stressor are not usually distinguished from chronic adaptation to training. Finally, changes in tryptophan metabolism and gut microbial composition in relation to exercise has been investigated extensively in athlete populations but research in healthy sedentary individuals is scarce. By training previously sedentary individuals for 12-weeks at different intensity-levels and measuring both acute and chronic measures of the microbiota-gut-brain axis communication, we aimed to (1) to understand how different intensities of exercise shapes immune function, gastrointestinal permeability and HPA axis reactivity, key pillars of the gut-brain axis communication; (2) distinguish between acute transient effect of exercise to chronic adaptation in an intensity-dependent manner and (3) understand how different intensities of exercise remodels the gut microbiome, gastrointestinal function and relevant changes in host and microbial tryptophan metabolism in the circulation. Our results suggests that the moderate-intensity training as a positive modulator of the gut-brain axis without the detrimental effects of over-training associated with high-intensity exercise. However, the light-intensity training exhibited the most important shift in microbial composition and the high-intensity training increased peripheral serotonin production after a maximal exhaustion challenge. Overall, we discovered intensity-dependent alterations in the microbiota-gut-brain axis measurements in response to exercise in previously sedentary people.

Introduction

In an era where mental health burden is considered a global disability, exercise is emerging as an effective and overlooked means of maintaining and improving mental health(Chekroud et al., 2018). While exercise is a physical stressor per se, it contributes in the long-term to improvement of symptoms in psychiatric disorders such as anxiety, depression, and PTSD(Firth et al., 2020). Alterations in tryptophan metabolism are frequently reported in stress-related psychiatric and gastrointestinal disorders(Marx et al., 2021; Paul et al., 2022; Wilmes et al., 2021) and may represent a key neurobiological target for exercise interventions. Increased metabolism of tryptophan along the kynurenine pathway can be initiated by either immune or stress-related stimuli(Gheorghe et al., 2019). This can simultaneously reduce CNS tryptophan availability for serotonin production and increase the CNS availability of kynurenine for onward conversion into neurotoxic (quinolinic acid) or neuroprotective (kynurenic acid) metabolites with implication for mood and cognition.

Preclinical evidence demonstrates that exercise shifts systemic kynurenine pathway metabolism towards production of kynurenic acid, thereby limiting the access of kynurenine to the CNS for onward metabolism(Agudelo et al., 2014). Increases of systemic(Agudelo et al., 2014; Schlittler et al., 2016) and skeletal muscle kynurenic acid(Agudelo et al., 2014) following exercise in active participants was reported. Furthermore, tryptophan is an important precursor for several key signalling molecules along the microbiome-gut-brain axis. Tryptophan metabolism is also impacted by microbial production of indoles, thereby changing tryptophan availability for serotonin and kynurenine production(Gheorghe et al., 2019), while having important beneficial effects for gut permeability and function(Agus et al., 2018). The potential of exercise in mental health is well-recognised but the mechanisms underpinning its effects are poorly understood. Key pillars of the gut-brain axis may be responsive to either the acute or chronic effects of exercise, including the immune system(Docherty et al., 2022), the HPA axis(Duclos & Tabarin, 2016) and tryptophan metabolism(Cervenka et al., 2017). More recently, gut microbiota alterations have been noted in various athletes(Mailing et al., 2019; O'Brien et al., 2022). However, the impact of exercise-induced remodelling of the gut microbiome on gut-brain axis signalling pathways are currently unknown, as well as their associated implications for gastrointestinal physiology, brain function and behaviour.

A number of open questions remain pertaining to the nature of the interface between exercise, the microbiota-gut-brain axis and tryptophan metabolism. The intensity and duration of exercise needed to achieve beneficial effects for brain function and behaviour is still uncertain. Furthermore, it is not clear to what extent the acute physiological response to exercise adapts over time during a chronic exercise protocol. Moreover, changes in gut microbial composition in relation to exercise has been investigated extensively in athlete populations but research in sedentary individuals is scarce. This study is based on the hypothesis that exercise-induced remodelling of microbiota-gut-brain axis signalling mediates some of the beneficial effects of exercise on brain function and behaviour. Although changes in microbial composition and host tryptophan metabolism in the context of exercise has been independently investigated, we aim to understand the interface with microbial signatures of exercise-remodelled gut-brain axis communication.

The first aim of this study is to understand how different intensities of exercise shape immune function, gastrointestinal permeability, tryptophan metabolism and HPA axis reactivity, key pillars of the gut-brain axis communication. Secondly, we aim to distinguish between the acute transient effect of exercise and chronic adaptation in an intensity-dependent manner. To do so, we investigated differences in immune and gut markers, host and microbial tryptophan and fatty acid metabolism following acute (1h-post maximal exercise challenge) and chronic (12-week intervention) changes in microbiota-gut-brain axis biomarkers in healthy sedentary individuals (**Figure 1**).

Methods

Study Design and Participant Recruitment

73 healthy participants with a sedentary lifestyle (defined as an energy expenditure of <35 kcal/kg/day or exercise <3 days/week over the past month) were recruited (42 females and 21 males; mean age 40.3 ± 11.2 years; 19-59.6 years old). Participants were randomised into high dose, moderate dose, or light dose exercise administered over 3 sessions/week. Participants commenced the exercise intervention based on their individual VO_2 peak and their randomisation. Exercise dosages structured to elicit energy expenditures at a high dose (16 kcal per kilogram of body weight per week [KKW]), moderate dose (8-12KKW) or a light dose (4 KKW) with the results determining the precise exercise schedule used for subsequent objectives. These energy expenditures are designed to meet 50% to 150% of the National Institutes of Health Consensus Development Panel physical activity recommendations. Exact values and progression were determined based on individual fitness assessed during the baseline visit and adjusted to match the monthly change in fitness. Individuals with any of the following criteria were excluded: cardiovascular or respiratory disease, metabolic disorders, allergies, any infection, or autoimmune diseases. Controls were free from physical illness in the fortnight prior to participation in the study. Ambulatory activity was tracked using a pedometer. The participants were fully informed of the purposes and risks associated with the study design before providing written, informed consent. Participants' characteristics are summarised in Table 1. The study conformed to current local guidelines and the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospital (APC084).

Exercise intervention

At baseline, and then every month for three months, all participants were subject to a stepwise incremental cycle ergometry test to determine their individual fitness level (starting at 20 watts, resistance increased every 2 mins by 20 Watt until exhaustion). Oxygen consumption (VO_2 peak), heart rate, were recorded continuously, and blood lactate assessed before, during (every 2 minutes) and after exercise. Voluntary exhaustion was defined as reaching at least two of the following criteria: level off in VO2, RER ≥ 1.10, high blood lactate levels (≥8 mmol/L), RPE of

≥18, and/or HR of ±10 bpm of the age-predicted maximum. Participants randomised to the exercise groups were asked to cycle for at 60% of their individually derived VO_2 peak, using heart rate as a reference marker, based on their most current fitness assessment visit. Participants in the exercise groups were instructed to train three times per week for 12 weeks and were provided with membership of the Mardyke Arena, a sports and fitness facility affiliated with University College Cork.

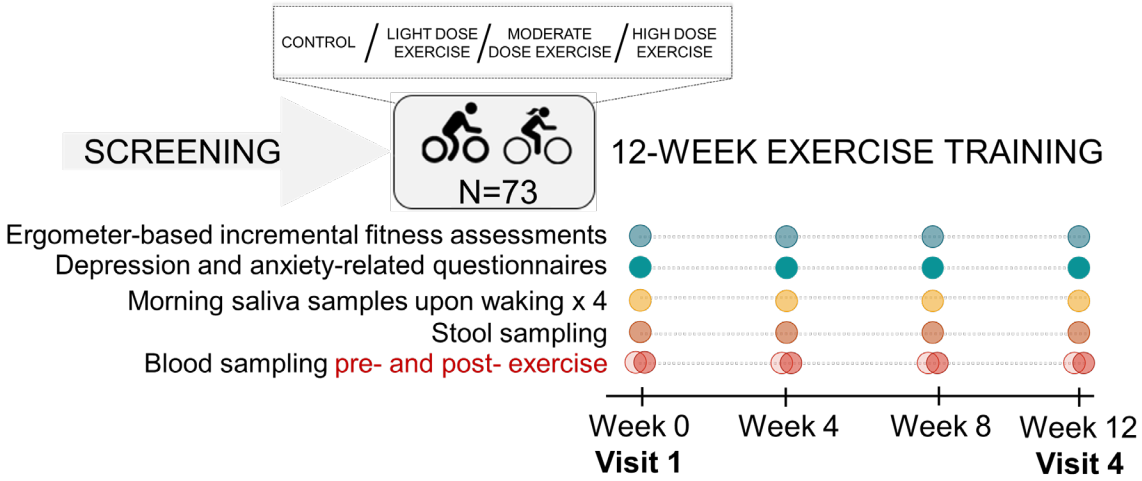

Figure 11. Experimental schematic. 73 healthy sedentary participants were recruited (see participants characteristics in Table 1). The study comprises 4 monthly visits where all participants performed an ergometer-based incremental exercise test to maximal voluntary exhaustion, that included blood withdrawn before and 1h-post exercise at each visit. At each visit, all participants filled out questionnaires to assess food frequency; levels of perceived depression- and anxiety; and provided samples of morning saliva (four), urine, and stool. Following the baseline assessment (visit 1) participants were randomised into one of the four groups: Three exercise training: high-intensity, moderate-intensity, or light-intensity performed over 3 sessions/week (cycling), or a no exercise control group. The control group were instructed to maintain their sedentary lifestyle, although they also performed an incremental voluntary maximum exhaustion test on an ergometer every month.

Sample collection and preparation

Whole blood was collected into 4-mL lithium-heparin containing tubes (Greiner Bio-One, 454029) and 3-mL K3EDTA tubes (Cruinn Diagnostics Limited, Dublin) 1-hour before exercise and 1-hour post exercise. Whole blood samples were centrifuged at 1500 g for 10 minutes minimum at 4°C. The supernatants were aliquoted, and then stored at −80 °C for later analysis.

Faecal sample collection

Freshly voided faecal samples were collected from participants into plastic containers containing an AnaeroGen sachet (Oxoid AGS AnaeroGen Compact, Fischer Scientific, Dublin),

and kept refrigerated (~8°C) until delivery to the laboratory using a cool pack. Upon arrival at the study site, the sample was placed in refrigeration (~8°C) until collected by the researcher processing the sample. The sample was homogenised, aliquoted, and then stored at −80°C for later analysis. Participants were instructed to collect the faecal sample as close to the study visit as possible, preferably on the day of the visit buttypically within a 24 hour window surrounding the study visit.

Immunoassays detection in blood

Cytokines levels from serum samples were quantified using U-plex® Metabolic Group 1 Multiplex (human) assays were used to measure BDNF (#K2516WK-2) levels and U-plex® Proinflam Panel 4 (human) Multiplex assays were used to measure IL1-β, IL-6, IL-8, TNF-α levels (#K15053K-2) according to manufacturer's instructions.

LBP and sCD14 levels from plasma samples were quantified using the LBP Hycult®Biotech (Human LBP) Kit (Hycult®Biotech, HK315-01) and sCD14 Hycult®Biotech (Human sCD14) Kit (Hycult®Biotech, HK320). LBP and sCD14 quantification were done according to the manufacturer's guidelines with 1:10 followed by 1:100 dilution (final dilution: 1:1000) or adjusted dilution where appropriate.

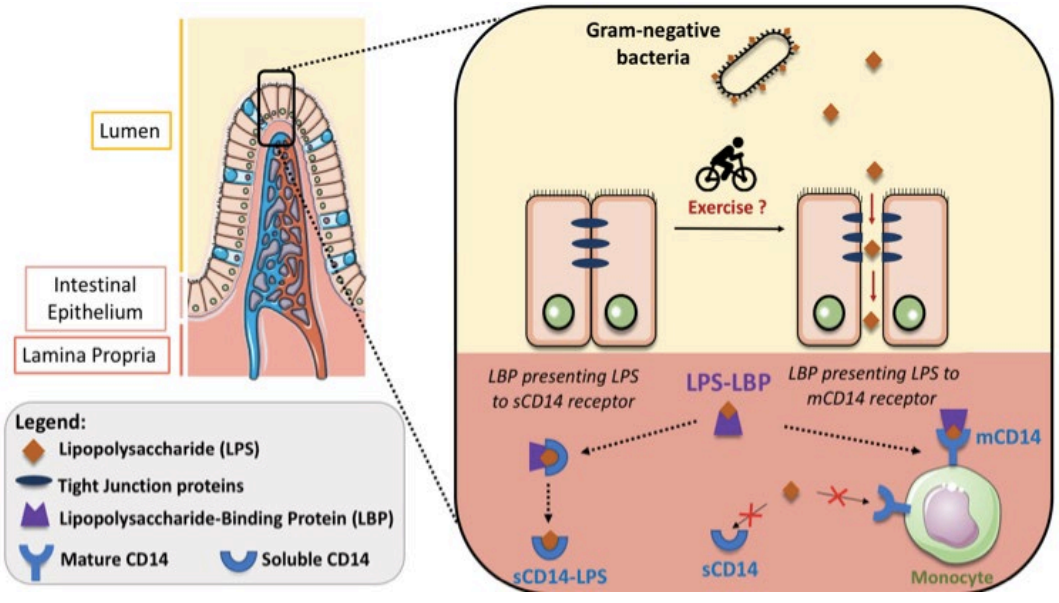

Figure 2. Altered intestinal permeability can be assessed using sCD14 and LPS-binding protein (LBP). Intense exercise training might induce intestinal permeability damage via disrupting tight junctions' proteins following ischaemia and/or hyperthermia. As a result, lipopolysaccharide (LPS, endotoxins), from gram-negative bacteria, are entering systemic circulation triggering an immune response. Indeed, sCD14 and LBP are both LPS recognition proteins. By binding to LPS, LBP is able to

present this endotoxin to sCD14 and mCD14 and generate immune activation by several mechanisms (Zuhl et al., 2014)

UHPLC-ESI-MS/MS – targeted metabolomics in serum

Samples were processed according to previously published protocol for tryptophan metabolites (Anesi et al., 2022) and short- and medium- chain fatty acids (Lotti et al., 2017).

Short-and medium-chain fatty acids

In short, 100µL of blood plasma were individually placed on 2 mL centrifuge tubs (Sarsted, Nümbrecht, Germany). Afterwards, 10 µL of acidified water, 15% phosphoric acid, and 10µL of internal standard were added and vigorously mixed up. Consecutively, a LLE was performed using 140µL of MTBE. The extraction was assisted by an orbital shaker (Multi RS-60; BioSan, Latvia). At this point, tubes were centrifuged at 36,670g at 5°C during 5 min. Finally, the organic phase aliquot (approx. of 150 µL plasma) was transferred into the vial prior to GC–MS analysis. A GC–MS/MS system consisted of Trace GC Ultragas chromatograph (Thermo Fisher Scientific, San Jose, CA,USA), equipped with an autosampler PAL combi-xt autosampler (CTC, Zwingen, Switzerland) coupled to a TSQ Quantum XLS tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A fused silica Stabilwax®-DA column(30m×0.25mmi.d.×0.25µm) (Restek Corporation, Bellefonte, USA) was used for the chromato-graphic separation. The MS detection operated on full-scan mode (EI at 70 eV, ion source temperature at 250°C, m/z values ranged from 40 to 300 Da and acquisition scan time 0.2 s) and multiple reaction monitoring (MRM) acquisition mode (Argon collision gas pressure of 1.2mTorr, a scan time 50 ms for each transition and time window of 1 min). The GC–MS data processing was performed using a qualitative and quantitative software package, XCALIBUR™2.2 software (Thermo Fisher Scientific, San Jose, CA, USA).

Tryptophan and amino acid metabolomics

Briefly, serum samples were thawed overnight in fridge. Aliquots (25 µl) were loaded on Ostro 96-well plates (Waters, Milan, Italy) and equal volume of ISTD in methanol was added. Three volumes of ice-cold acetonitrile acidified with formic acid (1% v/v) were added and plates were shaken for 5 min at 500 rpm on an Eppendorf shaker (Eppendorf, Milan, Italy). Plates were filtered by using a positive pressure 96-manifold (Water, Milan, Italy) for 5 min at 3 psi. The

procedure was repeated by adding 75 µl of ice-cold acidified acetonitrile. Samples were brought to dryness at 37°C using a gentle stream of nitrogen and then re-suspended twice by using 100 µl of water:acetonitrile 95:5, 0.5% formic acid, 1 mM ammonium formate (final volume: 200 µl). UHPLC-ESI-MS/MS were conducted on an AB Sciex 6500+ Triple Quadrupole (ABSCiex, Milan, Italy) coupled to a Shimadzu LC-30 AD pump. Chromatographic separation was carried out on Acquity Premier HSST3 2.1 x 5 mm (1.8 µM particle size) column using water 0.1% formic acid and acetonitrile 0.1% formic acid as mobile phases (A and B respectively). The gradient, at constant flow of 0.5 ml/min, started at 1%B, reached 5%B at 1 min, 12.5%B at 1.5min, 30%B at 2 min, 45%B at 3 min, 5%B at 3.5 min, 75%B at 4 min and then 95%B at 95%B. Total analysis time with column re-equilibration was 8 min. Oven temperature was set at 40°C. R3 solvent was *n*-propanol. The injection volume was 2 µl. QC pooled samples were injected every 20 samples to ensure system stability. Data were processed by using MultiQuant 3.0 software (AB Sciex, Milan, Italy)

Saliva collection and cortisol analysis

Morning saliva samples were collected using the Salivette system (Sarstedt, Germany). Participants were instructed to collect the saliva samples in the morning of the day of their study visit. A total of four samples was collected, with the first one collected upon wakening, the second one 30 min later, and the third and fourth one in 15 min increments. Participants were instructed to not brush their teeth until after all saliva samples were collected, to not eat or drink anything prior to the first sample, and to avoid eating and drinking 15 min prior to the remaining samples. Saliva samples were centrifuged at 1500 g for 10 minutes minimum at 4°C, aliquoted, and then stored at − 80 °C for later analysis. Cortisol analysis Salivary cortisol (CAR) was analysed using the ENZO Life Sciences enzyme-linked immunosorbent assay (ELISA) kits (Exeter, UK) per manufacturer's instructions. Cortisol quantifications were done according to the manufacturer's guidelines with a 1:3 dilution or adjusted dilution where appropriate.

Self-report questionnaires during study visits

Participants completed paper-based self-reported questionnaires to assess perceived stress (Cohen's perceived stress scale (PSS) (Cohen et al. 1983)), depression and anxiety levels (Beck's Depression Inventory II (BDI-II) (Beck et al. 1996)), Positive Affect and Negative Affect Schedule

(PANAS) (Watson et al., 1988), and State-Trait Anxiety Inventory questionnaire (Spielberger et al., 1999).

Statistical analysis

All data were analysed using RStudio (R-4.2.2). Outliers from metabolomic and myokines data were identified by at least ±3 standard deviations and consideration was given to removal for extreme outliers. Normality of residuals of metabolomic analysis were examined on R. Chronic measures were analysed using General Linear Mixed Effect Model on R using the following model: value ~ exercise * timepoint + (1|participant_ID). This model allowed us to analyse changes across the four visits (V1, V2, V3, V4; i.e., time) for 12-week of training across four experimental groups (control, light-, moderate- or high- intensity training). Acute measures, comparing difference before and 1-hour post exercise for each experiment group were computed separately on R using a two-way repeated measure ANOVA design in lme4 in R (Bates et al., 2015). This allowed us to compare differences of the 2 factors: (pre/post) * (V1/V4) for each experimental group. For cortisol measurements, area under the curve were calculated with respect to ground (AUCg). Data is expressed as mean ± SD unless otherwise indicated.

Whole genome sequencing

DNA was extracted using the Qiagen Power Faecal Pro kit (Qiagen) using the manufacturer's instructions. Following extraction, DNA was quantified using the Qubit high Sensitivity Assay (Thermo Fisher) and sequencing libraries were prepared using the Illumina DNA preparation kit (Illumina) as outlined by the manufacturer. Unique dual indices (UDIs) from IDT (Integrated DNA technologies) were used to barcode each sample. Barcoded samples were cleaned and pooled as described in the Illumina DNA preparation kit instructions. The final pool was then sequenced according to Illumina guidelines on a NovaSeq 6000 using S4 v1.5 reagents (Illumina). bFastq reads files were assessed for quality using fastqc (available online at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Taxonomic characterization of the gut microbiome

Sequences were aligned using Bowtie2 v2.2.5 (Langmead & Salzberg, 2012) against the Web Of Life reference database (Q. Zhu et al., 2019) downloaded the 20th of April 2021, with the "--very sensitive" and "--no-unal" flags. Alignments were then taxonomically annotated at the

species level using Woltka v0.1.3 (Q. Zhu et al., 2022) SOP, available in the software's github. Downstream statistical analysis of the count table was handled in R (v4.2.2) with Rstudio/Posit (v2022.12.0.353). The iNEXT library (Hsieh et al., 2016) was used to compute alpha diversity (Chao1 and Simpson Index). Differences in alpha diversity were assessed using linear mixed effect models using the lme4 package (Bates et al., 2015). For beta diversity and differential feature abundance, only taxa prevalent in >60% of samples were considered.

Principal component analysis was performed on centered log-ratio transformed (clr) values as a visual companion to the beta diversity analysis. Zeroes were imputed using the maximum likelihood estimator method for compositional count data discussed by Erb (Erb, 2023). Beta diversity was computed in terms of Aitchison distance (Euclidean distance of clr-transformed counts) and differences in beta diversity were assessed using the PERMANOVA implementation from the vegan library using 1000 permutations. Microbial volatility was measured as the Aitchison distance between visit 1 and visit 4 (Bastiaanssen et al., 2021). Differential abundance analysis was performed for each taxon in the count table using lme4 using the formula: "Taxon ~ Exercise Group * Time of visit + (1|participant)". p-values were adjusted using the Benjamini-Hochberg FDR procedure.

Functional characterization of the gut microbiome

The same alignments produced with Bowtie2 were analyzed with Woltka with the flags "--rank ko" and "--stratify", to perform the functional annotation of the genomes using Kegg Orthologs as reference database. The 177 strains with statistically significant results in the differential abundance analysis were filtered and the presence or absence of GMMs for those genomes was determined with a detection threshold of more than 50% coverage of each module using the R package "omixerRpm" v0.3.3 (Darzi et al., 2016). GMM abundance was calculated as the median of KO abundance in the pathway with maximum coverage (i.e., the default method).

Genomic investment analysis

Genomic investment was defined as the sum of the abundances of GMMs associated with a metabolic trait divided by the number of GMMs in that group, as described previously (Vieira-Silva et al., 2016a). The genomic investment was defined as the number of proteolytic modules

(hierarchically labelled as “amino acid degradation”), saccharolytic (“carbohydrate degradation”) and lipolytic (“lipid degradation”), divided by the total number of modules within each category (36, 22 and 6, respectively). Hierarchical groups were obtained from the original GMMs publication (Vieira-Silva et al., 2016a). The CLR-transformed count table was used to determine in which visit each of the previously defined 177 differentially abundant strains was enriched. Per subject, the log2 of the ratio between the abundance of each strain at visit 4 and visit 1 was calculated. Then, the mean and standard deviation of the distribution of log2(V4/V1) ratios of all the individuals was calculated. Per exercise group and species, the mean log2(V4/V1) was calculated. The distribution of all the ratios was divided in thirds, and the ratio of each species per exercise group was determined in the distribution. The central third was considered as no changes in abundance of the strain between visits. The third with the highest ratios suggests an increase of the abundance of that strain for the specific exercise group at visit 4. The lowest third suggests the opposite. For the triplot representation, the R packages “ggplot2” v3.4.1 and “ggtern” v3.4.1 were used.

Results

12 weeks of moderate- or high-intensity training improves overall fitness in previously sedentary individuals.

Healthy sedentary individuals were randomised into one of the four experimental groups: control (no exercise), light-, moderate-, or high-intensity training for 12 weeks (n=73). Exercise groups were instructed to exercise for 12-weeks, 3 times per week, at a prescribed intensity based on an individual fitness (**Figure 1**). All participants came monthly to the laboratory to undertake a combined fitness and psychological assessment that included an incremental exercise test to voluntary exhaustion to assess improvements in fitness (V1 (baseline), V2, V3, and V4 (12-week post training)). **Table 1** summarises participant characteristics and psychological assessment at baseline (V1) and following 12-weeks of exercise (V4) for all experimental groups. Generalised linear mixed-effects model analysis were used to compare all experimental groups over the course of the four visits (12-week) to assess the level of improvement, displayed as group*timepoint effects on VO_2 peak, relative peak power, and respiratory exchange ratio (RER). These are key measures of cardiorespiratory fitness, representing the peak maximal oxygen uptake during exercise, the highest average power output (watts) over a given amount of time relative to body weight, and the ratio in respired air (i.e., CO_2 production and O_2 consumption), respectively. Follow-up analyses revealed overall differences comparing the control to the moderate-intensity group in VO_2 peak ($p<0.05$) and relative peak power ($p<0.01$); and to high-intensity training in VO_2 peak ($p<0.01$) and RER ($p<0.01$). Interestingly, moderate- and high-intensity training were also significantly different in terms of RER ($p<0.05$)). However, our analyses show no differences in subjective measures of anxiety, stress, and depressive feelings after 12-week of training (**Table 1**). Supplementary **Table S1** display details measures of fitness across the four visits with complementary measures of fitness.

Table 4. Participant characteristics before and after a 12-week exercise intervention reflecting improvement in the overall measures of fitness and perceived measures of stress and anxiety.

Characteristics	Control		Light-intensity training		Moderate-intensity training		High-intensity training		GLMER (group *time)	GLMER (time)	GLMER (group)
	Baseline	12-week post	Baseline	12-week post	Baseline	12-week post	Baseline	12-week post			
Gender	8F		10F		12F		12F				
Age	36.2 (10.4)		44.5 (11.7)		40.7 (8.6)		40 (11.7)				
N	18	17	18	14	17	14	20	14			
BMI	24 (3.5)	23.6 (3.8)	25.7 (4.1)	25.5 (4.6)	25.2 (3.1)	24.3 (2.8)	25.2 (5.0)	26.5 (5.0)	ns	ns	ns
Body fat (%)	24.6 (12.5)	21.4 (11.6)	33.5 (14.2)	32.4 (13)	25.9 (8.7)	24.2 (8.9)	25.3 (11.7)	26.5 (11.4)	ns	ns	ns
Skinfold 7-sites (mm)	90.2 (24.3)	80.2 (21.3)	97.6 (19.4)	94.2 (22.2)	94.2 (22.1)	84.8 (21.1)	95.2 (29.8)	91.8 (25.0)	ns	**<0.01**	ns
VO_2 peak (L/min)	2.9 (0.9)	2.9 (1.0)	2.5 (0.7)	2.4 (0.5)	2.5 (0.8)	2.8 (0.8)	2.6 (0.5)	2.9 (0.6)	**<0.01**	ns	ns
Peak Lactate (mmol/L)	9.6 (5.5)	13.7 (3.0)	10.6 (4.3)	12.8 (3.5)	12.1 (3.8)	14.7 (2.9)	11.4 (5.0)	14.2 (3.2)	ns	**0.014**	ns
Relative Peak Power (Watt/kg)	2.8 (0.6)	2.9 (0.6)	2.4 (0.3)	2.6 (0.5)	2.3 (0.5)	2.7 (0.5)	2.7 (0.6)	3.0 (0.8)	**0.03**	*0.056*	0.067
Respiratory Exchange Ratio	1.6 (0.3)	1.8 (0.4)	1.6 (0.3)	1.7 (0.5)	1.6 (0.2)	1.8 (0.3)	1.8 (0.5)	1.6 (0.2)	**0.02**	**0.04**	**<0.01**
BDI score	4.7 (4.9)	3.2 (2.9)	6.9 (5.3)	5.4 (5.4)	6.4 (5.3)	4.1 (5.4)	4.4 (4.8)	2.1 (1.7)	ns	**0.04**	ns
PANAS positive	27.2 (3.6)	24 (5.4)	25.4 (6.0)	22.8 (5.1)	24.2 (4.1)	23 (3.6)	26.3 (4.0)	25.8 (2.9)	ns	**<0.01**	ns
PANAS negative	21.8 (3.3)	20.8 (5.2)	19.8 (5.7)	18.9 (5.8)	18.4 (3.3)	17.2 (3.0)	20.1 (3.5)	19.8 (2.5)	ns	ns	ns
STAI State anxiety score	22.8 (7.8)	20.6 (6.4)	21.7 (5.6)	19.3 (7.4)	24.5 (3.8)	22.8 (4.0)	25.1 (5.6)	25.2 (3.7)	ns	ns	ns
STAI Trait anxiety score	19.5 (6.4)	17.8 (6.2)	17.8 (5.5)	17.3 (6.3)	19.8 (4.8)	17.8 (4.7)	21.8 (4.6)	21.2 (4.2)	ns	ns	ns
PSS10	12.4 (7.7)	11.2 (6.8)	14.1 (6.6)	12.5 (7.3)	11.2 (5.2)	11.5 (7.0)	10.2 (5.8)	7.5 (5.2)	ns	ns	ns

Data expressed as mean +/- SD. P-value depicts significance in generalized linear mixed-effects model comparing all experimental groups across the 4 visits: V1 (Baseline), V2, V3, V4 (12-week post exercise)

Skinfold represents the sum of subcutaneous fat in mm measured at 7 sites: bicep, tricep, subscapular, suprailiac, mid-abdominal, thigh and calf; VO_2peak (L/min) represents the maximum rate of oxygen uptake during the voluntary maximal exhaustion test; Peak Lactate (mmol/L) represents the maximum blood lactate after the end stage of the incremental exercise test; Relative Peak Power represents the maximum power output in watts divided by the body weight in kg; Respiratory Exchange Ratio represents the ratio between the metabolic

production of carbon dioxide (CO_2) and the uptake of oxygen (O_2). BDI-II,Beck's Depression Inventory (range 0–63; scores below 10 are considered normal mood changes); PANAS positive (range from 10 – 50; with higher scores representing higher levels of positive affect); PANAS negative (range from 10 – 50; with lower scores representing lower levels of negative affect); STAI State anxiety score (range from 20 – 80; with higher scores indicate greater anxiety); STAI Trait anxiety score (range from 20 – 80; with higher scores indicate greater anxiety); PSS,Cohen's Perceived Stress Scale (range 0–40; with scores less than 13 considered low stress levels).

High-intensity training for 12 weeks increases the Cortisol Awakening Response

Cortisol awakening response was assessed to evaluate the impact of an active lifestyle in previously sedentary individuals on HPA axis reactivity. Exercise training requires the activation of the HPA axis and autonomic nervous system to adapt to a higher energy demand(Ball, 2015; Sohail et al., 2019), a key component of the gut-brain axis communication. In trained individuals, it has been reported that repeated acute increases in glucocorticoid secretion leads to chronic adaptation of HPA axis activation(Duclos et al., 1997, 2003; Duclos & Tabarin, 2016). We aimed to understand changes in chronic adaptation of HPA axis activation in previously sedentary individuals trained at different intensity, using cortisol awakening response (CAR), a key measure used in physical and mental health research (Stalder et al., 2016b). Statistical analyses were performed on the calculation of the Area Under the Curve with respect to ground (AUCg). Generalized linear mixed-effects model analysis comparing all experimental groups over the course of the four visits (12-week) display a group*time effect on AUCg ($p<0.01$). Follow-up analysis revealed differences in high-intensity training groups compared to control ($p<0.05$) and to light-intensity training ($p<0.01$) (**Figure 3A**) and appears to be the most increased over time (**Figure 3B**). Interestingly, in all exercise groups, we can observe a higher CAR the first weeks of exercise training (Visit 2 and 3) that returns to baseline level in light- and moderate- group but not in the high-intensity training group (**Figure 3B**). From our analyses, the moderate-intensity group appears to be the ideal exercise intensity for the cortisol awakening response to remain steady and have a positive adaptation in measures of fitness to exercise training.

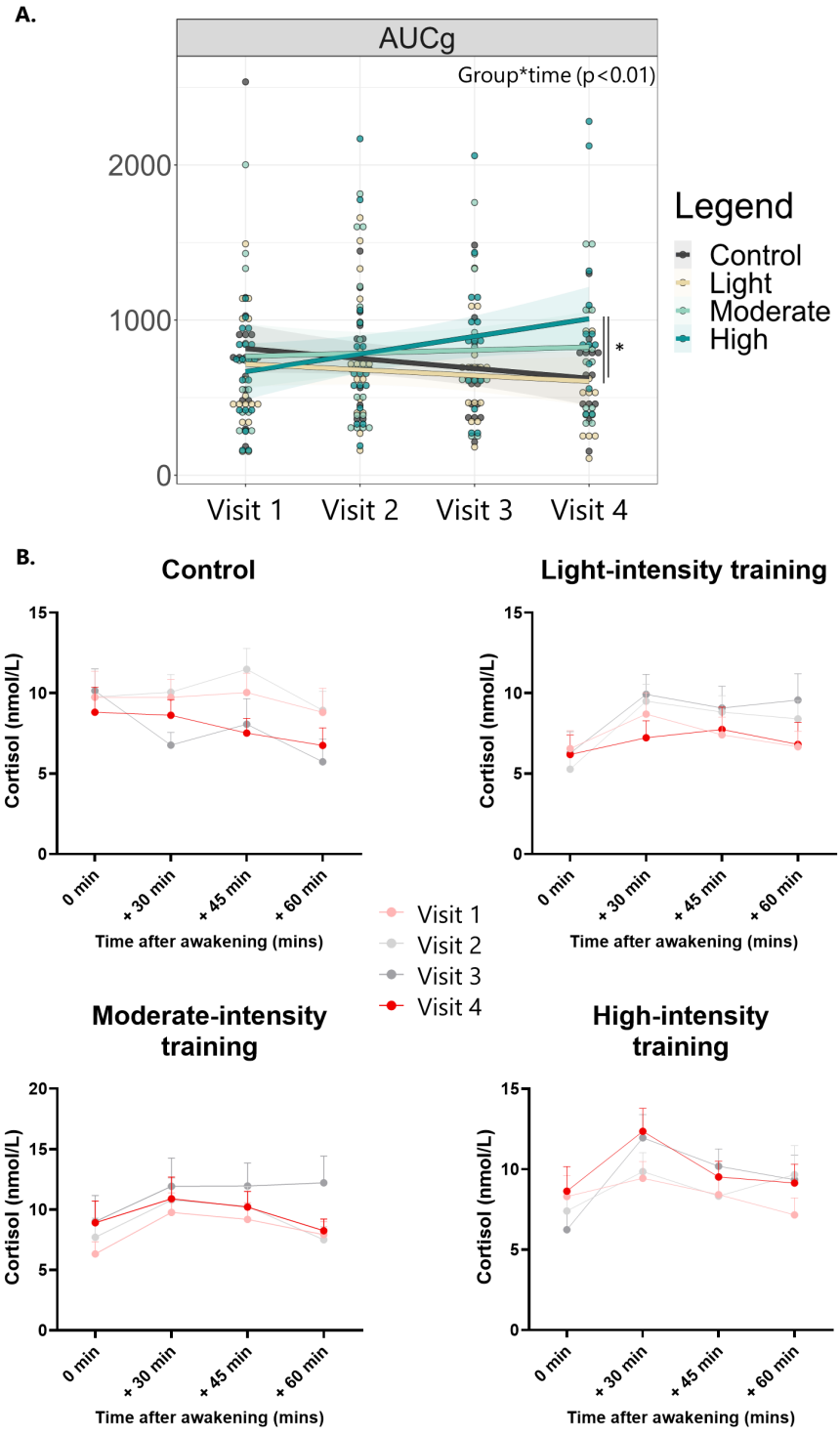

Figure 3. High-intensity training for 12 weeks remodels cortisol awakening response represented by AUCg.

(A) Cortisol Awakening Response (CAR) is represented by morning salivary cortisol (awakening, +30min, +45mins, +1hour) at each visit as measured by the area under the curve with respect to ground (AUCg). Data expressed as mean +/- SD. P-value depicts significance in generalized linear mixed-effects model comparing all experimental groups across the 4 visits: V1 (Baseline), V2, V3, V4 (12-week post exercise) (B) Cortisol Awakening Response visualisation in nmol/L of the different experimental groups at each visit.

12-week exercise had a modest impact on marker of intestinal permeability LBP and sCD14

Over the years, the study of acute and chronic effect of exercise on gut permeability has led to conflicting results mainly due to differences in exercise regiment, intensity and duration. Exercise-induced changes in gut permeability could lead to an impairment in gut-brain axis communication by increasing intestinal permeability, potentially leading to systemic inflammation(Costa et al., 2017). In this context, we measured blood markers of intestinal permeability (**Figure 2**) to understand the impact different intensity-training regimen on gut permeability and whether an adaptation in gut permeability could be found 12-week post-exercise. We found a modest impact on LBP level chronically with a group ($p<0.05$)) and time effect ($p<0.05$) and a time effect ($p<0.05$)) on sCD14 levels (**Figure 4**). Interestingly, follow-up analyses demonstrate differences in high-intensity training when compared to control (LBP, $p=0.06$; sCD14, $p=0.08$) or moderate-intensity training (LBP, $p<0.05$, sCD14, $p=0.07$).However, in the moderate intensity-group, we can observe a subtle decrease in both markers of intestinal permeability over time, suggesting potentially a positive adaptation with less LPS in the systemic circulation. Overall, it seems like 12-week exercise training had a modest impact on blood markers of intestinal permeability.

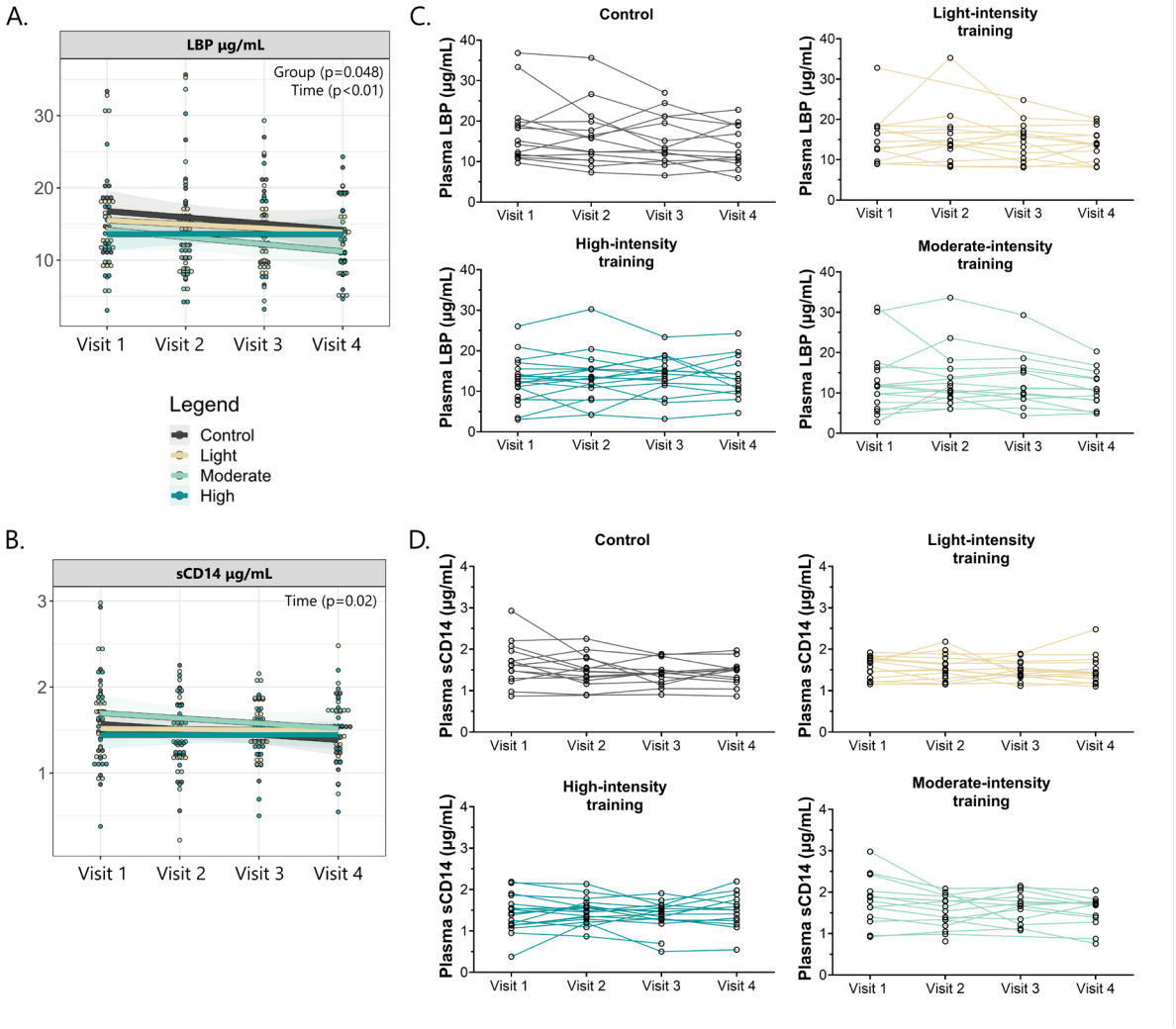

Figure 4. 12-week exercise had a modest impact on marker of intestinal permeability LBP and sCD14. (A-B) Lipopolysaccharide Binding Protein (LBP) and soluble CD14 were measured in the plasma to estimate changes in gut permeability, characterised by LPS translocation in the blood and the recruitment of LBP and sCD14 by immune activation. LBP had a group (p=0.048) and time (p<0.01) effect whilst sCD14 had a time effect (p=0.02) suggesting a modest impact on gut permeability over time. *(C-D)* Visualisation of individual differences in plasma sCD14 in µg/mL of the different experimental groups at each visit.

Modest changes in level of circulating myokines following a maximal exhaustion test and over a 12-week of training.

One main goal of our study was to compare reported acute and transient changes in inflammation markers (IL-1 β, IL-6, IL-8, TNF-α), gut permeability markers (LBP, sCD14) and BDNF to chronic adaptation of exercise. Acutely, neither three-way ANOVA comparing experimental groups, V1/V4 and pre/post measures nor two-way ANOVA within an experimental group, showed significant changes acutely. Chronically, we observed significant time*group effect in IL1-β (p<0.05) and BDNF (p<0.05)) over 12-weeks of exercise training

(**Table 2**). Follow-up analyses revealed that IL1-β levels (*p=0.066*) were most changed compared to controls in the moderate-intensity training only while BDNF levels were significantly different when comparing controls to light- ($p<0.05$), moderate- ($p<0.01$) or high- ($p<0.05$) intensity training. However, BDNF changes seemed driven by baseline differences rather than exercise training.

Table 2. Acute and chronic changes in circulating myokines chronically or following a maximal exhaustion test.

Characteristics	timepoint	Control		Light-intensity training		Moderate-intensity training		High-intensity training		GLMER (group*time) p-value	GLMER (time) p-value	GLMER (group) p-value
		Baseline	12-week post	Baseline	12-week post	Baseline	12-week post	Baseline	12-week post			
BDNF (pg/mL)	Pre	7749.4 (2989.3)	9434.1 (2449.4)	9246.8 (1587.3)	8443.6 (2559.3)	9991.8 (1894.9)	8894.1 (2480.1)	9822.0 (1911.8)	9507.7 (1785.4)	**.02**	ns	.03
	1h-post	7982.3 (3250.3)	7644.2 (3004.7)	9235.4 (2276.9)	8538.8 (2807.5)	8905.5 (2235.6)	9046.9 (2464.4)	8868.8 (1852.7)	8471.8 (1796.8)			
IL-1β (pg/mL)	Pre	0.11 (0.08)	0.059 (0.06)	0.15 (0.098)	0.12 (0.03)	0.09 (0.06)	0.11 (0.07)	0.19 (0.14)	0.20 (0.16)	**.04**	*.067*	ns
	1h-post	0.090 (0.08)	0.044 (0.04)	0.09 (0.10)	0.09 (0.068)	0.07 (0.052)	0.05 (0.03)	0.12 (0.15)	0.13 (0.14)			
IL-6 (pg/mL)	Pre	0.58 (0.58)	0.53 (0.31)	0.86 (0.66)	0.76 (0.41)	0.75 (0.44)	0.55 (0.28)	0.82 (0.72)	1.36 (0.88)	ns	ns	ns
	1h-post	0.77 (0.72)	0.86 (0.41)	1.24 (0.55)	1.0 (0.64)	1.20 (1.0)	0.78 (0.37)	0.91 (0.83)	1.03 (0.82)			
IL-8 (pg/mL)	Pre	10.50 (5.44)	10.76 (4.45)	12.62 (5.01)	12.24 (5.58)	10.23 (5.02)	9.72 (4.99)	10.19 (4.52)	11.04 (4.92)	ns	ns	ns
	1h-post	9.64 (4.29)	8.96 (4.29)	12.36 (4.75)	12.93 (4.67)	9.55 (5.22)	8.40 (4.24)	10.09 (3.51)	10.81 (6.18)			
TNF-α (pg/mL)	Pre	0.31 (0.28)	0.27 (0.25)	0.58 (0.45)	0.48 (0.30)	0.40 (0.23)	0.33 (0.23)	0.49 (0.31)	0.59 (0.34)	ns	*.07*	ns
	1h-post	0.37 (0.28)	0.26 (0.21)	0.45 (0.47)	0.55 (0.39)	0.28 (0.27)	0.28 (0.14)	0.37 (0.33)	0.56 (0.53)			
LBP (μg/mL)	Pre	16.47 (6.37)	13.83 (5.18)	15.63 (6.06)	13.74 (4.14)	14.04 (7.88)	10.91 (4.6)	13.65 (5.77)	13.46 (5.16)	ns	<0.01	0.04
	1h-post	16.75 (7.9)	15.32 (6.74)	14.89 (6.0)	14.84 (5.64)	11.78 (7.76)	12.61 (6.35)	12.26 (5.29)	12.14 (3.87)			
sCD14 (μg/mL)	Pre	1.65 (0.53)	1.44 (0.29)	1.56 (0.26)	1.52 (0.35)	1.79 (0.59)	1.53 (0.38)	1.45 (0.49)	1.49 (0.42)	ns	0.02	ns
	1h-post	1.37 (0.58)	1.50 (0.37)	1.51 (0.35)	1.66 (0.46)	1.57 (0.54)	1.48 (0.48)	1.28 (0.43)	1.33 (0.38)			

Data expressed as mean ± SD; pvalue depicts time*group interaction, time and group interaction in respective column analysed by general mixed effect models across the 4 visits: V1 (Baseline), V2, V3, V4 (12-week post exercise) comparing the four experimental groups.

Exercise influenced fermentation potential of exercise-responsive bacteria in a dose-dependent manner.

Since exercise training was reported to influence compositional and functional features of the gut microbiome (Shahar et al., 2020) and host metabolism (ref), we wanted to understand the potential impact of exercise-induced changes on host and microbial metabolites in the blood. To determine whether 12-weeks of exercise would alter gut microbiome diversity over time, we performed metagenomic sequencing at species level resolution. All experimental groups had no significant changes in α-diversity expressed by Chao1 and Simpson's index; meaning that the richness or diversity of the microbiome were unaltered by exercise (**Figure 5A**). β-diversity, assessing the degree of difference abundance (at a species-level) between microbiome at each visit for each experimental group, was unaltered (**Figure 5B**). To assess dynamic changes in gut microbiome over time with exercise, we assessed volatility(Bastiaanssen et al., 2021). No significant differences in volatility were found, implying that the gut microbiomes of control and exercising participants were similarly stable over time (**Figure 5C**).

However, we observed significant group*time effect on the relative abundance of 177 species following 12-week exercise ($q<0.2$), referred to as exercise-responsive species. When compared to the control group, light-intensity training group had the most significant differences in species characterised by a decrease in *Staphylococcus schleiferi, Staphylococcus succinus, Clostridium beijerinckii, Corynebacterium vitaeruminis, Staphylococcus equorum, Clostridium sartagoforme, Clostridium saccharobutylicum and Clostridium sp. BL8*; and an increase in *Bacteroidales bacterium H10, Eubacterium limosum, Holdemanella biformis* and *Bacteroides pectinophilus CAG:437*. The high-intensity group had a significant increase in *Clostridium sp. CAG:288* and *Selenomonas sp. ND2010* compared to the control group. The moderate-intensity group showed no significant differences when compared to the control group.

One main adaptation in microbial community of athletes compared to the general population was functional changes on microbial substrate degradation(L. M. Petersen et al., 2017b; Scheiman et al., 2019; Shahar et al., 2020). Hence, we aimed to understand whether different intensity training led to differences in saccharolytic, proteolytic and lipolytic potential(Vieira-

Silva et al., 2016a) of exercise-responsive bacteria based on their genomic investment profiles (**Figure 5D**). Each dot on the triplots represent the fermentation potential of a specific strain of bacteria from their genome. The colour represents relative abundance alterations of strain at a specific visit (blue, more abundant at V1; red, more abundant at V4; grey, equally abundant at V1 and V4). Interestingly, all-exercise group had enrichments in the relative abundance of different bacteria with specific fermentation profiles: the light-intensity training group had a higher abundance of strict proteolytic or saccharolytic strains at V4, while the moderate-intensity display an enrichment of exercise-responsive bacteria strains through a gradient of saccharolytic to proteolytic at V4. Most interestingly, the exercise-responsive bacteria in the high-intensity training group had a high proteolytic potential compared to V1, so the relative abundance of exercise-responsive strains with saccharolytic potential is decreased by a 12-week exercise training at V4. This result suggests that there is a dose-dependent effect of exercise on relative abundance of exercise-sensitive microbes: the more intense the exercise, the lower relative abundance of strains with saccharolytic potential.

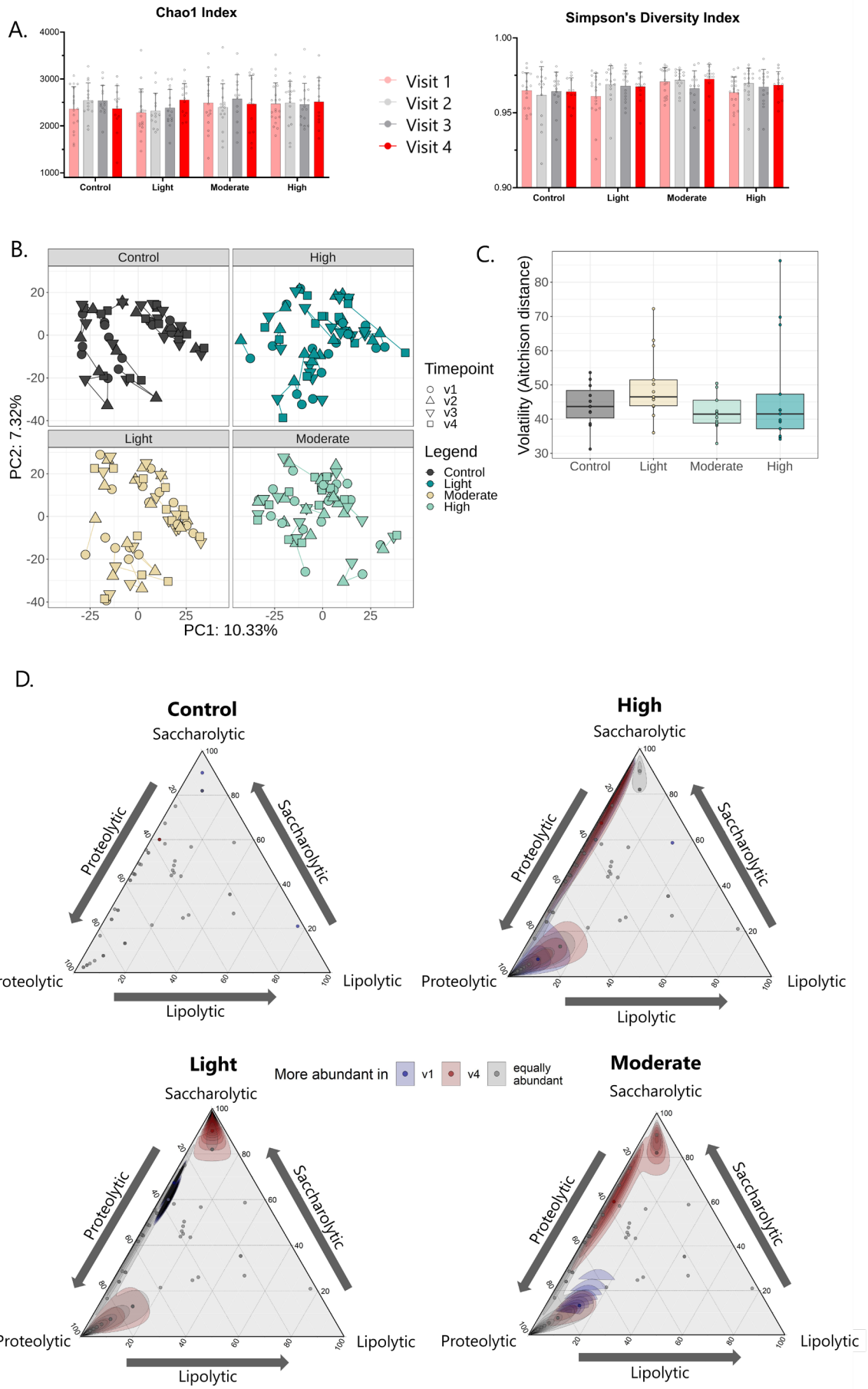
A.
Chao1 Index
4000
3000
2000
1000
Control
Light
Moderate
High
Visit 1
Visit 2
Visit 3
Visit 4
Simpson's Diversity Index
1.00
0.95
0.90
Control
Light
Moderate
High
B.
Control
High
Light
Moderate
PC2: 7.32%
PC1: 10.33%
Timepoint
v1
v2
v3
v4
Legend
Control
Light
Moderate
High
C.
Volatility (Aitchison distance)
Control
Light
Moderate
High
D.
Control
Saccharolytic
Proteolytic
Lipolytic
High
Light
Moderate
More abundant in
v1
v4
equally abundant

Figure 5. Exercise intensity differentially influenced the fermentation potential of exercise-responsive bacteria after 12-weeks of training. (A) Chao1 and Simpson's Index are measures of α-diversity that estimates richness within samples (total number of species) and diversity within a sample (number of individual bacteria from each of the bacterial species). (B) β- diversity is represented by Principal Component Analysis in terms of Aitchison distance (Euclidean distance of CLR-transformed data) and assesses difference between samples. (C) Volatility is the degree as compositional change of the microbial ecosystem over time and is calculated by intra-subject Aitchison distance between the species-level count table from the same subject at different timepoint using CLR-transformation. No significant difference was found in either of the microbiome parameters in response to a 12-week exercise training. (D) Triplots representation of the genomic investment in each of the three-fermentation potential (saccharolytic, proteolytic and lipolytic, defined as average copy number of gut metabolic modules) (Valles-Colomer et al., 2019b; Vieira-Silva et al., 2016a) in each experimental group. Each dot represents the fermentation profile for a given exercise-responsive species with: central dots representing equal investment in all three fermentation types (Generalism), while departure towards a corner corresponds to increased investment in that particular fermentation type (Proteolytic; Saccharolytic or Lipolytic). Red dots and shades represent highest V4/V1 ratios suggesting an increase of the abundance of that strain for the specific exercise group at visit 4. Blue dots and shades represent the lowest V4/V1 ratios, suggesting a decrease of the abundance of that strain for the specific exercise group at V4.

High-intensity training for 12 weeks increases peripheral serotonin production following a maximal exhaustion challenge.

One key goal of this study was to evaluate the effect of a maximal exhaustion test on host and microbial metabolites playing an important role in microbiota-gut-brain signalling and whether an adaptation in metabolism was observed after 12-weeks. More specifically, since microbial-responsive strains display intensity-depend effect on proteolytic potential, we were interested in potential changes in microbial degradation of tryptophan in the different exercise groups. We performed targeted metabolomics in serum samples to understand the effect of a maximal exercise challenge (pre and 1h-post) at the baseline visit (V1) and 12-weeks post-training (V4). Two-way repeated measures ANOVA within each group revealed changes in serotonin at high intensity training ($p<0.05$). Significant increase at V4 were found in serotonin production in the high-intensity group when comparing pre and one hour-post a maximal exercise challenge ($p<0.05$) (**Figure 6A**). Similarly, changes in phenylalanine levels were found in the light-intensity training group ($p<0.05$) and to a lesser extend in the high-intensity training group ($p=0.05$). The high-intensity training group appears to have an increase in KA/KYN ratio at V4 compared to all other experimental groups, suggesting a potential increase in KAT activity. Interestingly, we see a trend in increase in indole-3-lactic acid following a maximal exhaustion test at V4 in all the exercise groups which might suggest microbial proteolytic activity of tryptophan. Finally, we wanted to assess whether changes in exercise-responsive species (threshold ANOVA $F>5$) were correlated to changes in tryptophan

metabolism (threshold p-adjusted<0.1). A correlation matrix displaying trends in correlation between changes in species and host and microbial tryptophan metabolism visualised in **Figure 6B**. Overall, weak correlations were noted. However, it appears that there are several positive correlations between the kynurenic acid and indole-3-acetic acid level in the periphery and exercise-responsive microbes in the light-intensity group. Whereas several negative correlations appear in exercise-responsive microbes in serotonin level in the high-intensity training group. Acute and chronic changes in amino acids, and other tryptophan-related molecules can be found in supplementary **Table S3**.

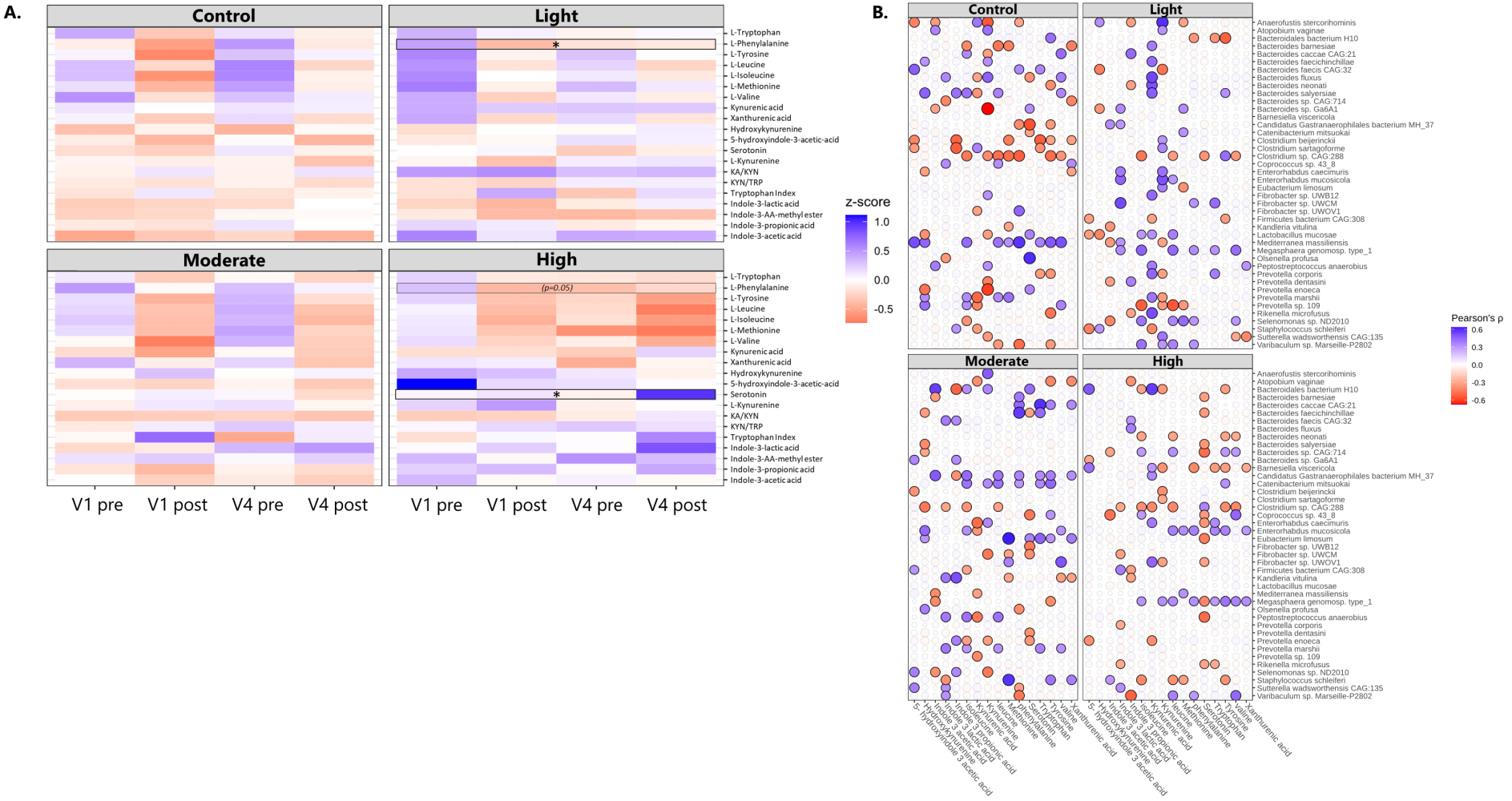

Figure 6. Heatmaps representing relative changes in host and microbial amino acid and tryptophan metabolism in response to a maximal exhaustion test at baseline and after 12-weeks of training and their correlation between exercise-responsive microbial species. (A) Heatmap representing changes in essential amino acids, branched chain

amino acids and host & microbial tryptophan metabolism following a maximal exhaustion test (pre/post) at baseline (V1) and 12-weeks after (V4). Z-scores for each metabolite were normalised by overall mean (including all participants at all visits). 2-way ANOVA comparing (pre*post) * (V1*V4) on the raw data were calculated for each group individually. Kyna: Kynurenic acid, Kyn: kynurenine, Trp: Tryptophan, Tryptophan Index (Tryptophan*100/LNAA1), LNAA1: sum of tyrosine, valine, phenylalanine, leucine and isoleucine level in plasma, competing with tryptophan for entry in blood brain barrier. (B) Correlation analysis between species significantly altered by exercise (threshold anova $F>5$; Benjamini-Hochberg adjusted $q<0.1$ for all cases.) and circulating tryptophan-related metabolites. Colour depicts effect size, with red indicating negative Pearson correlation coefficient and purple indicating positive correlation coefficient. Opaque points show correlations significant at the $p<0.05$ level. No significant correlation found after FDR correction.

Light-intensity training for 12 weeks decreases acetic acid production following a maximal exhaustion challenge.

Since microbial-responsive strains display intensity-depend effect on saccharolytic potential, we were interested in potential changes in microbial degradation of tryptophan in the different exercise groups. Microbial short-chain fatty acid (SCFA) production was shown to be an adaptation to exercise (Shahar et al., 2020), we investigated whether some changes in SCFAs happened one hour after a maximal exhaustion challenge and after12-weeks of training. Two-way repeated measures ANOVA revealed a trend in acetic acid changes in the light intensity training group ($p=0.05$): at baseline showing an increase following maximal exhaustion whereas a decrease is observed following a maximal exhaustion 12 weeks post training (**Figure 7A**). Interestingly, all exercise groups seem to have a decrease in circulating SCFAs (butyric, acetic and propionic acid) following a maximal exhaustion challenge which seems more pronounced at V4 compared to V1.

As above, we aimed to see whether changes in exercise-responsive species (threshold ANOVA $F>5$) were correlated to changes in fatty acids (threshold p-adjusted<0.1). A correlation matrix displaying trends in correlation between changes in species and host and microbial fatty acid metabolism is visualised in **Figure 7B**. Although weak correlations were found, control and high-intensity training exercise seem to have positive correlations with acetic acid level, while the moderate intensity training have a trend in negative correlation in propionic and butyric acid production. Interestingly, there is weaker and fewer correlations in the exercise groups compared to control which might suggest that control groups use more saccharolytic breakdown to cope with exercise demand while exercise group might shift to another source of energy (proteolytic for example). Acute and chronic changes in medium- and short-chain fatty acids can be found in supplementary Table S4.

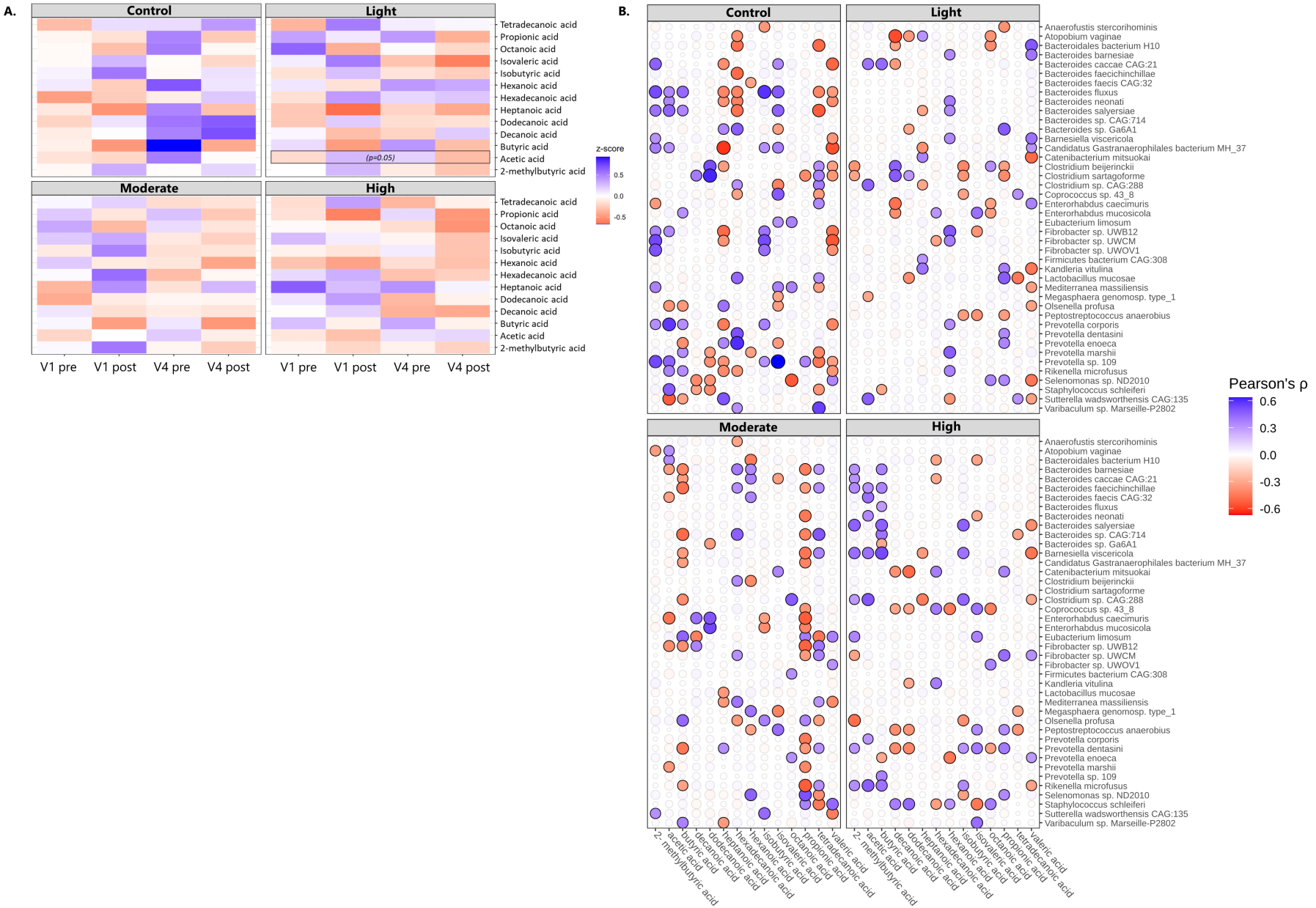
A.
Control
Light
Moderate
High
Tetradecanoic acid
Propionic acid
Octanoic acid
Isovaleric acid
Isobutyric acid
Hexanoic acid
Hexadecanoic acid
Heptanoic acid
Dodecanoic acid
Decanoic acid
Butyric acid
Acetic acid
2-methylbutyric acid
(p=0.05)
z-score
0.5
0.0
-0.5
V1 pre
V1 post
V4 pre
V4 post
B.
Control
Light
Moderate
High
Anaerofustis stercorihominis
Atopobium vaginae
Bacteroidales bacterium H10
Bacteroides barnesiae
Bacteroides caccae CAG:21
Bacteroides faecichinchillae
Bacteroides faecis CAG:32
Bacteroides fluxus
Bacteroides neonati
Bacteroides salyersiae
Bacteroides sp. CAG:714
Bacteroides sp. Ga6A1
Barnesiella viscericola
Candidatus Gastranaerophilales bacterium MH_37
Catenibacterium mitsuokai
Clostridium beijerinckii
Clostridium sartagoforme
Clostridium sp. CAG:288
Coprococcus sp. 43_8
Enterorhabdus caecimuris
Enterorhabdus mucosicola
Eubacterium limosum
Fibrobacter sp. UWB12
Fibrobacter sp. UWCM
Fibrobacter sp. UWOV1
Firmicutes bacterium CAG:308
Kandleria vitulina
Lactobacillus mucosae
Mediterranea massiliensis
Megasphaera genomosp. type_1
Olsenella profusa
Peptostreptococcus anaerobius
Prevotella corporis
Prevotella dentasini
Prevotella enoeca
Prevotella marshii
Prevotella sp. 109
Rikenella microfusus
Selenomonas sp. ND2010
Staphylococcus schleiferi
Sutterella wadsworthensis CAG:135
Varibaculum sp. Marseille-P2802
2-methylbutyric acid
acetic acid
butyric acid
decanoic acid
dodecanoic acid
heptanoic acid
hexadecanoic acid
hexanoic acid
isobutyric acid
isovaleric acid
octanoic acid
propionic acid
tetradecanoic acid
valeric acid
Pearson's ρ
0.6
0.3
0.0
-0.3
-0.6

Figure 7. Heatmaps representing relative changes in medium- and short-chain fatty acids in response to a maximal exhaustion test at baseline and after 12-weeks of training and their correlation between exercise-responsive microbial species. (A) Heatmap representing changes in host and microbial short-chain fatty acid production following a maximal exhaustion test (pre/post) at baseline (V1) and 12-weeks after (V4). Z-scores for each metabolite were normalised by overall mean (including all participants at all visits). 2-way ANOVA comparing (pre*post) * (V1*V4) on the raw data were calculated for each group individually. (B) Correlation analysis between species significantly altered by exercise (threshold anova F>5; Benjamini-Hochberg adjusted q<0.1 for all cases.) and circulating fatty acids. Colour depicts effect size, with red indicating negative Pearson correlation coefficient and purple indicating positive correlation coefficient. Benjamini-Hochberg adjusted q < 0.1 for all cases. Opaque points show correlations significant at the p<0.05 level. No significant correlation found after FDR correction. (B) Correlation analysis between species significantly altered by exercise (threshold anova F>5; Benjamini-Hochberg adjusted q<0.1 for all cases.) and circulating fatty acids. Colour depicts effect size, with red indicating negative Pearson correlation coefficient and purple indicating positive correlation coefficient. Opaque points show correlations significant at the p<0.05 level. No significant correlation found after FDR correction.

Discussion

The purpose of the study was to (1) to understand how different intensities of exercise shapes immune function, gastrointestinal permeability and HPA axis reactivity, key pillars of the gut-brain axis communication, (2) distinguishing between acute transient effect of exercise to chronic adaptation in an intensity-dependent manner and (3) understand how different intensities of exercise remodels the gut microbiome, gastrointestinal function and relevant changes in host and microbial tryptophan metabolism in the circulation.

Global indices of gut microbial changes following a 12-week exercise, α- and β- diversity, were unaltered, which is in accordance with some studies looking at the effect of exercise on these parameters in a shorter 3-week single intensity intervention(Rettedal et al., 2020) or pre- and post- a single bout of intense exercise(Sato & Suzuki, 2022; Tabone et al., 2021; X. Zhao et al., 2018). Changes in α-diversity were only reported in studies comparing athletes and non-athlete populations with a higher diversity noted in trained individuals(Barton et al., 2018b; S. F. Clarke et al., 2014; H. Morita et al., 2023). Similarly, no significant change in volatility, was found with exercise. To our knowledge, this is the first study to assess the degree of compositional change of the microbiome over time in response to exercise training. Microbial volatility is an important measure in longitudinal studies investigating stress or HPA axis activation, as it has been positively associated with cortisol and corticosterone production after chronic stress in humans and mice(Bastiaanssen et al., 2021).

Overall, our analysis revealed 20 genera having a significant group*time effect with no significant changes when comparing control to light-, moderate- or high-intensity training (data not shown). Interestingly, 5/20 genera changes by exercise are reported to be altered in psychiatric disorders (Bacteroides, Alistipes, Coprobacillus, Paraprevotella and Sutterella)(Nikolova et al., 2021). More specifically, the relative abundance of Sutterella is decreased in MDD and increased in light- and high-intensity training; Bacteroides is increased in anxiety with inconsistent finding in MDD and our study showed a decrease in moderate-intensity training. Alistipe and Paraprevotella have inconsistent changes in MDD but are increased in high- and light- intensity training with a decrease in moderate-intensity training. Finally the relative abundance of Coprobacillus is increased in schizophrenia but decreased in high-intensity training.

In addition, we assessed strain-level relative abundance alterations after 12-week exercise, to determine the effect of the exercise at the highest possible resolution: we found that 177 species that had a significant group*time effect following 12-week exercise. Interestingly, the light-intensity training group had the highest number of exercise-responsive bacteria compared to control, suggesting that the light-intensity training remodels the gut microbiome to a higher degree in our study. From a functional perspective, the compositional alterations observed were not associated with changes in the abundance of gut-brain and gut-metabolic modules associated with tryptophan metabolites. However, we provide novel insights into intensity-dependent effect of exercise on microbial fermentation profile at V4 when focusing on exercise-responsive strains of bacteria. This suggests that different intensity training could shape functional changes in the gut microbiome by orienting towards proteolytic fermentation during more vigorous exercise. Studies looking at changes in microbial composition are rarely assessed at a species level, and none of the studies, to our knowledge, observed changes in the species mentioned above or changes in relative abundance of exercise-responsive bacteria with specific fermentation profile.

Our results reveal that exercise impacts on tryptophan metabolism at a number of levels in a dose- and time-dependent manner and depending on the evaluation of resting state or response features. In particular, 12 weeks of high-intensity training was associated with increased circulating levels of serotonin after a maximal exhaustion challenge. From a compositional perspective, light-exercise training led to the most important relative abundance changes in exercise-responsive microbes with increases in the relative abundance of known tryptophan-metabolising species *Holdemanella biformis* and *Eubacterium limosum*. *Eubacterium limosum* was positively correlated with kynurenine level in the blood of light-intensity trained individuals and positively correlated with methionine, serotonin, tryptophan, tyrosine and xanthurenic acid in moderate-intensity training group. However, this strain was negatively correlated with serotonin production in the high-intensity training group. Interestingly, **Figure 6** suggests an increase in indole-3-lactic acid production following maximal exhaustion in all exercise groups after 12-weeks of training, suggesting microbial breakdown of tryptophan metabolism as an adaptation to exercise. Although it was shown that microbiome-related adaptation to exercise training is associated with an increase in

circulating SCFAs (Mach & Fuster-Botella, 2017), our study provides evidence that a host physical stressor like exercise can acutely change microbial metabolite production.

Exercise may impact host tryptophan metabolism via a number of mechanisms. Previous work suggested that changes in tryptophan metabolism mediated by skeletal KAT enzymes should be reflected one hour post exercise by reduced serum kynurenic acid levels (Schlittler et al., 2016). We did not detect any alterations in circulating kynurenine or kynurenic acid, although there was some evidence for increased KAT activity after a maximal exercise challenge (KA/KYN ratio) following 12 weeks of a high exercise dose (**Figure 6B**). The dynamics of this response may be more complicated that previously envisaged. However, Schlitter et al. found an increase in KYNA and decrease in QUIN/KYNA ratio one hour after 150-km road cycling exercise in clinical setting, no changes were found following eccentric exercise (100 drop jumps)(Schlittler et al., 2016). This suggests that an intense effort leading to potential muscle damage triggers those changes, perhaps on a timescale that is different to the transcriptional regulation of muscle KAT enzymes previously reported(Agudelo et al., 2014; Schlittler et al., 2016). However, we saw no significant changes in host or microbial tryptophan-related metabolism which could be explained by the fact that our participants reached exhaustion in the incremental voluntary ergometer fitness assessment at around 20 mins (lower intensity compared to a 150-km road cycling exercise). Another study with less intense endurance training paradigm in healthy participants, showed changes in KYNA levels immediately post-exercise but returned to baseline onehour-post exercise which could explain that we did not see the expected changes onehour post exercise training(Joisten, Kummerhoff, et al., 2020).

Many of the subsequent studies looking at changes in tryptophan metabolism that display changes, analysed blood samples immediately post-exercise(Bansi et al., 2018; Lewis et al., 2010; Mieszkowski et al., 2022; Strasser et al., 2016); therefore changes in tryptophan metabolism in response to exercise might be transient. Other studies looking at chronic changes in kynurenine, QUIN or KYNA following exercise reported no changes (D. J. Allison et al., 2019; Bizjak et al., 2021). Bizjak et al. hypothesised that kynurenine levels might be increased in participants suffering from overtraining syndrome(Bizjak et al., 2021), which is an interesting hypothesis since most of the studies looking at the effect of exercise on tryptophan metabolism study athlete population or participants having a very active lifestyle(Hudson et al., 2021; Lewis et al., 2010; Mieszkowski et al., 2022; Schlittler et al., 2016; Strasser et al., 2016).

We might not see an effect on tryptophan metabolism along the kynurenine pathway since our population was previously sedentary. Although we observed changes in serotonin production in the periphery in response to high-intensity training and this might lead to serotonin-mediated peripheral changes, serotonin cannot cross the blood brain barrier(Gheorghe et al., 2019), therefore is unlikely to lead directly to beneficial effects in the CNS. Interestingly, Millischer et al. applied an exercise training protocol in MDD patients for 12-week at 3 different intensities and found no differences long-lasting changes in plasma levels of kynurenine or KYNA before and after training(Millischer et al., 2017). This suggests that the resilience to stress-induced depression found in preclinical results due to a shift in kynurenine metabolism by Agudelo et al. might not be applicable in humans in terms of stress-related changes, but more research is needed to reach reliable conclusions. Moreover, transcriptional changes in skeletal muscle regulation by KAT enzymes induces by exercise(Agudelo et al., 2014; Schlittler et al., 2016) are not expected to be reflected immediately post-exercise in circulating kynurenine levels, suggesting that other mechanisms implicated in the regulation tryptophan metabolism could be recruited such as inflammation orhepatic TDO activity via glucocorticoid release.

The cortisol awakening response reflects mainly circadian regulation of cortisol secretion and is often used as a measure of HPA axis reactivity in psychiatric conditions(Chida & Steptoe, 2009). This work demonstrates that high-intensity training, but not light- and moderate-intensity training, increases chronically the cortisol awakening response compared to control. However, no significant changes were observed in tryptophan conversion towards the kynurenine pathway in the high-intensity group, which could have been expected since this pathway is influenced by immune- and stress-related mediators(Gheorghe et al., 2019).

This might be explained by the fact that the light-intensity training group did not show significant improvement in overall fitness while the moderate-intensity training elicited a lower physiological “stress” response chronically when compared to the high-intensity training group. Transient changes in cortisol secretion pattern have been demonstrated in trained individuals; however resting conditions are similar(Duclos & Tabarin, 2016). A systematic review reported that a certain threshold of exercise might be required to alter HPA axis and influence CAR(T. Anderson & Wideman, 2017), our study suggests that moderate-intensity training is ideal to have beneficial fitness improvement of exercise without chronically

impacting CAR (**Figure 3**). This study suggests that there is a dose-dependent effect of exercise on CAR. A meta-analysis in psychiatric-related study demonstrate that CAR response was positively associated with stress whereas it was negatively correlated with fatigue and exhaustion(Chida & Steptoe, 2009). CAR has been suggested to be used in exercise-related study as a sign of overtraining(T. Anderson & Wideman, 2017), although exercise could act chronically by decreasing pituitary sensitivity to negative feedback(Duclos et al., 1997, 2003). Our CAR results are consistent with previous research suggesting that the CAR response to training is influenced by intensity(T. Anderson & Wideman, 2017).

When looking at myokines in the circulation, we found that IL1-β, a pro-inflammatory cytokine often increased by exercise due to cellular damage (Docherty 2022), exhibited baseline changes following 12-week exercise suggesting a physiological adaptation to exercise. IL1- β were most different in the moderate-intensity training when compared to control. However, the IL1- β data need to be interpreted with caution due to low n number of detected IL1-β in some cases (**Table S2**). Interestingly, increased levels of IL-β and IL-6 have been reported in a number of stress-related psychiatric conditions, leading to neuroinflammation and production of kynurenic acid in the brain(Erhardt et al., 2017). Both IL-β and IL-6 are known to be increased in the circulation following exercise, maybe shifting tryptophan metabolism in the periphery towards kynurenic acid production and not in the brain. Changes in KAT enzymes function in the periphery due to exercise has been hypothesised to mediate resilience to stress in a mouse model of depression(Agudelo et al., 2014).

Alterations in gut permeability can drive immune system changes that might activate host metabolism of tryptophan along the kynurenine pathway. We found no effect of exercise on blood markers of gut permeability following a maximal exhaustion challenge. LBP was significant reduced in the moderate-intensity training compared to controls at visit four, and there was also a trend for a chronic decrease in both blood markers of intestinal permeability in this group which could imply that exercise training, at a relevant dose, has subtle benefits gut barrier function. The reduction in gut permeability following 12 weeks of moderate intensity exercise was not associated with significant reductions in inflammatory markers (**Table 2**). Exercise training has been shown to disrupt gut barrier function at an intense

training level and is usually observed in regular and marathon runners(Chantler et al., 2021; Stuempfle & Hoffman, 2015), which was not observed in our highest intensity training group.

When looking at changes in SCFAs in response to exercise, we found that light-intensity training leads to a decrease in acetic acid production after a maximal exhaustion test in trained individuals but not at baseline (**Figure 7**). A previous study reported changes in increased faecal concentration of SCFAs in a 6-week intervention study in lean but not obese individuals, suggesting a chronic adaptation to exercise(J. M. Allen et al., 2018). It would be interesting to assess faecal level of SCFAs to understand differences between circulating and excreted metabolites in response to acute exercise.

Hippocrates famously claimed: "*If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health*". Since then, specific mechanisms underlying the adequate dose of exercise necessary to promote its beneficial effects remain to be discovered. By training previously sedentary individuals at different intensity for 12-weeks, we found intensity-dependent effects on different pillars of the microbiota-gut-brain axis, with high-intensity training increasing the CAR. Moreover, we report an intensity-dependent effect of exercise on host and microbial tryptophan and fatty acid metabolism, showing that even light-intensity exercise can lead to marked compositional alterations at the species level. Our work provides the first piece of evidence that the relative abundance of exercise-responsive bacteria with saccharolytic potential decreases with exercise intensity**.** The functional implications and mechanistic underpinnings of these observations for host-microbe dialogue require additional evaluation to understand how best to prescribe exercise for precision control of key signalling pathways.

Supplementary tables:

Table S1B. Summary of chronic changes in fitness level across different exercise groups

Measurement		Control			Light			Moderate			High					
	Visit	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	GLMER (group*time)	GLMER (group)	GLMER (time)
Body Mass Index	1	23.8	3.4	14	25.7	4.4	14	24.7	2.8	14	26.5	5.5	14	ns	ns	ns
	2	25.4	8.4	14	25.7	4.5	14	24.5	2.8	14	26.4	5.2	14			
	3	23.6	3.7	14	25.8	4.3	14	22.3	6.9	14	26.3	5.0	14			
	4	23.6	3.8	14	25.5	4.6	14	24.3	2.8	14	26.5	5.0	14			
Body Fat (%)	1	21.0	9.1	14	33.9	14.4	14	25.3	8.5	14	27.0	11.7	14	ns	ns	ns
	2	21.2	8.7	14	33.6	13.7	14	24.7	8.9	14	27.5	11.5	14			
	3	21.6	8.4	14	33.4	13.1	14	22.4	10.8	14	27.1	11.6	14			
	4	21.4	11.6	14	32.4	13.0	14	24.2	8.9	14	26.5	11.4	14			
Heart Rate beats/min	1	152.1	63.3	14	147.8	59.5	13	157.1	46.9	14	161.4	35.2	14	ns	ns	ns
	2	169.6	44.3	14	171.0	14.5	13	140.3	66.1	14	172.1	11.9	14			
	3	177.4	9.2	14	160.9	45.4	13	151.8	58.9	13	162.3	43.9	14			
	4	157.7	59.6	14	149.2	58.5	13	143.9	67.4	14	150.5	57.2	14			
Height (cm)	1	162.8	43.4	14	158.1	43.9	13	151.6	57.4	14	170.1	7.2	14	ns	ns	ns
	2	162.9	43.5	14	158.7	42.3	14	173.8	8.0	14	158.5	40.8	14			
	3	162.9	43.6	14	157.4	44.0	13	163.1	44.6	13	158.5	40.8	14			
	4	162.9	43.6	14	158.2	43.8	13	163.7	43.0	14	158.6	40.9	14			
Peak Lactate (mmol/L)	1	10.2	5.8	14	11.1	4.2	13	12.1	4.2	14	11.0	5.7	14	ns	ns	0.01
	2	11.5	5.9	14	11.9	3.0	13	11.7	3.6	14	11.8	4.0	14			
	3	11.1	5.1	14	13.2	3.0	12	13.1	4.3	13	12.5	4.0	14			

	4	13.7	3.0	14	12.8	3.5	13	14.7	2.9	14	14.2	3.2	14		
Respiratory Exchange Ratio	1	1.6	0.3	14	1.7	0.4	13	1.6	0.2	14	1.8	0.5	14	0.02	<0.01	0.04
	2	1.6	0.2	14	1.6	0.4	13	1.7	0.3	14	1.9	0.5	14			
	3	1.5	0.2	14	1.6	0.2	13	1.7	0.4	13	1.7	0.4	14			
	4	1.8	0.4	14	1.7	0.5	13	1.8	0.3	14	1.6	0.2	14			
Resting Heart Rate	1	62.2	7.8	14	66.4	7.8	14	64.7	9.8	13	63.3	6.1	14	ns	ns	ns
	2	64.5	6.6	15	68.3	8.4	15	66.8	6.7	13	64.6	8.5	17			
	3	62.1	8.7	14	65.8	8.8	15	66.9	8.4	13	63.3	11.4	17			
	4	64.9	7.6	14	65.6	6.0	14	66.5	6.0	13	67.5	6.8	14			
Skinfold - Sum of 4 sites (mm)	1	45.3	14.3	14	54.5	18.9	14	48.2	13.0	14	51.6	17.0	14	ns	ns	*0.05*
	2	46.9	13.8	16	52.7	11.3	17	46.8	12.3	16	50.9	19.6	19			
	3	49.3	18.5	14	51.1	12.0	15	43.5	11.7	13	48.0	15.8	17			
	4	41.0	13.6	14	49.4	13.3	14	44.2	11.4	14	49.5	15.5	14			
Skinfold - Sum of 7 sites (mm)	1	87.4	24.0	14	99.3	19.5	14	91.7	22.5	14	100.3	30.4	14	ns	ns	<0.01
	2	86.6	23.6	14	98.5	20.4	14	89.2	22.7	14	94.3	24.4	14			
	3	84.7	20.7	14	97.2	18.9	14	84.6	21.2	13	96.4	25.5	14			
	4	80.2	21.3	14	94.2	22.2	14	84.8	21.1	14	91.8	25.0	14			
Time to exhaustion (min)	1	17.5	7.7	14	16.7	5.3	13	18.7	4.2	14	15.9	8.1	14	ns	ns	ns
	2	15.3	9.6	14	17.4	5.1	13	18.9	6.6	14	18.9	7.7	14			
	3	18.4	8.6	14	16.3	7.0	13	18.1	8.4	13	18.3	9.2	14			
	4	18.2	8.2	14	16.5	7.0	13	18.0	7.7	14	21.3	6.2	14			
VCO2 L/min	1	4.1	1.7	14	3.3	1.3	13	3.5	1.1	14	3.8	0.9	14	<0.01	ns	ns
	2	3.7	1.3	14	3.3	0.9	13	3.6	1.1	14	3.9	1.0	14			
	3	3.7	1.0	14	3.3	0.8	13	3.9	1.2	13	3.7	0.9	14			
	4	4.1	1.3	14	3.3	0.6	13	3.9	1.1	14	3.9	1.0	14			

Ve BTPS L/min	1	92.2	49.7	14	83.2	30.0	13	88.8	40.6	14	68.8	47.3	14	ns	ns	ns
	2	90.1	47.5	14	67.2	40.2	13	85.1	44.0	14	73.3	51.7	14			
	3	89.2	44.8	14	83.7	32.2	13	77.1	52.4	13	56.9	48.5	14			
	4	83.1	54.0	14	72.6	30.9	13	88.4	50.4	14	85.0	50.9	14			
VO2 L/min	1	3.0	1.0	14	2.4	0.7	13	2.5	0.8	14	2.5	0.4	14	ns	ns	ns
	2	2.8	1.0	14	2.3	0.5	13	2.7	0.8	14	2.6	0.4	14			
	3	2.9	0.9	14	2.5	0.6	13	2.7	0.9	13	2.6	0.5	14			
	4	2.9	1.0	14	2.4	0.5	13	2.8	0.8	14	2.9	0.6	14			
Work Rate (Watts)	1	20.3	8.3	14	15.5	8.0	13	17.1	4.3	14	13.0	9.1	14	0.04	ns	*0.06*
	2	15.7	10.6	14	11.8	7.1	13	18.7	4.5	14	14.6	8.8	14			
	3	15.3	9.6	14	16.5	5.4	13	17.8	6.7	13	17.9	7.3	14			
	4	16.2	10.8	14	14.0	7.3	13	17.4	7.9	14	16.2	10.2	14			
Watt/kg	1	2.8	0.6	14	2.4	0.4	13	2.3	0.5	14	2.6	0.6	14	0.03	ns	*0.06*
	2	2.8	0.8	14	2.4	0.3	13	2.5	0.4	14	2.9	0.7	14			
	3	2.8	0.6	14	2.5	0.4	13	2.7	0.5	13	2.9	0.7	14			
	4	2.9	0.6	14	2.6	0.5	13	2.7	0.5	14	3.0	0.8	14			
Weight (kg)	1	72.5	16.6	14	73.9	17.0	14	74.7	11.7	14	77.2	18.8	14	ns	ns	ns
	2	77.3	33.8	16	76.2	17.4	17	74.2	11.9	16	73.2	17.2	19			
	3	67.1	24.4	15	73.8	16.3	15	73.2	12.3	13	73.6	17.6	17			
	4	72.0	16.6	14	73.3	17.6	14	73.8	11.7	14	77.0	17.7	14			

Table S2. Acute and chronic changes in circulating myokines chronically or following a maximal exhaustion test.

Metabolite (μM)	Timepoint	Visit	Control			Light			Moderate			High			GLMER (group*time)	GLMER (group)	GLMER (time)
			MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N			
BDNF pg/mL	Pre	1	7749.4	2989.3	14	9246.8	1587.3	14	9991.8	1894.9	14	9822.0	1911.8	14	0.02	0.03	ns
		2	9370.6	2355.0	16	9304.8	2555.2	17	9152.1	1403.8	16	9429.6	1884.5	19			
		3	8930.2	2267.5	13	9623.5	2044.6	14	9466.5	2242.7	13	9486.3	1404.2	16			
		4	9434.1	2449.4	14	8443.6	2559.3	14	8894.1	2480.1	14	9507.7	1785.4	14			
BDNF pg/mL	Post	1	7982.3	3250.3	15	9235.4	2277.0	14	8905.5	2235.6	13	8868.8	1852.7	15	ns	ns	ns
		2	8892.8	3300.4	12	8945.5	2201.1	13	9769.4	2330.8	13	7944.0	1732.2	16			
		3	7229.8	2812.9	10	8307.4	1798.6	12	9355.4	2804.3	10	8408.9	2157.5	14			
		4	7644.2	3004.7	11	8538.8	2807.5	11	9046.9	2464.4	10	8471.8	1796.8	11			
IL1β pg/mL	Pre	1	0.11	0.08	5	0.15	0.10	6	0.09	0.06	8	0.19	0.14	6	0.04	ns	ns
		2	0.08	0.07	6	0.09	0.05	9	0.07	0.05	9	0.14	0.12	10			
		3	0.09	0.07	2	0.09	0.08	8	0.07	0.05	7	0.19	0.15	9			
		4	0.06	0.06	5	0.12	0.03	6	0.11	0.07	8	0.20	0.16	6			
IL1β pg/mL	Post	1	0.10	0.08	8	0.09	0.10	7	0.07	0.05	8	0.12	0.15	6	ns	ns	ns
		2	0.07	0.09	3	0.11	0.06	4	0.05	0.04	6	0.14	0.14	8			
		3	0.03	0.02	3	0.14	0.12	5	0.06	0.03	5	0.17	0.13	7			
		4	0.04	0.04	2	0.09	0.07	6	0.05	0.03	7	0.13	0.14	4			
IL6 pg/mL	Pre	1	0.58	0.58	5	0.86	0.66	9	0.75	0.44	9	0.82	0.72	7	ns	ns	ns
		2	0.61	0.51	7	0.70	0.65	13	0.79	0.59	13	1.02	0.84	14			
		3	0.61	0.61	7	0.51	0.36	11	0.60	0.39	9	0.97	0.78	12			
		4	0.53	0.31	5	0.76	0.41	9	0.55	0.28	9	1.36	0.88	7			
IL6 pg/mL	Post	1	0.77	0.72	15	1.24	0.55	11	1.20	1.00	13	0.91	0.83	12	ns	ns	ns
		2	0.57	0.34	10	1.09	0.82	9	0.76	0.41	11	1.49	1.05	10			
		3	0.58	0.42	8	0.83	0.62	9	0.77	0.52	8	1.16	0.87	12			
		4	0.86	0.41	7	1.00	0.64	10	0.78	0.37	10	1.03	0.82	9			
IL8 pg/mL	Pre	1	10.50	5.44	14	12.62	5.01	14	10.23	5.02	14	10.19	4.52	13	ns	ns	ns
		2	11.36	4.48	16	10.11	4.19	17	9.58	4.41	15	10.94	4.48	19			
		3	10.74	3.74	13	12.62	5.13	15	9.25	4.70	13	10.81	4.84	15			
		4	10.76	4.45	14	12.24	5.58	14	9.72	4.99	14	11.04	4.92	13			
IL8 pg/mL	Post	1	9.64	4.29	15	12.36	4.75	14	9.55	5.22	14	10.09	3.51	15	ns	ns	ns
		2	11.22	5.68	12	11.38	3.83	13	9.51	4.46	13	11.72	4.76	15			
		3	10.43	4.68	10	12.64	5.78	12	8.43	5.08	10	12.20	5.82	12			
		4	8.96	4.29	10	12.93	4.67	11	8.40	4.24	11	10.81	6.18	10			
TNFα pg/mL	Pre	1	0.31	0.28	12	0.58	0.45	12	0.40	0.23	14	0.49	0.31	10	ns	ns	ns

		2	0.30	0.27	12	0.40	0.32	17	0.34	0.16	13	0.44	0.38	16		
		3	0.26	0.25	9	0.41	0.32	13	0.33	0.15	12	0.52	0.31	14		
		4	0.27	0.25	12	0.48	0.30	12	0.33	0.23	14	0.59	0.34	10		
TNFα pg/mL	Post	1	0.31	0.28	14	0.45	0.47	13	0.28	0.27	12	0.37	0.33	12	ns	ns	ns
		2	0.20	0.22	9	0.39	0.40	13	0.29	0.13	11	0.49	0.47	14		
		3	0.22	0.17	8	0.43	0.40	12	0.32	0.30	9	0.46	0.36	12		
		4	0.26	0.21	10	0.55	0.39	11	0.28	0.14	11	0.56	0.53	7		
LBP µg/mL	Pre	1	16.5	6.4	13	15.6	6.1	14	14.0	7.9	14	13.7	5.8	14	ns	0.048	<0.01
		2	16.2	7.5	15	14.7	6.7	16	13.2	7.4	15	13.5	6.0	18			
		3	15.4	6.2	14	14.7	4.7	15	12.7	6.5	13	13.9	4.8	17			
		4	13.8	5.2	13	13.7	4.1	14	10.9	4.6	14	13.5	5.2	14			
LBP µg/mL	Post	1	16.8	7.9	14	14.9	6.0	14	11.8	7.8	13	12.3	5.3	16	ns	ns	ns
		2	14.9	5.6	12	15.2	6.8	13	14.0	6.3	13	13.2	6.2	15			
		3	15.6	6.7	10	15.4	4.8	12	13.9	8.6	10	12.9	5.8	14			
		4	15.3	6.7	11	14.8	5.6	11	12.6	6.3	11	12.1	3.9	10			
sCD14 µg/mL	Pre	1	1.65	0.53	14	1.56	0.26	14	1.79	0.59	13	1.45	0.49	14	ns	ns	0.02
		2	1.41	0.43	16	1.45	0.44	17	1.48	0.38	15	1.47	0.31	18			
		3	1.42	0.29	14	1.51	0.24	15	1.65	0.36	13	1.38	0.35	17			
		4	1.44	0.29	14	1.51	0.35	14	1.53	0.38	13	1.49	0.42	14			
sCD14 µg/mL	Post	1	1.37	0.59	15	1.52	0.35	14	1.57	0.54	13	1.29	0.43	16	ns	ns	ns
		2	1.39	0.35	12	1.38	0.38	12	1.56	0.44	13	1.39	0.35	15			
		3	1.47	0.42	11	1.43	0.48	12	1.84	0.46	10	1.38	0.28	14			
		4	1.50	0.38	11	1.66	0.46	11	1.48	0.48	11	1.33	0.38	10			

Table S3. Summary changes in acute and chronic changes in circulating amino acid and related-host and -microbial metabolites following exercise.

Metabolite (μM)	Timepoint	Visit	Control			Light			Moderate			High			GLMER (group*time)	GLMER (group)	GLMER (time)
			MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N			
3HK*100/KYN	Pre	1	2.87	1.46	13	3.40	1.88	13	3.44	1.19	13	3.89	1.20	14	ns	ns	ns
		2	3.17	1.20	13	3.76	1.91	14	3.49	1.14	14	4.47	1.05	14			
		3	3.00	1.87	13	3.39	2.02	14	3.85	2.04	13	4.16	1.59	14			
		4	2.74	1.70	13	3.55	1.72	13	3.62	1.37	13	3.91	1.09	14			
	Post	1	3.28	1.82	11	4.29	2.41	10	3.78	1.28	11	3.55	0.82	11			
		2	3.19	1.62	9	3.33	1.72	10	3.75	0.70	11	4.03	1.05	11			
		3	3.42	1.71	10	3.50	2.17	11	3.76	1.63	10	4.00	1.01	11			
		4	4.14	1.68	11	3.67	2.13	11	3.95	1.24	11	3.58	1.48	10			
Cinnamoyle glycine	Pre	1	0.09	0.08	13	0.14	0.11	14	0.09	0.09	14	0.11	0.08	14	ns	ns	ns
		2	0.12	0.16	13	0.14	0.11	14	0.07	0.05	14	0.10	0.04	14			
		3	0.12	0.15	12	0.14	0.17	14	0.09	0.07	13	0.12	0.11	14			
		4	0.15	0.13	13	0.15	0.11	14	0.08	0.08	14	0.15	0.16	14			
	Post	1	0.12	0.11	11	0.16	0.07	9	0.09	0.09	11	0.16	0.14	11			
		2	0.12	0.10	10	0.20	0.20	9	0.09	0.07	11	0.11	0.05	11			
		3	0.17	0.18	10	0.16	0.11	11	0.12	0.12	10	0.15	0.11	11			
		4	0.17	0.15	11	0.18	0.12	11	0.09	0.07	11	0.21	0.15	10			
Hippuric acid	Pre	1	4.69	2.92	13	6.73	4.25	14	4.78	3.70	14	6.61	4.15	14	ns	ns	ns
		2	6.69	8.83	13	7.40	7.04	14	6.03	3.41	14	8.12	10.10	14			
		3	4.47	3.01	13	6.23	6.39	14	6.35	6.13	13	5.72	3.19	14			
		4	5.50	3.94	13	7.87	6.34	14	6.38	5.19	14	5.21	1.80	14			
	Post	1	3.74	1.90	10	5.01	4.30	10	7.92	8.99	11	6.04	4.13	11			
		2	6.28	5.57	10	7.08	3.24	10	4.29	2.55	11	8.52	10.58	11			

		3	5.37	3.11	10	4.59	2.83	11	5.11	4.08	10	8.58	7.01	11		
		4	4.92	3.65	11	6.69	5.71	11	5.03	3.48	11	4.80	2.86	10		
Histidine	Pre	1	10.32	2.10	13	11.93	3.25	13	9.64	2.22	14	9.83	2.38	14	ns	ns	*0.06*
		2	10.70	3.39	13	9.52	2.65	14	10.12	2.16	14	9.80	2.16	14		
		3	9.45	3.20	13	9.93	3.04	14	10.41	3.00	13	10.41	2.04	14		
		4	10.09	2.99	13	9.36	3.20	14	10.15	3.35	14	9.20	3.30	14		
	Post	1	10.21	2.91	11	9.35	3.21	10	8.63	3.04	11	10.28	3.16	11		
		2	10.36	3.95	10	9.75	3.82	10	9.96	1.31	11	10.03	3.58	11		
		3	8.61	2.36	10	9.92	2.66	11	9.61	2.05	10	9.88	2.72	11		
		4	10.04	3.13	11	11.20	3.97	11	9.60	3.17	11	10.33	2.55	10		
Hydroxykynurenine	Pre	1	0.01	0.01	13	0.01	0.01	12	0.01	0.01	14	0.02	0.01	14	ns	**0.01**	ns
		2	0.01	0.01	13	0.01	0.01	14	0.02	0.01	14	0.02	0.01	14		
		3	0.01	0.01	13	0.01	0.01	14	0.02	0.01	13	0.01	0.01	14		
		4	0.01	0.01	13	0.01	0.01	12	0.01	0.00	14	0.01	0.01	14		
	Post	1	0.01	0.01	11	0.01	0.01	10	0.02	0.01	11	0.02	0.01	11		
		2	0.01	0.01	10	0.01	0.01	10	0.02	0.01	11	0.02	0.01	11		
		3	0.01	0.01	10	0.01	0.01	11	0.01	0.01	10	0.02	0.01	11		
		4	0.01	0.01	11	0.01	0.01	11	0.01	0.01	11	0.01	0.01	10		
Indole-3-Acetic Acid Methyl ester	Pre	1	0.01	0.01	13	0.01	0.01	14	0.01	0.01	13	0.02	0.01	14	ns	ns	ns
		2	0.01	0.01	13	0.01	0.01	14	0.02	0.01	14	0.02	0.01	14		
		3	0.01	0.01	13	0.01	0.01	14	0.02	0.01	13	0.02	0.01	14		
		4	0.01	0.01	13	0.01	0.01	14	0.01	0.01	13	0.02	0.01	14		
	Post	1	0.01	0.01	11	0.01	0.01	10	0.02	0.00	11	0.01	0.00	11		
		2	0.01	0.01	10	0.01	0.01	10	0.02	0.00	11	0.02	0.01	11		
		3	0.01	0.01	10	0.01	0.01	11	0.02	0.01	10	0.02	0.01	11		
		4	0.01	0.01	11	0.01	0.01	11	0.02	0.00	11	0.02	0.01	10		
Indole-3-acetic acid	Pre	1	8.29	3.50	12	14.68	6.86	14	11.15	5.00	14	13.19	7.24	13	ns	*0.05*	ns
		2	8.12	5.10	13	14.32	6.65	14	11.74	5.28	14	13.66	7.93	14		
		3	9.62	7.57	13	11.63	4.60	14	14.11	5.93	13	13.79	8.72	14		

		4	9.89	6.63	12	13.65	7.71	14	10.50	4.42	14	11.60	6.93	13		
	Post	1	9.37	5.41	10	11.95	4.86	10	9.66	4.96	11	11.01	5.20	11		
		2	6.90	4.90	10	13.52	5.79	10	11.46	3.44	11	11.13	6.35	11		
		3	9.93	6.24	10	11.79	3.42	11	11.57	2.84	10	10.85	6.54	11		
		4	8.68	5.24	11	13.53	5.35	11	9.60	2.79	11	11.03	5.81	10		
Indole-3-acryolylglycine	Pre	1	0.02	0.02	13	0.02	0.01	14	0.02	0.01	14	0.02	0.01	14	ns	ns	ns
		2	0.02	0.02	13	0.02	0.01	13	0.02	0.01	14	0.03	0.02	14		
		3	0.02	0.01	13	0.02	0.02	14	0.01	0.01	13	0.02	0.01	13		
		4	0.02	0.01	13	0.02	0.01	14	0.02	0.01	14	0.02	0.01	14		
	Post	1	0.02	0.01	11	0.02	0.01	9	0.02	0.01	11	0.03	0.02	10		
		2	0.02	0.01	10	0.03	0.02	10	0.01	0.01	11	0.02	0.01	11		
		3	0.02	0.02	10	0.02	0.01	11	0.01	0.01	10	0.03	0.02	11		
		4	0.02	0.01	11	0.02	0.01	10	0.02	0.01	11	0.03	0.02	9		
Indole-3-lactic acid	Pre	1	2.32	0.72	12	2.36	0.75	14	2.39	0.69	14	2.61	0.75	13	ns	ns	ns
		2	2.49	1.24	13	2.20	0.80	14	2.63	0.67	14	2.96	0.94	14		
		3	2.31	1.13	13	2.52	0.98	14	3.14	1.64	13	2.70	0.50	14		
		4	2.39	0.81	12	2.48	1.51	14	2.90	0.74	14	2.60	0.58	13		
	Post	1	2.36	1.18	11	2.20	0.78	10	2.48	0.69	11	2.80	0.53	11		
		2	2.96	1.19	10	2.24	0.74	10	2.97	0.72	11	3.00	0.54	11		
		3	2.36	1.17	10	2.19	0.56	11	3.26	1.33	10	2.90	0.71	11		
		4	2.59	0.98	11	2.66	1.21	11	3.04	0.70	11	3.35	1.26	10		
Indole-3-propionic acid	Pre	1	1.22	1.27	12	1.54	1.03	14	1.19	0.83	14	1.57	1.07	14	ns	ns	ns
		2	1.57	1.46	13	1.25	0.81	13	1.05	0.89	14	1.37	0.62	14		
		3	1.13	0.84	13	1.50	1.62	14	1.08	0.51	13	1.60	1.24	14		
		4	1.48	1.17	12	1.23	0.93	14	1.27	0.79	14	1.31	0.87	14		
	Post	1	1.18	0.95	11	1.45	1.12	10	0.95	0.44	11	1.67	1.30	11		
		2	1.35	1.00	10	1.60	1.08	10	0.87	0.56	11	1.18	0.58	11		
		3	0.96	0.68	10	1.09	0.96	11	1.05	0.66	10	1.72	1.37	11		
		4	1.34	1.00	11	1.29	1.05	11	1.14	0.44	11	1.77	1.57	10		

Indoxyl-sulfate	Pre	1	4.25	2.04	13	7.14	3.92	14	6.49	4.41	14	4.36	1.82	14	ns	ns	ns
		2	3.74	2.20	13	5.69	3.14	14	6.98	3.96	14	5.42	2.11	14			
		3	4.86	3.39	13	6.70	4.17	14	8.40	4.23	12	5.05	2.70	14			
		4	4.30	2.05	13	5.69	2.94	14	11.11	15.17	14	5.37	1.92	14			
	Post	1	3.46	2.20	11	5.21	2.96	10	5.62	3.70	11	4.27	1.73	11			
		2	2.84	1.52	10	5.01	2.49	10	7.38	5.49	11	4.73	2.38	11			
		3	3.73	2.32	10	5.59	3.75	11	7.08	2.53	9	4.36	2.48	11			
		4	4.71	2.46	11	5.48	2.77	11	5.96	2.27	10	5.22	2.09	10			
KYNA*100/KYN	Pre	1	42.13	33.57	13	66.30	59.65	14	32.27	31.19	13	32.68	30.67	14	ns	ns	ns
		2	47.09	44.09	13	60.56	54.61	14	25.43	22.13	14	32.09	29.36	14			
		3	49.82	44.45	13	51.56	39.71	14	41.01	38.76	13	34.05	28.18	14			
		4	41.99	36.92	13	58.76	46.84	14	34.62	28.86	13	40.53	53.23	14			
	Post	1	50.88	46.46	11	69.05	53.32	10	32.99	43.09	11	34.26	39.02	11			
		2	48.68	40.84	9	64.64	70.26	10	17.79	17.40	11	35.92	43.69	11			
		3	56.42	46.38	10	72.18	60.11	11	34.66	34.67	10	33.71	32.05	11			
		4	51.31	47.36	11	60.43	50.52	11	30.86	28.52	11	50.19	44.82	10			
KYNA*1000/TRP Breakdown	Pre	1	2.28	1.84	13	2.89	2.30	14	1.94	1.57	14	1.89	1.41	14	ns	ns	ns
		2	2.40	2.18	13	2.95	2.29	14	1.85	1.51	14	1.94	1.33	14			
		3	2.68	2.52	13	2.66	1.74	14	2.31	1.66	13	1.96	1.49	14			
		4	2.35	2.22	13	2.96	2.01	14	2.24	1.40	14	1.87	1.38	14			
	Post	1	2.57	2.03	11	2.87	2.38	10	1.44	1.08	11	1.98	1.62	11			
		2	2.69	2.29	10	3.03	2.26	10	1.44	1.39	11	2.00	1.69	11			
		3	2.75	1.81	10	2.29	1.76	10	1.81	1.41	10	2.08	1.75	11			
		4	2.51	2.23	11	2.95	2.29	11	1.82	1.63	11	2.75	2.33	10			
Kynurenic acid	Pre	1	0.13	0.10	13	0.16	0.12	14	0.10	0.08	14	0.10	0.08	14	ns	ns	ns
		2	0.14	0.12	13	0.15	0.10	14	0.10	0.08	14	0.11	0.07	14			
		3	0.13	0.12	13	0.14	0.08	14	0.13	0.09	13	0.10	0.08	14			
		4	0.13	0.12	13	0.15	0.09	14	0.12	0.08	14	0.09	0.06	14			
	Post	1	0.12	0.09	11	0.14	0.11	10	0.07	0.07	11	0.10	0.09	11			

		2	0.13	0.11	10	0.15	0.12	10	0.07	0.07	11	0.10	0.08	11		
		3	0.13	0.09	10	0.13	0.11	11	0.10	0.08	10	0.11	0.09	11		
		4	0.13	0.10	11	0.15	0.10	11	0.09	0.09	11	0.14	0.11	10		
L-Isoleucine	Pre	1	68.34	19.51	13	72.52	13.64	13	67.43	11.39	14	65.09	13.72	14	ns	ns	ns
		2	70.28	16.43	12	68.18	21.82	14	65.05	9.59	13	67.25	12.29	14		
		3	63.29	23.54	12	69.45	17.18	13	70.82	21.30	13	62.66	19.04	13		
		4	72.69	16.77	13	65.04	10.48	13	69.03	14.90	14	60.42	14.81	14		
	Post	1	53.40	13.33	10	63.35	19.71	10	57.30	21.36	11	56.09	11.48	11		
		2	55.20	19.97	10	53.30	13.13	10	56.11	7.53	11	54.15	10.32	11		
		3	55.78	17.79	10	49.48	12.00	11	58.30	17.68	10	55.65	14.20	11		
		4	61.99	21.98	11	61.14	15.89	11	56.83	12.28	11	54.21	13.26	10		
L-Kynurenine	Pre	1	0.35	0.13	13	0.36	0.19	14	0.35	0.17	14	0.40	0.15	13	ns	ns	ns
		2	0.36	0.15	13	0.35	0.18	14	0.45	0.15	14	0.45	0.17	14		
		3	0.32	0.14	13	0.39	0.19	14	0.41	0.20	12	0.38	0.18	14		
		4	0.35	0.10	13	0.38	0.23	14	0.39	0.15	14	0.35	0.13	13		
	Post	1	0.35	0.15	11	0.31	0.16	10	0.38	0.20	11	0.44	0.17	11		
		2	0.28	0.14	10	0.35	0.18	10	0.42	0.12	11	0.38	0.12	11		
		3	0.31	0.14	10	0.27	0.16	11	0.39	0.18	10	0.40	0.15	11		
		4	0.31	0.11	11	0.38	0.22	11	0.35	0.16	11	0.36	0.16	10		
L-Leucine	Pre	1	117.87	27.23	13	122.68	16.42	14	115.27	14.97	14	112.43	18.72	14	ns	ns	ns
		2	124.10	27.39	13	114.08	29.30	14	114.45	21.25	14	112.89	19.51	14		
		3	112.65	34.39	12	120.37	20.44	13	121.04	29.09	13	114.70	30.19	14		
		4	124.22	21.71	13	115.51	23.39	14	119.64	20.74	14	106.21	24.79	14		
	Post	1	105.77	31.23	11	107.28	24.47	10	103.08	20.82	10	102.35	15.55	11		
		2	100.30	26.91	10	96.28	19.66	10	101.62	8.02	11	98.31	19.80	11		
		3	100.51	24.18	10	90.09	14.05	11	105.66	23.00	10	97.78	17.42	11		
		4	105.71	28.47	11	105.39	20.60	11	102.93	14.31	11	94.71	17.61	10		
L-Methionine	Pre	1	23.07	6.40	13	26.02	6.71	14	23.21	4.55	14	22.86	3.84	14	ns	ns	ns
		2	26.30	7.82	13	24.40	7.72	14	23.54	7.31	14	21.12	4.68	14		

		3	23.75	6.92	13	27.58	7.76	14	25.05	7.59	13	22.14	8.36	14		
		4	25.03	7.30	13	24.19	4.95	14	24.83	6.21	14	18.24	5.59	14		
	Post	1	19.50	5.45	11	21.54	6.44	10	19.76	5.92	11	20.03	3.45	11		
		2	20.82	5.39	10	19.13	4.36	10	21.43	5.48	11	18.77	3.95	11		
		3	19.02	3.91	10	18.84	3.60	11	20.68	5.86	10	19.44	3.82	11		
		4	21.46	7.73	11	22.77	8.24	11	20.53	7.02	11	17.52	3.18	10		
L-Phenylalanine	Pre	1	53.76	10.40	13	58.69	7.16	14	59.03	7.96	14	57.36	5.94	14	ns	ns	ns
		2	55.81	10.00	13	55.17	10.10	14	55.73	9.51	13	54.70	8.32	14		
		3	50.88	9.91	13	58.70	4.69	14	56.55	9.84	13	56.40	11.11	14		
		4	59.17	10.25	13	53.19	4.96	14	57.41	9.52	14	51.40	8.52	14		
	Post	1	49.75	7.35	11	51.13	8.30	10	54.37	10.56	10	50.97	7.90	11		
		2	50.36	8.94	10	49.36	6.55	10	55.34	8.48	11	51.28	11.00	11		
		3	46.88	5.11	10	48.39	5.24	11	54.77	10.42	10	52.75	9.01	11		
		4	53.51	11.75	11	53.46	6.35	11	55.26	12.33	11	52.66	9.38	10		
L-Tryptophan	Pre	1	56.76	9.92	12	56.39	7.87	14	53.91	8.95	14	54.85	6.66	14	ns	ns	ns
		2	57.47	11.50	13	55.04	15.19	14	51.58	9.16	13	55.71	10.19	14		
		3	50.76	18.92	13	56.97	9.34	14	55.98	10.87	13	54.20	11.95	14		
		4	54.27	9.19	12	52.24	9.29	14	52.53	6.89	14	51.56	10.29	14		
	Post	1	49.95	7.91	11	53.60	11.71	10	50.11	12.56	11	51.70	7.99	11		
		2	51.30	9.48	10	47.07	9.39	10	50.35	11.01	11	50.38	6.51	11		
		3	46.09	9.62	10	46.58	6.50	11	54.47	8.95	10	52.48	8.53	11		
		4	52.06	13.18	11	53.24	8.85	11	50.55	7.98	11	51.02	11.63	9		
L-Tyrosine	Pre	1	58.29	17.29	13	65.66	14.21	14	59.74	8.80	14	60.34	13.05	14	ns	ns	ns
		2	60.21	14.15	12	63.42	16.71	14	57.47	11.88	13	58.00	12.25	14		
		3	55.10	18.46	13	67.04	12.15	13	58.71	12.26	13	59.24	16.58	14		
		4	61.55	14.54	13	61.59	14.30	14	62.35	13.54	14	54.18	13.90	14		
	Post	1	48.19	10.47	11	56.53	15.42	10	51.35	13.30	11	52.62	13.52	11		
		2	50.94	13.48	10	51.43	12.27	10	52.85	9.36	11	48.55	10.89	11		
		3	47.51	9.37	10	53.46	9.00	11	52.07	11.59	10	49.71	8.70	11		

		4	58.42	17.97	11	57.66	8.40	11	54.83	14.10	11	50.00	11.33	10		
L-valine	Pre	1	519.52	124.10	13	507.54	70.58	14	464.63	59.71	14	484.52	81.25	14	ns	ns	ns
		2	538.94	96.93	13	472.16	114.90	14	457.50	71.84	13	510.61	86.71	14			
		3	450.91	171.27	11	535.00	92.26	14	526.73	127.02	13	483.52	114.34	13			
		4	497.47	98.94	13	479.36	54.18	14	502.97	79.55	14	458.80	103.38	14			
	Post	1	450.74	122.91	11	442.44	111.03	10	399.35	118.70	11	444.27	83.84	11			
		2	435.61	108.67	10	407.81	97.12	10	438.81	65.45	11	445.22	122.57	11			
		3	423.06	120.28	10	403.29	62.27	11	445.89	100.13	10	421.76	96.15	11			
		4	477.78	138.28	11	458.82	81.10	11	441.05	76.33	11	423.52	93.44	10			
Norepinephrine	Pre	1	0.09	0.04	13	0.11	0.03	14	0.10	0.03	14	0.09	0.02	14	ns	ns	ns
		2	0.09	0.03	13	0.11	0.04	14	0.11	0.03	14	0.10	0.03	14			
		3	0.09	0.04	12	0.11	0.04	14	0.11	0.03	13	0.10	0.03	14			
		4	0.10	0.04	13	0.10	0.03	14	0.11	0.03	14	0.09	0.03	14			
	Post	1	0.09	0.04	11	0.09	0.03	10	0.09	0.02	10	0.09	0.03	11			
		2	0.09	0.03	10	0.10	0.04	10	0.10	0.03	11	0.09	0.03	11			
		3	0.11	0.05	10	0.10	0.03	11	0.10	0.03	10	0.10	0.02	11			
		4	0.10	0.02	10	0.10	0.03	11	0.12	0.04	11	0.11	0.04	10			
Ornithine	Pre	1	12.96	3.79	13	15.26	5.06	13	14.44	2.67	14	13.90	5.62	14	ns	ns	ns
		2	14.86	4.50	13	14.17	6.09	14	13.12	3.61	14	14.34	5.00	14			
		3	12.94	5.25	13	14.38	3.91	14	13.14	2.91	13	14.11	4.34	14			
		4	14.68	4.36	13	12.44	4.11	13	13.36	2.96	14	12.88	4.57	14			
	Post	1	10.40	4.46	11	10.60	5.05	10	9.80	3.12	11	10.36	3.09	11			
		2	9.83	4.49	10	9.89	3.84	10	10.85	2.46	11	9.66	3.72	11			
		3	9.02	4.37	10	9.43	3.39	11	10.10	3.28	10	10.20	3.93	11			
		4	10.92	3.94	11	12.69	4.41	11	9.95	2.46	11	8.78	3.98	10			
p-cresol glucuronide	Pre	1	0.06	0.07	13	0.06	0.07	14	0.05	0.04	14	0.05	0.05	14	ns	ns	ns
		2	0.04	0.05	13	0.09	0.18	14	0.08	0.05	14	0.04	0.03	14			
		3	0.08	0.10	13	0.06	0.06	14	0.07	0.06	12	0.04	0.04	14			
		4	0.08	0.12	13	0.05	0.05	14	0.08	0.07	14	0.04	0.02	14			

	Post	1	0.04	0.05	11	0.05	0.05	10	0.04	0.03	11	0.05	0.04	11		
		2	0.03	0.04	10	0.06	0.07	10	0.09	0.07	11	0.04	0.02	11		
		3	0.06	0.05	10	0.05	0.06	11	0.09	0.08	9	0.03	0.02	11		
		4	0.05	0.04	11	0.08	0.09	11	0.07	0.04	10	0.06	0.05	10		
p-cresol sulfate	Pre	1	561.96	581.11	11	688.79	517.56	14	767.16	377.15	13	627.80	393.18	14	ns	ns	ns
		2	492.87	442.70	13	498.68	321.56	13	743.68	368.92	13	612.97	389.31	14		
		3	573.45	324.98	12	666.26	480.68	14	951.59	451.23	12	659.70	495.16	14		
		4	434.36	241.16	11	526.07	420.68	14	776.42	461.83	13	580.38	338.65	14		
	Post	1	430.64	324.54	11	648.79	505.64	10	574.28	348.51	11	604.61	322.33	11		
		2	331.75	341.33	10	586.17	466.17	10	700.59	300.94	10	630.33	419.78	11		
		3	587.25	362.03	10	614.34	434.27	11	1077.30	807.75	9	509.43	393.95	11		
		4	441.33	333.15	11	575.14	509.67	10	791.19	475.93	10	760.94	540.71	10		
Phenylalanine/Tyrosine ratio	Pre	1	0.96	0.16	13	0.92	0.13	14	1.00	0.16	14	0.94	0.14	13	ns	ns	ns
		2	0.90	0.16	13	0.90	0.17	14	0.98	0.20	14	0.97	0.18	14		
		3	0.97	0.19	13	0.87	0.16	14	0.98	0.18	13	0.99	0.20	14		
		4	0.98	0.17	13	0.90	0.17	14	0.95	0.20	14	0.99	0.20	13		
	Post	1	1.05	0.15	11	0.94	0.19	10	0.99	0.15	11	1.01	0.20	11		
		2	1.02	0.19	10	0.99	0.15	10	1.06	0.17	11	1.07	0.18	11		
		3	1.01	0.18	10	0.93	0.16	11	1.06	0.15	10	1.07	0.15	11		
		4	0.95	0.15	11	0.93	0.07	11	1.03	0.21	11	1.08	0.20	10		
Phenylacetyl-l-glutamine	Pre	1	1.73	1.30	13	1.88	1.12	14	1.90	0.95	14	1.96	1.28	14	ns	ns	ns
		2	1.32	0.98	13	1.85	1.32	14	2.19	0.59	14	1.88	1.00	13		
		3	1.84	1.33	13	1.83	1.18	14	2.59	1.13	13	1.79	1.35	14		
		4	1.45	0.63	13	1.76	1.30	14	2.31	0.91	14	2.13	1.12	14		
	Post	1	1.52	0.96	11	2.09	1.54	10	1.69	1.01	11	1.59	0.89	11		
		2	1.01	0.89	10	1.98	1.61	10	2.07	0.40	11	1.78	1.14	11		
		3	1.25	0.73	10	1.62	1.03	11	2.59	1.12	10	1.48	0.99	11		
		4	1.50	0.80	11	2.13	1.58	11	2.25	0.93	11	2.04	1.03	10		
Phenyllactic acid	Pre	1	0.67	0.39	13	0.73	0.63	14	0.61	0.33	14	0.61	0.31	14	ns	ns	ns

		2	0.71	0.47	13	0.67	0.56	14	0.56	0.24	14	0.49	0.20	14		
		3	0.85	0.64	13	1.12	1.08	14	0.75	0.52	12	0.57	0.24	14		
		4	0.77	0.66	12	0.51	0.27	14	0.95	0.77	14	0.56	0.27	14		
	Post	1	0.66	0.28	11	0.65	0.41	10	0.59	0.37	11	0.71	0.25	11		
		2	0.89	0.60	10	0.68	0.35	10	0.78	0.43	11	0.61	0.19	11		
		3	0.93	0.81	10	0.91	0.60	11	0.98	1.14	9	0.69	0.25	11		
		4	0.70	0.24	11	1.05	0.64	11	0.92	0.80	10	0.94	0.68	10		
LNAA1 (Tyr + Val + Phe + Leu + Iso)	Pre	1	817.78	176.81	13	826.98	99.55	14	766.10	67.99	14	779.75	114.81	14	ns	ns	ns
		2	858.12	162.60	13	773.01	182.66	14	745.51	95.36	13	803.45	121.47	14		
		3	719.73	243.21	11	862.28	145.18	14	833.85	192.09	13	769.89	167.07	13		
		4	815.10	149.06	13	778.35	96.58	14	811.40	123.02	14	731.01	151.77	14		
	Post	1	713.72	169.85	11	720.72	168.05	10	697.76	133.63	10	706.29	121.48	11		
		2	629.46	263.41	11	658.18	139.27	10	704.73	86.18	11	697.51	157.18	11		
		3	673.73	164.15	10	644.70	90.44	11	716.69	151.91	10	677.64	136.01	11		
		4	757.41	201.64	11	736.47	115.90	11	710.91	110.41	11	675.10	130.45	10		
LNAA2 (Trp + Val + Phe + Leu + Iso)	Pre	1	817.07	172.86	13	817.71	93.47	14	760.27	76.36	14	774.25	109.85	14	ns	ns	ns
		2	850.41	151.75	13	764.63	179.99	14	778.81	172.58	14	801.16	121.45	14		
		3	813.22	329.47	13	849.56	142.77	14	831.11	192.31	13	800.94	206.75	14		
		4	810.51	150.73	13	769.00	97.15	14	801.58	117.60	14	728.39	147.94	14		
	Post	1	715.48	169.72	11	717.79	164.52	10	696.19	134.45	10	705.37	115.13	11		
		2	692.76	162.48	10	653.83	135.53	10	702.23	88.69	11	699.34	154.21	11		
		3	672.32	166.22	10	637.82	89.77	11	719.09	147.59	10	680.41	133.23	11		
		4	751.05	198.85	11	732.05	119.51	11	706.63	107.68	11	679.86	129.28	10		
Putrescine	Pre	1	0.13	0.05	13	0.13	0.04	14	0.16	0.06	14	0.13	0.05	14	ns	ns	ns
		2	0.13	0.05	13	0.12	0.04	14	0.14	0.05	14	0.13	0.05	14		
		3	0.13	0.06	13	0.12	0.04	14	0.15	0.06	13	0.14	0.05	13		
		4	0.14	0.05	13	0.12	0.03	14	0.15	0.05	14	0.14	0.05	14		
	Post	1	0.14	0.05	10	0.12	0.03	10	0.15	0.05	11	0.17	0.04	11		
		2	0.15	0.07	10	0.13	0.05	10	0.16	0.06	11	0.14	0.06	11		

		3	0.12	0.05	10	0.12	0.04	11	0.16	0.06	10	0.15	0.05	10		
		4	0.16	0.06	11	0.14	0.04	11	0.14	0.04	10	0.15	0.07	10		
Serotonin	Pre	1	11.73	5.44	13	12.74	3.37	14	12.64	3.92	14	12.99	5.53	13	ns	ns	ns
		2	12.52	4.57	13	12.49	3.65	14	12.73	4.79	14	13.82	5.72	14		
		3	11.36	3.84	13	12.77	5.05	14	13.94	5.83	13	13.12	4.60	14		
		4	13.33	4.60	13	11.25	4.31	14	12.95	4.79	14	12.30	4.83	13		
	Post	1	11.35	3.57	11	12.94	4.43	10	13.13	5.97	11	13.36	6.34	11		
		2	12.23	5.39	10	14.51	5.60	10	12.03	4.70	11	14.16	5.84	11		
		3	11.26	3.94	10	12.56	4.09	11	13.21	5.23	10	14.44	6.11	11		
		4	12.30	5.70	11	12.23	6.09	11	11.65	4.83	11	17.65	6.95	10		
TMA	Pre	1	1.31	0.39	13	1.23	0.38	13	1.30	0.42	14	1.28	0.40	13	ns	ns	ns
		2	1.31	0.28	13	1.31	0.34	14	1.50	0.50	14	1.43	0.44	14		
		3	1.21	0.33	12	1.22	0.35	14	1.47	0.43	13	1.41	0.33	14		
		4	1.27	0.27	13	1.17	0.29	14	1.27	0.38	14	1.39	0.48	13		
	Post	1	1.20	0.50	10	1.21	0.41	10	1.19	0.29	11	1.22	0.29	11		
		2	1.28	0.40	10	1.28	0.40	10	1.42	0.38	11	1.21	0.42	10		
		3	1.21	0.33	10	1.05	0.31	11	1.26	0.34	10	1.23	0.35	11		
		4	1.24	0.40	11	1.29	0.36	11	1.22	0.32	11	1.26	0.45	10		
TMAO	Pre	1	0.81	1.09	12	0.59	0.46	14	0.91	0.97	14	0.69	0.52	14	ns	ns	ns
		2	0.44	0.21	13	0.68	0.58	13	0.93	1.07	14	0.78	0.46	14		
		3	1.19	1.80	13	1.03	0.80	14	1.09	0.90	13	1.04	1.01	14		
		4	0.62	0.42	12	1.05	1.09	14	0.95	1.37	14	1.03	0.75	14		
	Post	1	0.61	0.54	11	0.50	0.38	10	0.81	0.68	11	0.59	0.33	11		
		2	0.39	0.19	10	0.64	0.62	10	0.84	0.59	11	0.79	0.38	11		
		3	0.88	1.32	10	0.93	0.75	11	0.90	0.55	10	0.91	0.60	11		
		4	1.20	1.53	11	0.65	0.55	11	1.10	1.28	11	0.89	0.67	10		
Tryptophan Index (Trp*100/LNAA1)	Pre	1	6.90	1.08	12	6.89	1.13	14	7.03	1.00	14	6.87	0.94	13	ns	ns	ns
		2	6.81	1.51	13	7.23	1.67	14	7.05	1.27	14	6.99	1.09	14		
		3	6.38	1.42	13	6.70	1.16	14	6.82	0.97	13	6.93	1.50	14		

		4	6.89	0.79	12	6.72	0.93	14	6.54	0.86	14	7.07	1.19	13		
	Post	1	7.21	1.36	11	7.53	1.10	10	7.86	1.40	11	7.12	0.82	10		
		2	7.63	1.63	10	6.91	1.12	9	7.10	1.06	11	7.44	1.32	11		
		3	7.04	1.67	10	7.29	0.98	11	7.44	1.42	9	7.87	1.20	11		
		4	7.00	1.14	11	7.27	0.88	11	7.18	1.08	11	7.72	1.60	9		
Tyramine	Pre	1	0.14	0.03	13	0.16	0.04	14	0.15	0.03	14	0.14	0.03	14	ns	ns	ns
		2	0.15	0.03	12	0.14	0.04	14	0.13	0.04	13	0.13	0.04	14		
		3	0.14	0.04	13	0.16	0.04	14	0.14	0.03	13	0.14	0.03	13		
		4	0.16	0.04	13	0.14	0.04	14	0.15	0.04	14	0.12	0.04	14		
	Post	1	0.10	0.03	11	0.00	0.04	10	0.12	0.04	11	0.14	0.04	11		
		2	0.12	0.03	10	0.11	0.03	10	0.11	0.03	11	0.13	0.04	11		
		3	0.13	0.03	10	0.13	0.03	11	0.13	0.03	10	0.12	0.03	10		
		4	0.14	0.04	11	0.13	0.03	11	0.13	0.04	11	0.13	0.07	10		
Tyrosine Index (Tyr*100/LNAA2)	Pre	1	7.24	1.87	13	8.04	1.55	14	7.97	1.59	14	7.84	1.63	14	ns	ns	ns
		2	7.59	1.78	13	8.42	1.82	14	7.84	1.49	14	7.30	1.40	14		
		3	7.17	1.71	13	8.30	1.80	14	7.19	1.18	13	7.54	1.69	14		
		4	7.67	1.50	13	8.04	1.83	14	7.79	1.34	14	7.43	1.19	14		
	Post	1	6.94	1.53	11	7.88	1.36	10	8.01	1.33	11	7.42	1.29	11		
		2	7.41	1.16	10	7.94	1.34	10	7.53	0.92	11	7.08	1.52	11		
		3	7.34	1.80	10	8.47	1.55	11	7.32	1.31	10	7.36	0.81	11		
		4	7.91	1.84	11	7.99	1.34	11	7.80	1.72	11	7.34	0.86	11		
3,4-hydroxyphenyl lactic acid	Pre	1	2.52	1.26	12	3.25	3.58	14	2.24	0.88	14	2.47	1.22	14	ns	ns	ns
		2	3.14	2.78	13	3.16	2.22	14	2.49	0.87	14	2.11	0.84	14		
		3	3.39	2.69	13	4.48	3.80	14	2.98	1.43	12	2.40	0.67	14		
		4	3.09	2.63	12	2.59	1.40	14	3.59	3.14	14	2.46	0.71	14		
	Post	1	3.31	1.41	11	3.31	2.23	10	2.95	1.18	11	3.35	1.37	11		
		2	4.46	3.05	10	3.66	1.47	10	3.40	1.25	11	2.85	1.44	11		
		3	4.59	4.36	10	4.47	2.95	11	4.69	4.49	9	3.28	1.07	11		
		4	3.39	1.34	11	4.45	2.57	11	4.32	2.94	10	4.37	2.78	10		

3-methoxy-p-tyramine	Pre	1	0.12	0.02	13	0.14	0.04	14	0.14	0.03	13	0.12	0.03	14	ns	ns	ns
		2	0.13	0.03	13	0.14	0.03	14	0.12	0.02	13	0.13	0.03	14			
		3	0.12	0.03	13	0.13	0.03	12	0.12	0.02	12	0.13	0.03	14			
		4	0.13	0.03	13	0.13	0.04	14	0.12	0.02	13	0.12	0.03	14			
	Post	1	0.13	0.02	11	0.14	0.03	10	0.11	0.04	11	0.12	0.02	11			
		2	0.12	0.03	10	0.14	0.03	10	0.12	0.02	10	0.13	0.03	11			
		3	0.13	0.02	10	0.13	0.03	11	0.12	0.03	9	0.13	0.03	11			
		4	0.14	0.02	11	0.13	0.03	11	0.13	0.03	10	0.14	0.03	10			
5-hydroxyindole-3-acetic acid	Pre	1	0.39	0.29	13	0.38	0.13	14	0.36	0.16	14	0.78	0.63	14	ns	ns	ns
		2	0.70	0.80	13	0.46	0.38	14	0.45	0.41	14	0.49	0.53	14			
		3	0.35	0.22	13	0.62	0.55	14	0.43	0.39	13	0.43	0.30	14			
		4	0.42	0.45	13	0.47	0.32	14	0.40	0.32	14	0.47	0.42	14			
	Post	1	0.29	0.10	11	0.41	0.34	10	0.34	0.19	11	0.48	0.22	11			
		2	0.31	0.11	10	0.36	0.22	10	0.29	0.08	11	0.35	0.10	11			
		3	0.30	0.10	10	0.44	0.33	11	0.29	0.07	10	0.38	0.20	11			
		4	0.29	0.10	11	0.45	0.24	11	0.32	0.11	11	0.43	0.16	10			
Xanthurenic acid	Pre	1	0.32	0.07	13	0.35	0.07	13	0.34	0.07	13	0.33	0.05	14	ns	ns	ns
		2	0.32	0.08	13	0.32	0.10	14	0.33	0.12	14	0.34	0.06	14			
		3	0.28	0.11	12	0.33	0.06	14	0.35	0.08	13	0.33	0.06	14			
		4	0.33	0.08	13	0.33	0.07	13	0.33	0.06	13	0.28	0.07	14			
	Post	1	0.30	0.06	11	0.30	0.09	10	0.31	0.08	10	0.32	0.05	11			
		2	0.30	0.06	10	0.29	0.06	10	0.30	0.06	11	0.31	0.05	11			
		3	0.27	0.06	10	0.26	0.03	11	0.34	0.08	10	0.31	0.05	11			
		4	0.30	0.08	11	0.31	0.07	11	0.29	0.07	11	0.31	0.05	10			

Table S4. Summary changes in acute and chronic changes in circulating host and microbial SCFAs production.

Metabolite (μM)	Timepoint	Visit	Control			Light			Moderate			High			GLMER (group*time)	GLMER (group)	GLMER (time)
			MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N			
2-methylbutyric acid	Pre	1	0.44	0.22	14	0.45	0.20	14	0.45	0.22	14	0.44	0.24	14	ns	ns	ns
		2	0.54	0.26	14	0.36	0.11	14	0.55	0.35	14	0.45	0.18	14			
		3	0.41	0.18	14	0.39	0.20	14	0.42	0.15	13	0.42	0.27	14			
		4	0.45	0.21	14	0.43	0.19	14	0.44	0.25	14	0.43	0.18	14			
	Post	1	1	0.26	11	0.51	0.29	10	0.56	0.35	11	0.41	0.13	11			
		2	0.42	0.09	10	0.49	0.21	10	0.48	0.18	11	0.39	0.10	11			
		3	0.45	0.29	10	0.35	0.15	11	0.52	0.18	10	0.42	0.12	11			
		4	0.48	0.22	11	0.39	0.17	11	0.40	0.09	11	0.40	0.13	10			
acetic acid	Pre	1	33.34	17.66	14	32.57	19.53	14	31.97	11.41	14	33.71	17.79	14	ns	ns	ns
		2	40.03	22.18	14	37.39	19.01	14	38.52	11.90	14	32.85	16.20	13			
		3	36.52	28.99	14	25.38	11.40	14	39.78	20.27	12	47.00	31.26	14			
		4	47.77	29.84	14	40.34	28.26	14	40.92	22.37	14	39.01	22.72	14			
	Post	1	32.22	12.80	11	42.45	28.79	10	36.43	22.07	11	29.91	9.43	11			
		2	37.24	24.34	10	34.54	21.34	10	26.68	8.76	11	33.49	12.07	11			
		3	29.30	8.98	10	27.52	8.72	11	40.15	19.39	10	37.40	17.04	11			
		4	35.96	25.76	11	29.30	24.85	11	34.61	20.79	11	39.85	26.81	10			
butyric acid	Pre	1	1.17	0.68	14	1.35	0.85	14	1.26	0.56	13	1.44	0.60	14	ns	ns	**0.04**
		2	1.60	0.98	14	1.35	1.31	14	1.39	0.74	14	1.47	1.17	14			
		3	1.39	1.23	14	0.88	0.52	14	1.37	0.82	13	1.43	1.13	14			
		4	2.00	1.59	14	1.58	0.88	14	1.31	0.94	13	1.52	1.04	14			
	Post	1	0.82	0.43	11	0.84	0.57	10	0.84	0.28	11	1.17	0.51	11			
		2	0.98	0.44	10	0.76	0.33	10	0.70	0.39	11	1.04	0.36	11			
		3	0.76	0.32	9	0.63	0.35	11	0.94	0.35	10	1.09	0.91	11			
		4	0.88	0.49	11	0.97	0.63	11	0.81	0.58	11	1.17	0.73	10			
decanoic acid	Pre	1	1.34	1.20	14	1.52	1.21	14	1.61	1.67	14	1.46	1.41	14	**0.03**	ns	ns

		2	1.38	0.92	14	1.31	0.99	14	1.09	0.80	14	0.97	0.72	14		
		3	1.43	1.20	14	0.93	0.74	13	0.90	0.51	13	1.25	1.67	14		
		4	2.18	1.53	14	1.18	0.87	14	1.34	1.12	14	0.96	0.55	14		
	Post	1	1.49	0.79	11	1.03	0.77	10	1.25	1.06	11	1.72	1.93	11		
		2	1.53	0.76	10	1.03	0.70	10	1.09	0.57	11	0.83	0.45	11		
		3	1.50	0.96	10	0.83	0.56	11	1.11	0.99	10	0.88	0.49	11		
		4	2.45	2.72	11	1.76	2.09	11	1.29	1.02	11	0.90	0.43	10		
dodecanoic acid	Pre	1	1.17	1.09	14	1.10	1.32	14	0.93	0.54	14	1.56	1.38	14	**0.02**	ns	**0.02**
		2	1.57	1.26	13	1.59	1.74	14	1.11	0.68	14	1.53	2.13	14		
		3	1.73	1.32	14	0.68	0.42	14	0.95	0.56	13	1.20	1.24	14		
		4	2.11	1.88	14	1.23	1.10	14	1.38	1.65	14	1.01	0.67	14		
	Post	1	1.56	0.59	11	1.68	1.17	10	1.31	0.84	11	1.92	1.24	11		
		2	2.40	1.69	10	1.83	0.94	10	1.21	0.53	11	1.49	1.42	11		
		3	1.94	1.19	10	0.94	0.41	11	1.60	1.78	10	1.44	0.94	11		
		4	2.24	1.80	11	1.25	1.13	11	1.37	1.09	11	1.35	0.90	10		
heptanoic acid	Pre	1	0.06	0.04	9	0.05	0.07	7	0.05	0.04	9	0.10	0.06	9	**0.03**	**<0.01**	**0.02**
		2	0.06	0.03	11	0.04	0.06	9	0.07	0.04	11	0.11	0.09	12		
		3	0.08	0.06	9	0.09	0.10	7	0.05	0.04	12	0.08	0.06	12		
		4	0.10	0.08	9	0.06	0.05	7	0.06	0.04	9	0.09	0.08	9		
	Post	1	0.04	0.03	9	0.03	0.02	9	0.09	0.05	7	0.08	0.05	11		
		2	0.06	0.06	7	0.04	0.02	8	0.06	0.03	7	0.07	0.06	10		
		3	0.04	0.04	5	0.04	0.04	5	0.05	0.03	6	0.09	0.08	9		
		4	0.05	0.02	6	0.05	0.02	4	0.08	0.06	7	0.07	0.05	8		
hexadecanoic acid	Pre	1	4.24	2.58	14	5.70	4.09	14	6.51	6.25	13	7.10	5.32	14	*0.06*	ns	ns
		2	7.97	6.97	14	8.62	7.38	14	5.47	3.06	14	6.15	2.85	14		
		3	8.83	8.30	14	4.69	3.05	14	5.12	3.59	13	5.48	6.12	14		
		4	5.99	4.18	14	7.17	4.36	14	4.91	2.32	13	5.03	3.37	14		
	Post	1	5.23	2.05	11	8.30	6.19	10	9.17	7.17	11	7.91	4.38	11		
		2	7.83	5.19	10	7.92	4.53	10	6.60	3.89	11	6.50	4.95	11		

		3	7.95	4.77	10	4.79	2.23	11	6.40	3.63	10	7.22	5.00	11			
		4	7.43	4.61	11	7.46	7.27	11	6.21	4.76	11	5.32	2.84	10			
hexanoic acid	Pre	1	0.38	0.24	14	0.39	0.21	14	0.42	0.35	14	0.28	0.22	14	ns	ns	0.048
		2	0.41	0.20	14	0.37	0.32	13	0.33	0.20	14	0.30	0.25	14			
		3	0.50	0.38	14	0.31	0.20	14	0.37	0.24	13	0.36	0.34	14			
		4	0.55	0.35	14	0.47	0.53	14	0.33	0.19	14	0.32	0.21	14			
	Post	1	0.30	0.14	11	0.32	0.14	10	0.34	0.28	11	0.24	0.14	11			
		2	0.29	0.16	10	0.29	0.13	10	0.33	0.20	11	0.20	0.12	11			
		3	0.34	0.17	10	0.26	0.11	11	0.32	0.22	10	0.21	0.18	11			
		4	0.39	0.29	11	0.46	0.25	11	0.24	0.15	11	0.28	0.16	10			
isobutyric acid	Pre	1	0.47	0.26	14	0.43	0.19	14	0.44	0.19	14	0.45	0.19	14	ns	ns	ns
		2	0.57	0.27	14	0.40	0.16	14	0.50	0.25	14	0.50	0.24	14			
		3	0.46	0.21	14	0.37	0.14	14	0.43	0.13	13	0.41	0.20	14			
		4	0.46	0.19	14	0.43	0.16	14	0.43	0.18	14	0.48	0.21	14			
	Post	1	0.58	0.35	11	0.51	0.28	10	0.56	0.33	11	0.44	0.13	11			
		2	0.48	0.11	10	0.49	0.17	10	0.53	0.25	11	0.41	0.09	11			
		3	0.52	0.32	10	0.42	0.16	11	0.50	0.18	10	0.46	0.17	11			
		4	0.49	0.18	11	0.41	0.14	11	0.43	0.11	11	0.40	0.12	10			
isovaleric acid	Pre	1	1.06	0.76	14	1.15	1.04	14	1.25	0.88	14	1.30	0.70	14	ns	ns	ns
		2	1.44	1.15	14	0.91	0.49	14	1.42	0.92	14	1.26	0.78	14			
		3	0.90	0.76	14	0.93	0.57	14	1.14	0.63	13	1.15	0.86	14			
		4	1.07	0.94	14	0.86	0.53	14	1.00	0.72	14	1.04	0.76	14			
	Post	1	1.32	0.75	11	1.50	1.64	10	1.36	0.76	11	1.14	0.58	11			
		2	1.01	0.46	10	1.10	0.70	10	1.05	0.32	11	1.04	0.58	11			
		3	1.09	0.73	9	0.66	0.36	11	1.19	0.31	10	1.00	0.35	11			
		4	0.93	0.24	10	0.62	0.43	11	0.91	0.36	11	0.86	0.47	10			
octanoic acid	Pre	1	1.22	0.92	14	1.96	2.17	14	1.65	1.80	14	1.23	1.08	14	ns	ns	ns
		2	1.24	0.86	14	1.04	0.90	14	1.24	0.96	14	1.00	0.76	14			
		3	1.08	0.83	14	1.08	0.82	14	0.91	0.63	13	1.10	0.93	14			

		4	1.85	1.24	14	1.28	1.03	14	1.41	1.09	14	1.07	0.78	14			
	Post	1	0.90	0.50	11	0.81	0.42	10	0.88	0.54	11	1.19	1.11	11			
		2	0.87	0.29	10	0.61	0.28	10	0.87	0.38	11	0.62	0.32	11			
		3	0.80	0.42	10	0.69	0.65	11	0.76	0.30	10	0.65	0.36	11			
		4	1.21	1.01	11	1.04	0.66	10	1.11	0.73	11	0.67	0.36	10			
propionic acid	Pre	1	1.37	0.67	13	1.64	0.79	14	1.54	0.71	14	1.31	0.70	13	ns	ns	ns
		2	1.79	0.83	13	1.34	0.53	14	1.57	0.73	14	1.26	0.57	13			
		3	1.57	1.08	13	1.31	0.37	14	1.58	0.50	13	1.38	1.06	13			
		4	1.72	0.74	13	1.67	0.63	14	1.56	0.65	14	1.50	1.05	13			
	Post	1	1.30	0.38	11	1.46	0.88	10	1.32	0.51	11	1.02	0.35	11			
		2	1.23	0.38	10	1.11	0.33	10	1.19	0.47	11	0.92	0.37	11			
		3	1.09	0.40	10	0.90	0.25	11	1.63	0.45	10	1.23	0.70	11			
		4	1.24	0.34	11	1.16	0.33	11	1.23	0.39	11	1.08	0.33	10			
tetradecanoic acid	Pre	1	1.23	0.94	14	1.19	1.05	14	1.68	1.83	14	1.42	0.83	14	ns	ns	ns
		2	1.60	0.98	14	2.17	2.37	14	1.09	0.58	14	1.36	0.96	14			
		3	2.09	1.88	14	0.91	0.67	14	1.16	0.69	13	1.54	1.99	14			
		4	1.81	1.29	14	1.72	1.61	14	1.42	1.32	14	1.20	0.84	14			
	Post	1	1.81	0.71	11	2.30	2.12	10	1.87	0.91	11	2.13	1.10	11			
		2	2.19	0.85	10	2.06	1.08	10	1.40	0.86	11	1.65	0.98	11			
		3	1.95	0.84	10	1.20	0.63	11	1.77	1.79	10	1.73	1.30	11			
		4	2.15	1.26	11	1.68	1.54	11	1.46	1.05	11	1.54	0.91	10			

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Chapter 5

General Discussion

I. Overview and Summary

In this work, we explored host and microbial tryptophan metabolism in response to a psychological and physical stressor along the microbiota-gut-brain axis as an adaptation to HPA axis activation. These findings contribute to a deeper understanding of underlying mechanisms facilitating stress-coping and physiological adaptation in response to different types of stress exposure. This may provide new avenues to avoid the emergence of chronic stress and subsequent stress-related disorders.

In ***Chapter 2***, we identified novel region- and sex-dependent effects of the microbiota in modulating serotonergic responses to acute stress at both terminals of the gut-brain axis communication network. Firstly, GF status led to higher corticosterone levels both at baseline and in response to an acute stressor while colonised GF animals had similar corticosterone levels to control animals. More specifically, we demonstrate that the presence of gut microbes influences gastrointestinal response to acute stress differently in the ileum and colon in a sex-dependent manner. Notably, the stress-induced increase in colonic serotonin of male conventional mice was absent in male GF mice, an effect that was restored following colonization. This effect of stress on the serotonergic system was not observed in the colon of female mice. Central serotonergic response to acute stress, measured in key regions impacted by stress (hypothalamus, hippocampus and frontal cortex), were more pronounced in females. Importantly, this study provides evidence for sex-specific gastrointestinal and central serotonergic systems, highlighting the need for more emphasis on sex as a biological variable in stress-related gastrointestinal and psychiatric conditions. Together, these data provide evidence that colonisation of GF mice led to a partial restoration of gastrointestinal and central serotonergic systems comparable to conventional mice, suggesting that microbial manipulation could be exploited to modulate host serotonergic responsivity to acute stress.

In ***Chapter 3***, we focused in depth on the effects of HPA axis activation (following an acute stressor or by circadian influence) on tryptophan metabolism, and its impact on gut function and permeability. Our results provide novel evidence that acute stress alters GI microbial and host tryptophan metabolism and increases gut permeability *in vivo*. We showed that acute stress induces functional alterations in the ileum (*ex vivo* and *in vitro*) and alters tryptophan metabolism in the colon. At baseline, tryptophan-metabolizing bacteria and their predicted

metabolites exhibit circadian rhythmicity and gut microbiota depletion disrupts rhythmicity of host genes related to gut barrier function and for tryptophan enzymes. Finally, we observed that both microbial modulation and acute stress led to altered gut barrier function. Overall, we found that gastrointestinal response to acute stress is circadian-, microbial- and region-dependent. Our findings highlight a novel role for gut microbes, both at baseline and following acute stress, in regulating circadian rhythmicity of tryptophan metabolism and barrier function in the gut.

In ***Chapter 4***, we moved to humans and used exercise to evaluate the response and adaptation to a physical stressor, asking: (1) how different intensities of exercise shape key pillars of microbiota-gut-brain axis communication and (2) understand how different intensities of exercise remodel the gut microbiome, gut function as well as host and microbial tryptophan metabolism in previously sedentary individuals. Overall, we found intensity-dependent changes in measures of the microbiota-gut-brain axis, pointing towards moderate-intensity training as a positive modulator of the microbiota-gut-brain axis without the detrimental effects of over-training. More specifically, we found intensity-dependent changes in SCFAs production, tryptophan metabolism and differences in fermentation profiles in exercise-responsive species.

Taken together, these data illustrate that, by deciphering the underlying beneficial mechanisms of acute stress, either psychological or physical, rational approaches can be developed to prevent allostatic overload or maladaptive coping with intense or frequent stressors (**Figure 9**).

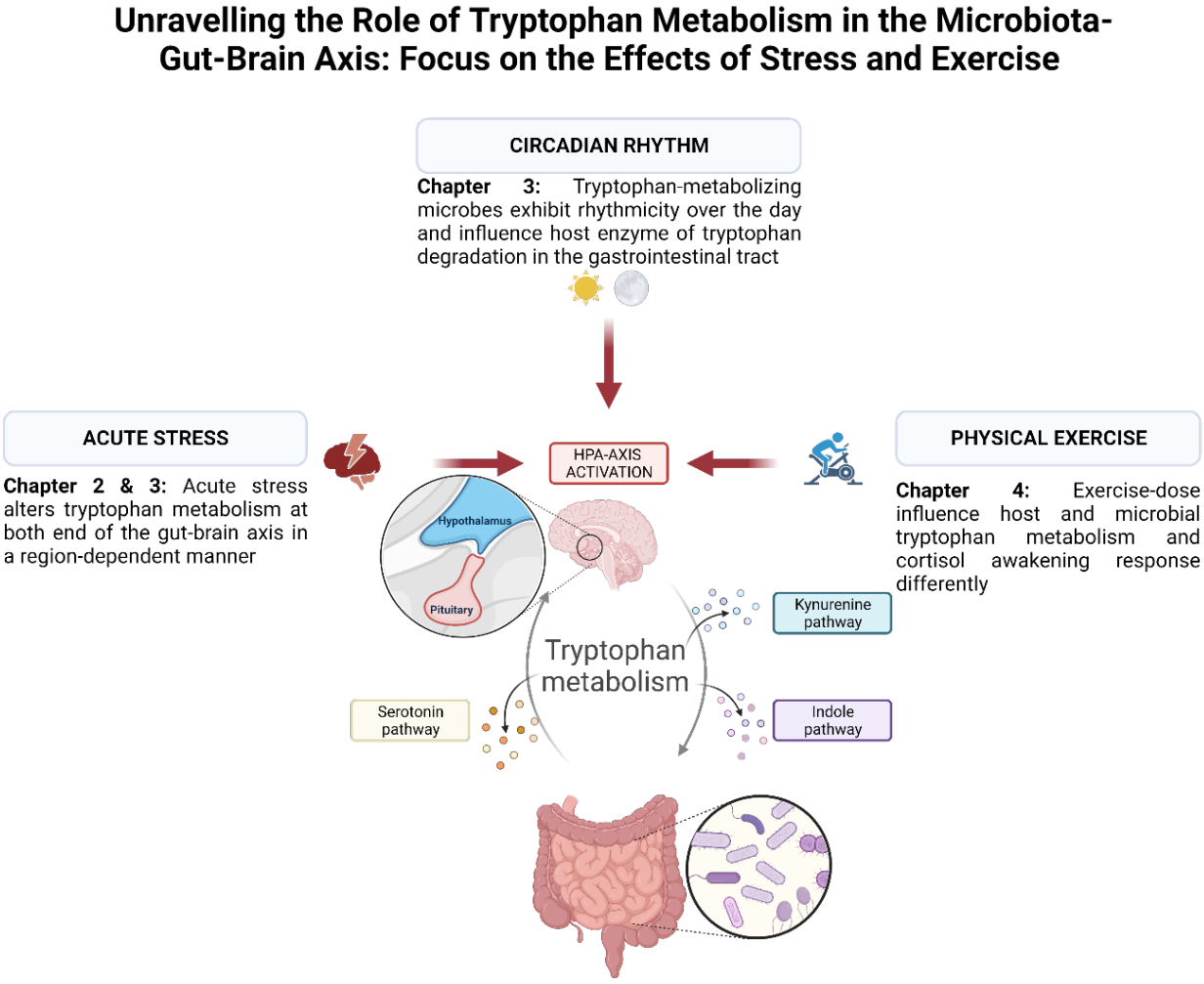

Figure 12. Summary findings

I. Germ-Free and Antibiotic-Depleted Mice as Animal Models to Study the Microbiota-Gut-Brain Axis

There is a clear need to gain causal insights into the role of the microbiome in brain and behaviour. Perhaps the most parsimonious way to examine this is to understand how the host functions in the absence of microbes. To this end, GF and antibiotic-treated animals are used as excellent proof-of-concept studies to understand the extent to which microbiome-host interactions shapes our physiology and behaviour (Luczynski et al., 2016). Pioneering studies enabled a paradigm shift in psychiatry by investigating the impact of the presence or absence of our tiny inhabitants on stress responsivity (Sudo et al., 2004), brain function and plasticity (G. Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011) and central serotonergic system (G. Clarke et al., 2013; Heijtz et al., 2011). Specifically, the microbial influence on central serotonergic levels in key brain regions implicated for stress-related disorders and important for emotional regulation and cognitive processes (such as in the striatum (Heijtz et al., 2011); the prefrontal cortex ***Chapter 2***; and the hippocampus, (G. Clarke et al., 2013)). Importantly, GF status or antibiotic-treated mice reduced anxiety-like behaviour in several studies (Bercik et al., 2011; G. Clarke et al., 2013; Desbonnet et al., 2015; Heijtz et al., 2011; Neufeld et al., 2011). This impairment in anxiety-like behaviour could be explained by a lack of "training" of the HPA axis by microbes that might cause differences in glucocorticoid sensitivity or differences in feedback-loop mechanisms in GF animals.

Since then, the influence of the intestinal microbiota in the regulation of neurotransmitters along the gut-brain axis has become evident (Mittal et al., 2017). In this work, we have expanded on previous research using GF and antibiotic-treated animals to characterise the influence of an acute stressor (***Chapter 2 & Chapter 3***) and circadian rhythm (***Chapter 3***) on GI tryptophan metabolism. Furthermore, we used colonised GF animals to distinguish mechanisms that might be driven by developmental abnormalities due to the absence of microbes during specific time windows (***Chapter 2 & Chapter 3***). In ***Chapter 3***, we employ targeted-antibiotic treatment to determine whether the differences we observed between animals treated with a broad-spectrum antibiotic cocktail and controls were caused by gram-positive or gram-negative bacteria. Overall, this work has allowed us to understand how microbes respond to the activation of the HPA axis under conditions of stress at different

points in the circadian rhythm cycle, which surely contributes to the adaptation of the host response a stress exposure.

Both antibiotic-depleted and GF mice are widely used in neuroscience and neurogastroenterology research, despite some limitations. Antibiotic treatments are deployed to circumvent neurodevelopmental deficits in GF animals (Luczynski 2016). While offering the ability to target specific time windows, antibiotics can cause temporary or, to a lesser extent, permanent alterations in the gut-brain axis, depending on when they are deployed (Gheorghe et al., 2021). On the other hand, the GF option does not allow for normal features of competition with commensal microbes, no similar condition exists in humans and GF physiological deficits are significant (Gheorghe et al., 2021; E. A. Kennedy et al., 2018; Luczynski et al., 2016). The use of animals born in a GF environment and subsequently colonised are a good control for abnormal development of GF mice. Both animal models provide a more comprehensive way to understand the involvement of the gut microbiota in health and disease.

In this work, we used these approaches to gain important converging insights about the interface between acute stress, the gut microbiome and tryptophan metabolism. Specifically, we shift the focus from the implication of the presence or absence of microbes for the host to investigate how microbes respond to stress exposure (***Chapter 2, Chapter 3***), potentially leading to benefits for the host. However, future work should investigate how specific antibiotic treatment (targeted or broad-spectrum) manipulate specific strains in the microbiome. Indeed, antibiotic treatment can lead to antibiotic-resistance or overgrowth of unwanted species and can even promote fungal and viral activity. In addition, we need to understand how different antibiotic regimen affect the different layers of the gut, to distinguish between antibiotic and microbiota-related dysfunction.

Both animal models have become the gold standard in microbiome research to assess complex host-microbiota interactions (Gheorghe et al., 2021). The study of acute (G. Clarke et al., 2013; Sudo et al., 2004), (***Chapter 2***) and chronic (Cryan & Dinan, 2012) stress in these models added a new layer of complexity, highlighting the importance of host-microbes crosstalk in shaping the response of our stress axes over time. In his early work, Sudo and colleagues demonstrated that gut microbiota influences HPA axis development, with an impairment in HPA axis

activation in GF animals, that could be normalised by mono-colonisation with a beneficial microbe or exacerbated by a pathogenic bacteria (Sudo et al., 2004). This was the first piece of evidence towards the "psychobiotic revolution" (S. Anderson et al., 2017), demonstrating strain-specific effects on stress-responsivity.

Exposure or colonisation of microbiota-deficient animals with specific strains of bacteria are an invaluable tool to understand the extent to which mono- or poly-colonisation can remodel several pathways of the microbiota-gut-brain axis (i.e. immune and hormone signalling, microbiome-host communication, gut function, tryptophan metabolism). In this work, we used an *in-silico* approaches to understand how circadian rhythms shape microbial tryptophan breakdown (***Chapter 3***). Future studies are needed in these animals to understand how host and microbial tryptophan degradation differs in response to an acute stressor at different timepoints across a 24-hour cycle. Similarly, it would be interesting to understand the impact of mono- or poly-colonisation of rhythmic tryptophan-metabolising bacteria in GF mice and investigate the GI response to an acute stressor over the day. The use of GF and antibiotic-treated animals continues to advance our understanding in the involvement of gut microbes in brain and gut function, which would not have been possible in humans due to ethical considerations.

Could the gut microbiota drive psychological well-being in humans and really help improve stress-related or GI symptoms Some studies attempt to provide answers by studying healthy volunteers: targeting host-microbiome interaction led to translatable results to benefit mental health (Berding et al., 2022; N. Zhang et al., 2020) and stress-responsivity (Dalile et al., 2020), even though some interventions did not (Kelly et al., 2017). In parallel, we are now understanding that psychiatric populations, such as MDD, have impairments in gut microbiota composition, potentially leading to functional changes. Causality in the microbiome-psychiatry field has been investigated with Faecal Microbiota Transplantation (FMT) studies, by transplanting a donor's microbiota into a recipient gastrointestinal tract. FMT studies from human clinical patients into healthy recipient animals allowed us to grasp a first layer of complexity in the involvement of gut microbiome in psychiatric disorders: FMT from depressed donors in recipient mice led to transmission of key symptoms in MDD (C. Huang et al., 2019; Kelly et al., 2016; S. Liu et al., 2020; Zheng et al., 2016), schizophrenia (F. Zhu et al., 2019, 2020), anorexia nervosa (Hata et al., 2019) and IBS patients with stress-related symptoms (De Palma

et al., 2017). Even more surprisingly, the first clinical studies emerged as potential therapeutic intervention for Major Depressive Disorder (Cai et al., 2019; De Clercq et al., 2019; Doll et al., 2022; Green et al., 2023) and IBS (H. L. Huang et al., 2019; Kurokawa et al., 2018; Mazzawi et al., 2018; Mizuno et al., 2017), all showing improvements in stress-related symptoms associated with their disorders.

II. Prescribing the exercise pill: Which dose is best and when should it be taken?

All this work combined suggests that progress in successfully interfacing the fields of microbiome science, gastroenterology and psychiatry requires targeted interventions for disease-specific functional imbalances that can be ameliorated by certain strains of microbes. Indeed, patients with various diseases exhibit varying degrees of imbalance, whether stress-related symptoms are the primary or secondary outcome of the disorder. The transition towards strain-specific interventions to precisely target the most relevant functional changes can be expedited by the systematic evaluation of newly identified candidates. For example, we found exercise-responsive microbes in ***Chapter 5*** with increased relative abundance of proteolytic bacteria in the highest exercise group, suggesting functional adaptations to exercise training. This dose also increased the cortisol awakening response, which may not always be desirable. Even light-intensity exercise substantially remodelled the gut microbiota at the species level, but with no significant changes in fitness level.

Future work could investigate the strain-specific benefits of these exercise-responsive microbes and lead to next generation probiotics to improve fitness (McDermott et al., 2022) and brain function (Eastwood et al., 2021). Furthermore, specific probiotics could be used for different type of training (resistance or endurance) or targeted for athletes or non-athletes' population to fit physiological needs to exercise demand. Similarly, while some probiotics could influence fitness improvement, like *Veillonella atypica* described as a performance-enhancing strain (Scheiman et al., 2019), others could potentially counteract the detrimental effects of intense exercise training on the GI tract, particularly in athletes? (De Oliveira & Burini, 2009). Candidate microbes or complex communities could be transplanted in GF mice, and to a later stage in patients with mental health issues, to see whether some of the beneficial effects of exercise could be transferred via the exercise-remodelled gut microbiome. Likewise,

additional studies could supplement tryptophan-metabolising or SCFAs producing microbes to MDD patients and see which ones confers the most benefits. This might result in adjuvant therapy for certain mental health conditions. The study of the crosstalk between the host and microbiome offers multiple avenues of research in the psychiatry field or disorders associated with psychiatric symptoms as secondary outcomes. Consideration also needs to be given not just to the intensity which should be prescribed but also to the optimal time of day to exercise, since we have shown that many of the key biological targets are influenced by circadian rhythms.

III. HPA-Axis & Tryptophan Metabolism: What is the Microbial Influence?

The extent to which the microbiota impacts central serotonin is a difficult concept to comprehend, although some pioneering studies in the field published proof-of-concept work demonstrating the impact of GF status on central tryptophan metabolism, brain development and behaviour (Bercik et al., 2011; G. Clarke et al., 2013; Desbonnet et al., 2015; Heijtz et al., 2011; Neufeld et al., 2011). However, no studies have investigated changes in the serotonergic response at both ends of gut-brain communication following acute stress as a transient response, that may lead to maladaptation in stress-related disorders. This research seems very pertinent given that psychiatric disorders are often linked to a dysregulation in central serotonergic system, and that peripheral tryptophan is essential for to central production of serotonin. Results from this work support the fact that not only microbes can breakdown tryptophan (Gheorghe et al., 2019), thereby shifting its availability for host degradation; but also influence host-mediated degradation of tryptophan in the colon (***Chapter 2, Chapter 3***). These are in alignment with the current literature on the topic showing that intestinal microbes can promote host serotonin degradation in enterochromaffin cells (Yano et al., 2015) or that microbiota metabolites can regulate both *IDO-1* expression (Martin-Gallausiaux et al., 2018) and serotonin production (Reigstad et al., 2015) in epithelial cells. These findings indicate an important and under investigated role of gut microbes in GI function, especially in the colon.

Since both host and microbial tryptophan metabolism influence greatly immune and barrier function of the gastrointestinal tract (Agus et al., 2018), further research is needed to fully grasp the extent of microbial influence. More specifically, the colon seems more affected by

the influence of microbes so region-specific studies should inform further how compositional and functional microbial changes impact different biogeographical regions and layers of the gut. To this day, there remains a deficit in understanding on how microbes influence the expression of host enzymes of tryptophan breakdown, except for butyrate production (Martin-Gallausiaux et al., 2018). Since IDO-1 expression seem to be downregulated by the presence of microbes (Martin-Gallausiaux et al., 2018), this could lead to important mechanism to reduce IDO-1 expression induced in GI disorders with colitis (Acovic et al., 2018). Conversely, cell culture experiments on a *TPH-1* reporter cell line would allow us to understand which microbial metabolites could regulate serotonin production in enterochromaffin cells. Since *IDO-1* expression and activity is important to maintain immune tolerance in the GI tract and TPH-1 leads to increases in serotonin thereby influencing gut function, further work should investigate the implication of their rhythmicity over the day in the GI tract. This would allow for novel avenues of microbiota-targeted intervention if some disorders demonstrated arrhythmicity or exaggerated rhythmicity in host-enzymes of tryptophan degradation.

IV. Tryptophan Metabolism along the Microbiome-Gut-Brain Axis Under Stress: Where Are We Going?

Given that GF animals have baseline impairments in central, peripheral and GI tryptophan metabolism (G. Clarke et al., 2013; Heijtz et al., 2011; Wikoff et al., 2009) (***Chapter 2***) and that an acute stressor can shift tryptophan metabolism in a region-dependant manner (***Chapter 2, Chapter 3***); we aimed at understanding subsequent functional changes in gut permeability in male mice. Indeed, host and microbial tryptophan ligands are key players in regulation of gut barrier function (Agus et al., 2018; Meynier et al., 2022) and immune signalling (Zelante et al., 2013). Several important questions remain to be answered in the field: (1) whether acute stress leads to transient changes in gastrointestinal host or microbial tryptophan metabolites and (2) if gut barrier function and permeability can be shaped by either the gut microbiota, or exposure to stress at different points in circadian rhythm cycles.

In ***Chapters 2*** & ***3***, we show that microbial status influence greatly the expression of the rate-limiting enzymes of host tryptophan breakdown in the colon of male mice. Similarly, we show in ***Chapter 3*** that GI permeability and function is impaired by an acute stressor (15-min) *in vivo*, *ex vivo* and *in vitro* in mice. Interestingly, acute stress led to changes in caecal microbial

and host tryptophan breakdown only in microbially-colonised but not GF mice (***Chapter 3***). Since activation of the HPA axis by stress can alter GI tryptophan metabolism, we wondered if regulation of its activation by circadian rhythms would also result in differences in GI tryptophan metabolism. In addition, there is some evidence that microbial abundance oscillates throughout the day (Thaiss et al., 2014) and that microbiota control of intestinal epithelial cells depends on functional circadian machinery (Mukherji et al., 2013). Therefore, we also wondered about the role of the gut microbiota in HPA-axis related modulation of GI tryptophan metabolism.

Interestingly, we show in ***Chapter 3***, that 61% of rhythmic bacteria in conventional mice were tryptophan-metabolizing. Based on our metagenomic data, we used the TrpNet database to estimate the predicted abundance of microbial tryptophan breakdown and found that inferred microbial tryptophan and kynurenine degradation exhibited differences depending on time-of-day. Both microbial and host tryptophan metabolism modulate gut barrier function and permeability through binding to receptors, the most well-known being the Aryl Hydrocarbon Receptor (AhR) (Agus et al., 2018). We then used cell culture technique with AhR reporter assay to understand whether caecal supernatants collected from stressed mice would increase AhR activity *in vitro*. *AhR*-mediated *Cyp1a1* transcription was not increased by caecal supernatants collected from stressed animals. However, AhR activity was reduced in a dose-dependent fashion by treatment with caecal supernatants from mice exposed to partial or full antibiotic treatment. AhR signalling is an important regulator of the immune response, especially in regulating gastrointestinal homeostasis (Agus et al., 2018).

A deeper understanding of microbial influence of AhR activity and signalling will allow us to appreciate the relevance of the impact of microbial metabolites. Investigation of AhR activity at a different resolution would be appreciated, as AhR is present in immune cells and epithelial cells, several specific layers of gut barrier function could be considered. For this work, flow cytometry experiments to isolate a specific type of immune cells in mucosal tissues, called Innate Lymphoid Cell 3 (ILC3s), would be very interesting in the context of circadian regulation of gut barrier function, as they are known to be regulated by AhR (S. Li et al., 2018). AhR-regulation of ILC3s play an important role in the development and progression of IBS and is modulated indirectly by microbial signals (Song et al., 2020). More interestingly, recent evidence suggests that the gut microbiota drives ILC3s rhythms in the small intestine, leading

to differences in resistance to infection over the day (Brooks et al., 2021). Other important receptors acting as modulators of gut barrier function through microbial signals could be investigated in reporter cell lines such as Trpa1 (Ye et al., 2020) and PXR (Dvořák et al., 2020; Illés et al., 2020b).

Taken together, our increasing knowledge from these studies has illuminated a number of research strands and an expanding range of therapeutic targets than can be pursued as we moved towards improved regulation of tryptophan metabolism in stress-related disorders. These strategies will need to account for the diversity of receptors and microbial enzymes involved as well as regional and temporal dynamics in the microbiota-gut-brain axis across the lifespan. Possible approaches could include precision editing to add or remove microbial strains of important for tryptophan metabolism (K. N. Lam et al., 2021). Alternatively, gene-targeted depletion in specific strains of bacteria could be carried out (K. N. Lam et al., 2021). For example, targeting clock-genes in tryptophan-metabolising bacteria or targeting microbial tryptophan-degrading enzymes could inform us on the functional role of tryptophan-metabolising bacteria on gut barrier function over the day. Alternately, formulation-based strategies could be developed to deliver microbial tryptophan metabolites to important host targets at distinct sites in the gut. A recent study administered colonic-delivered microbial by-products (SCFAs) in healthy men, which attenuated cortisol-responsivity to a psychosocial stress (Dalile et al., 2020). Since regional differences exists in the gut in terms of physiology and bacterial population, future work should target specific sites in human research, while pre-clinical work should delve specifically into understanding the mechanisms by which specific bacteria benefit us with the new tools that are emerging.

V. Open-Source Bioinformatic Tools to Understand the Microbiota-Gut-Brain Axis

Future research should take advantage of available and emerging tools to comprehend potential new avenues of research, often leading to more targeted research questions. As an example, in ***Chapter 5*** we observed unaltered α- and β-diversities or volatility but changes in the relative abundance of exercise-responsive microbes at the species only. Computational analysis of genomic investments of those exercise-responsive microbial species allowed us to understand potential changes in microbial saccharolytic and proteolytic degradation in an

intensity-dependent way. This could inform further studies to look at specific saccharolytic activity of gut microbes for low-intensity training (i.e. SCFAs production) and proteolytic degradation for moderate-to high-intensity training (i.e. microbial amino acid degradation). This opens new avenues of research in the exercise-microbiome field to see whether different exercise intensities can benefit from functional changes in the microbiome by specifically looking at gut-derived metabolites. Altogether, this entire thesis utilised cutting-edge computational and bioinformatic technologies to infer functional characteristics of gut microorganisms: such as gut-brain modules (Valles-Colomer et al., 2019a), gut-metabolic modules (Vieira-Silva et al., 2016b), inferred microbial degradation of tryptophan with TrpNet database (Lu et al., 2022) and genomic investment analysis to understand strain-specific fermentation substrates (Vieira-Silva et al., 2016a), to help us direct our research. To analyse our rhythmic data, we developed Kronos, a computational tool to facilitate biological rhythmicity analysis suitable for complex experimental designs (Bastiaanssen et al., 2023). These tools are very useful for saving time and providing the resources needed to answer hypothesis-driven questions.

VI. *How, When and Where Does Acute Stress Exposure Becomes a Chronic Problem?*

Understanding the impact of brief exposures to stress is fundamental to grasp adaptations to chronic stress and the implications for psychiatric disorders and stress-related gastrointestinal dysfunction. Indeed, transient stress exposure disturbs homeostasis to adapt to new state of energy demand. However, repeated, prolonged, inadequate or non-adaptive responses to stress can cause adverse psychological and physical disturbances over time. In psychiatric research, one key question to be answered is: when does acute stress become a chronic problem (Hammen et al., 2009)? Understanding the processes through which stressors cause depression may be hampered if the impact of acute versus chronic stress are not differentiated and elaborated. Similarly, understanding the beneficial effects of a healthy acute stress response, or HPA axis activation due to exercise could generate new therapeutic avenues.

Exercise is a perfect example of a positive, adaptive response to a physical stressor leading to positive adaptations, if practiced chronically but without excess. Indeed, overtraining syndrome in athletes are associated with mood, cognitive and behavioural changes like those

observed in depressed populations (Smith, 2000). These changes are accompanied by changes along the MGBA: chronic systemic inflammation, alterations in gut barrier function and changes in stress- axes (HPA and ANS). Therefore, in ***Chapter 5***, we sought to understand the optimal dose of exercise necessary for positively remodelling the microbiota-gut-brain axis without causing mood or chronic physiological imbalances. More specifically, we were interested in changes in tryptophan metabolism in the circulation, as it can differ in athletes compared to sedentary individuals (see ***Introduction – Section V-B***, Table 2. Clinical studies interested in acute or chronic effect of exercise on tryptophan metabolism: focus on healthy participants). Moreover, pre-clinical work suggests that tryptophan metabolism in skeletal muscle could mediate stress-resilient effect of exercise (Agudelo et al., 2014), we wondered if exercise, at a defined intensity, would remodel tryptophan degradation by microbes in humans. Interestingly, we found that circulating serotonin was higher directly after a maximal exhaustion in high-intensity trained individuals compared to their sedentary state. This suggests dose-dependent changes in exercise modulation of tryptophan metabolism. Interestingly, all exercise group following 12-weeks of training seem to have an increase in indole-3-lactic acid production after a maximal exhaustion test, a microbial tryptophan catabolite. Several studies are now highlighting the role for strain-specific microbes in enhancing performance through SCFAs production (L. Huang et al., 2022; H. Morita et al., 2023; Scheiman et al., 2019), however less was known about microbial tryptophan metabolites.

As the study of reciprocal interactions between exercise and the microbiome evolves, understanding GI adaptation to exercise training is necessary. This will allow us to understand how this new environment in shaping bacterial population: such as changes in immune signalling, pH, tolerance to heat stress and ischemia, mucus secretion, nutrients availability, energy metabolism, transit time (Mailing et al., 2019). For example, in ***Chapter 5***, we see differences in serotonin production following exercise in the high-intensity training group, which could very well influence gut motility and transit time. It would be interesting to investigate transient and chronic changes in bacterial population of mice receiving FMT of exercise-responsive bacteria and whether changes would be sustained with or without exercise. It would be logical to assume that adaptation to training leads to GI changes that would benefit the growth of exercise-responsive bacteria. This method would also help

determine the optimal strategy to use probiotics to maintain a healthy GI environment, thereby increasing the likelihood of providing beneficial effects.

On the other hand of this bidirectional communication, the gut is also subject to physiological challenges, such as stressors, rhythmicity from central and peripheral clocks and is highly impacted by gut microbes. The GI barrier is a selective but permeable barrier allowing the transport of molecule from the lumen of the intestine to the blood and through multiple mechanisms. In some circumstances, exposure to an intense psychological or physical stressor impairs gut permeability leading to the translocation of metabolites from the lumen in the blood (Kelly et al., 2015). Ultimately, the presence of unwanted molecules in the circulation could trigger low-grade systemic inflammation which has been linked in the past to depressive symptoms (Wium-Andersen et al., 2013). For this reason, the study of acute stress is necessary to understand how several exposures to stress could lead to transient or permanent damage of gut barrier function. In ***Chapter 3***, we first demonstrated that a 15-min acute stress is sufficient to alter paracellular permeability *in vivo* in both conventional and ABX-treated mice. Further experiments *ex-vivo* comparing the effect of this acute stressor on active or inactive phase of the circadian rhythm demonstrated that paracellular permeability is affected more in the ileum than the colon in a circadian and microbiota-dependent manner. In summary, we found that acute stress evokes functional changes in transepithelial resistance and interacts with time-of-day to induce impairments to paracellular permeability specifically in the ileum. Importantly, most preclinical and clinical studies investigating intestinal permeability impairment focuses on paracellular permeability – characterised by the transport of substances across the epithelium. However, other routes of GI permeability are also very relevant in GI disorders in the context of ion transport and transepithelial transport routes, especially in GI disorders such as celiac disease, inflammatory bowel disease and metabolic alcoholic metabolic liver disease (Vanuytsel et al., 2021).

Overall, when addressing the issue of gut permeability dysfunction, using a variety of techniques is necessary (***Introduction – Section IV-B***) to fully understand the extent to which the pathophysiology is changing gut barrier composition and function and the potential repercussions. This is true whether looking at barrier dysfunction as the primary outcome of a pathophysiology (relevant in GI disorders) or as a result of other physiological imbalance (relevant in stress-related disorders). We must refine our knowledge of regional variations in

how stress affects the gastrointestinal tract, since this seven-meter-long organ in humans is physiologically and functionally different along its length (Farré et al., 2020). On the other hand, bacterial distribution and abundance differ in a region-specific manner since the environment of the lumen shapes living conditions for microbes (Selber-Hnatiw et al., 2020). While we investigated *in vivo*, *ex vivo* and *in vitro* the impact of acute stress in microbially colonised or depleted animals on the ileum and colon (***Chapter 2 & 3***), future work is needed to investigate this at a higher resolution. More specifically, the GI tract is composed of several layers that all play a role in the bidirectional communication between the brain and the gut and that are impact in different ways by the presence or absence of microbes. Our results suggests that the presence of microbes influence important host receptors in the intestines, but they are also present in found in immune cells (Trpa1, AhR, PXR) and neurons (Trpa1) (Naert et al., 2021; Sun et al., 2022; Zhou, 2016), which could be investigated separately. Lastly, gut microbes have been shown to influence serotonin production (Yano et al., 2015) or IDO-1 expression (Martin-Gallausiaux et al., 2018) in enterochromaffin cells, another important regulatory mechanism that could be studied in a specifically investigated. Further work can examinate differences in epithelial or immune barrier function over the day in knock-out mice model for these specific receptors in conventional and microbially-depleted animals.

Microbes are also important in regulating mucus secretion, an important layer of gut barrier function (El-Aidy et al., 2013). In ***Chapter 3***, we show that the rhythmicity of the expression of tight junction- and mucus-related genes are impacted by different microbial status. We also demonstrate that paracellular permeability is altered differently, suggesting differences in the epithelial layer of gut barrier function. Interestingly, past studies investigated microbial metabolites acts on the intestinal barrier through G-coupled protein binding thereby indirectly regulating gene expression of mucin and tight junction related genes (Cheng et al., 2018) or increasing colonic secretion (Bhattarai et al., 2018). Future work could look at the rhythmicity of SCFA production, a by-product of microbial fermentation, and investigate whether it contributes to diurnal changes in GI functions in the host. Most importantly, the study of gastrointestinal disorders allowed us to understand that individual differences exist in stress resilience and susceptibility to GI disorders (Park et al., 2018). Understanding what characterises a stress-resilient gut will generate knowledge about positive adaptations to stressors and potentially shift our research question from a sickness to a wellbeing perspective.

In conclusion, the study of acute transient changes in the face of a stressor is necessary to understand adaptation and resilience to stress, allowing us to rethink our scientific questions and not only focus on disease-centred approaches.

VII. Overall Conclusions

This thesis aims at deepening our understanding on the influence of physical and psychological stressor on host and microbial degradation of tryptophan along the microbiome-gut-brain axis, both in pre-clinical and clinical settings. Advancing our understanding on peripheral tryptophan degradation allows a better understanding on its influence on gut barrier function and permeability but also understand how shifts in tryptophan degradation peripherally leads to differences at the central nervous system. This work complements other existing data reinforcing the importance of intestinal microbes in shaping tryptophan metabolism: either by microbial degradation (indole pathway) or influencing host rate-limiting enzymes of tryptophan degradation (serotonin or kynurenine pathways). Understanding gastrointestinal degradation of tryptophan, the main site of its metabolism, is required to comprehend central nervous system levels of its metabolites. Indeed, the majority of central tryptophan and its metabolites derives from the periphery. This work focuses on positive adaptation to exercise modelled by acute restraint stress in mice and exercise in healthy sedentary individuals which is complementary to the study of chronic or maladaptive stress, highlighting the importance of healthy transient changes leading to resiliency and homeostasis. Future work to distinguish transient acute changes from chronic maladaptive responses in human studies will be critical to understanding susceptibility and resilience in many areas of research.

"The active realist ideal is not truth or certainty, but a continual and pluralistic pursuit of knowledge."

Hasok Chang

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