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NUTRITIONAL STRATEGIES TO REDUCE FATIGUE IN ATHLETES AND ACTIVE PEOPLE

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Thesis contents

Like many children, I once dreamed of becoming a football player. That dream was strong enough to make a twelve-year-old boy leave his hometown of San Piero Patti and move to Ferrara, chasing the hope of a future in football. I can still remember saving money from my small job at a local bar to buy my first mobile phone—just so I could call my family.

Those were days of discipline and repetition: school in the morning, the team bus in the afternoon to training, then back home to study, have dinner, and rest—ready to live the same day again. When I returned to Messina for my final two years of high school and completed my diploma at the hospitality school, I carried with me not only a passion for football, but also a growing curiosity for food, flavor, and transformation.

After graduation, I began to travel across Europe, working in kitchens and pastry shops. Each transformation of food fascinated me—the alchemy of heat, the mystery of flavor, the subtle chemistry behind every ingredient. I began buying books—one after another—trying to understand more. Food chemistry laboratories soon became my favorite place, where curiosity met science. Yet, it still wasn't enough. I wanted to know how food affects the human body, how nutrition interacts with physiology. That desire led me to university.

Books, lectures, endless notes—each page deepened my fascination, especially with sports nutrition. My two theses on nutrition in football players marked the beginning of a new journey. In 2012, I began working with footballers and professional athletes, uniting my first dream with my scientific passion.

Curiosity has always guided me—questions, doubts, reflections that push me to never stop searching for answers. That same curiosity led me to apply for a PhD, a journey that has deeply enriched me and continues to challenge me to think critically, to question, to learn.

I am profoundly grateful to my supervisor, Professor Sam Marcora, who believes in me and inspires me every day. It is an honor and a privilege to have him as a mentor. My heartfelt thanks also go to Dott.ssa Daniela Pinto and Dott. Fabio Rinaldi, whose trust and support made this project possible.

I owe everything to my family—my mother, my sister Anna, and my brother Salvatore—who have always encouraged me to follow my dreams. To Salvo, La Frè, and Matteuccio, thank you for standing by my side every single day.

Finally, I dedicate this thesis to my Luanina, for her sweetness, kindness, and the light she brings into my life.

Tindaro

CHAPTER 1

GENERAL INTRODUCTION

BACKGROUND

On 12 October 2019, Eliud Kipchoge ran 26.2 miles (42.1 km) in 1:59:40 at the INEOS Challenge in Vienna, becoming the first man on earth to comfortably run a marathon in under two hours. When asked afterward what made the feat possible, he didn't talk about his shoes, his stride, or even his years of training. Instead, he simply said: *"I am running to make history, to show that no human is limited"*[1]. While Kipchoge was pointing to the mental dimension of performance, athletes across history have recognized, sometimes intuitively, the gut's importance. Ancient Olympians consumed fermented dairy products to sustain them through competition[2], and cyclists in the early Tour de France famously turned to yogurt and other cultured foods to keep digestion stable during grueling stages[3]. These anecdotes, once considered mere curiosities, now resonate with scientific findings that the human gut microbiome plays an important role in human health and physical performance[4].

A striking example comes from Scheiman et al. (2019), who discovered that elite marathon runners harbored higher levels of *Veillonella atypica* after competition, when transplanted into mice, this bacterium improved run-to-exhaustion times by metabolizing lactate into propionate, a short-chain fatty acid (SCFA) that enhances endurance[5]. This finding sparked a wave of interest in the potential of short chain fatty acids (SCFAs) and probiotics as allies in sport.

AIMS AND OUTLINE OF THE THESIS

The general aim of my doctoral research program was to investigate the potential for supplementation of Short Chain Fatty Acids (SCFAs) and probiotics to benefit athletic health, exercise performance and recovery. Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, are metabolites produced in the gut via microbial fermentation of dietary fibers referred to as microbiota-accessible carbohydrates (MACs). These bacterial metabolites, described also as postbiotics, have been observed to regulate host dietary nutrient metabolism, energy balance, and local and systemic immune functions[6]. In vitro and in vivo experiments have shown links between the presence of bacteria-derived SCFAs and host health through the blunting of inflammatory processes, as well as purported protection from the development of illness associated with respiratory infections. This bank of evidence suggests that SCFAs could be beneficial to enhance the athlete's immunity, as well as act to improve exercise recovery via anti-inflammatory activity and to provide additional energy substrates for exercise performance (reviewed in detail in **Chapter 2**). Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Probiotic administration has been linked to a multitude of health benefits, with gut and immune health being the most researched applications[7]. Therefore, in **Chapter 3** I present the results of a large-scale investigation into the influence of a novel elite athlete-derived probiotic, consisting of a multi-strain Lactobacillus consortium, on sleep quality, exercise recovery and gut microbiome composition in both elite football players and the general population. This two-phase study, included an open-label study followed by

a controlled longitudinal study in a professional football (soccer) team, allowed us to identify key interactions between probiotics, the gut microbiome, and the host. Although there is no established consensus regarding the most effective probiotic strains, dosing protocols, or supplementation in elite football, probiotic supplementation has gained increasing interest among sports science and medical practitioners. In **Chapter 4** I report the real-world perceptions and practices of stakeholders supporting elite soccer teams regarding probiotic supplementation. By capturing data across professional roles and countries, this study seeks to identify current trends, highlight areas of uncertainty, and provide a foundation for future work to inform evidence-based guidelines in professional football environments. A general discussion (**Chapter 5**) containing a summary of the main findings as the apply to the research question detailed in this general introduction (**Chapter 1**), their critical discussion and directions for future research concludes my thesis. Additional documents and information not included in these chapters are collected in the appendixes. Eventually, the three original studies included in my doctoral research program should advance our knowledge-base and motivate practitioners to prescribe probiotics and short chain fatty acids safe and effective interventions to elite athletes.

THESIS FORMAT

This thesis consists of one qualitative review of the literature and two clinical trials of which I am the principal investigator. Chapter 2 is based on a published review[8] in which I contributed through the conceptualisation of review framework, defining the key mechanisms linking short-chain fatty acids to athletic health, exercise

performance, and recovery. In addition, I was responsible for the translation of the reviewed scientific evidence into applied, practice-oriented considerations, with specific relevance for athletes and sport practitioners. The figure included in this chapter was adapted to support the narrative focus of the thesis.

Chapter 3 is based on a published two-phase study[9], which included an open-label study followed by a controlled longitudinal study in a professional soccer team, in which I am the first author. Chapter 4 provides insights into the implementation of probiotics supplementation in elite soccer players based on results of a cross-sectional, survey study design used to survey practitioners' beliefs and practices pertaining to probiotic supplementation in elite soccer.

All three manuscripts are written as stand-alone papers that have been either invited by or submitted to relevant international biomedical journals. For consistency I wrote all manuscripts in the style adopted by the School of Sport, Health and Exercise Sciences in Bangor which is described in the American Psychological Association Publication Manual (2001), the current recommendations of the University of Wales for theses preparation, and the CONSORT (Consolidated Standards of Reporting Trials) statement for improving the quality of reports of parallel-group randomized trials[10]. For the same reason I have included all cited references in a single list at the end of the thesis and numbered illustrations consecutively. However, to facilitate reading, I defined abbreviations at their first appearance within each chapter of the thesis. The contribution of the co-authors of each original manuscript is detailed in the acknowledgments. As all the manuscripts included in this thesis are independent but linked, at times there is a necessary overlap between chapters.

CHAPTER 2

SHORT-CHAIN FATTY ACIDS FOR ATHLETIC HEALTH, EXERCISE PERFORMANCE AND RECOVERY

Abstract

The gut microbiome is known to play an important role on physiological and homeostatic characteristics of the human host. These interactions have been demonstrated as important factors for the risk and progression of disease, as well as for the day-to-day health of the general population. It is, therefore, not surprising that there has been a growing interest surrounding the potential for an interplay between the gut microbiome and host to impact aspects of athlete health and performance. This has, in part, been driven by the consideration that gut bacteria by-products (i.e. metabolic waste) could be harnessed by the host and utilised for a beneficial outcome. This concept has led to a development in the theory of leveraging short-chain fatty acids (SCFAs, as by-products of fibre fermentation) to provide a novel strategy for enhancing athlete performance. At present, the available evidence from *in vitro* and *in vivo* studies indicates that administration of SCFAs offers a great potential to mitigate respiratory infections (i.e. immune function), blunt an inflammatory response (i.e. act as an anti-inflammatory effector), and alleviate fuel deficits (through modulation of energy metabolism). This review discusses the current literature investigating the effect of SCFA administration in cellular, animal and human models, with the aim to provide a narrative review of the current data. Practical implications and future considerations for SCFA-modulating behaviour in athletes are provided, alongside a conclusion on whether SCFAs could be used as a potential therapeutic/ergogenic aid to improve athletic health, performance, and recovery.

Introduction

The desire to improve and maximise training, performance, and recovery from competitive sport is shared by amateur and elite athletes alike. It is, therefore, no surprise that the marketplace is saturated with various nutritional supplements/aids looking to fuel performance (e.g., carbohydrate [CHO] drinks), support muscular hypertrophy and recovery (e.g. protein powders), and increase/maintain alertness (e.g. caffeine drinks).

The ability to maintain athletic performance and recovery is not only driven by the capacity to fuel and rebuild, but also an imperative need to maintain a high level of immunity to avoid illnesses that could disrupt training and competition. For this reason, there has been an increasing interest in the role that the gut microbiome, as a symbiotic partner, can play on the overall benefit to athlete physiology and immunity[1112]. Therefore, modulating the human gut microbiota could be a plausible gut-centric strategy to provide support for exercise performance and recovery. Bacteria resident within the gastrointestinal (GI) tract are able to utilise molecules associated with host dietary intake for their own metabolic purpose, providing an array of metabolic by-products that are then released into the gut lumen and subsequently pass into the systemic circulation[13]. Whilst the majority of the research attention to these metabolic by-products has been given to those considered as deleterious to health (e.g., trimethylamine N-oxide, p-cresol and indoxyl sulfate), there is also evidence that bacterial metabolites can provide a positive benefit to the human host[13]. One classification of metabolites, known collectively as short-chain fatty acids (SCFAs), are of particular interest through the preliminary evidence that

they provide a protective mechanism in disease[13], with notable benefits to renal conditions and infectious diseases[13,14].

SCFAs are small (C1-C5) aliphatic carboxylic acids that are predominantly produced by bacterial fermentation of dietary fibres and non-digestible CHO[6]. The three major SCFAs produced within the gut are acetic acid (acetate), propionic acid (propionate) and butyric acid (butyrate)[13]. To date, SCFAs have shown in cellular and animal research to provide measurable positive impact on the regulation of tissue damage, inflammation and immunity[13]. It is therefore unsurprising that there is a steady and growing interest in the idea that gut microbiome- and SCFA-focused research could play a key role in enhancing athlete health and sports performance.

Although SCFAs may be present in some food stuffs (e.g., acetate in vinegar and butyrate in cheese), the main source for their presence is via gut microbial metabolism[15]. Therefore, if we wish to consider the use of SCFAs as ergogenic aids, we must also consider the ways in which we are able to influence the level of SCFAs within the body. This could be done via a prebiotic (food for the gut bacteria), probiotic (delivery of optimal bacteria), or postbiotic (supplementation of bacterial metabolites) approach. Whichever 'delivery' method is chosen, we must investigate the science to understand if an increase in SCFAs is meaningfully observed, and whether this increase has any associations with improvements in athletic variables (i.e., performance and/or recovery).

The first step is to consider the potential benefits of SCFAs for athletes, at least in theory. Scientific research suggests that the primary ways SCFAs could be advantageous in sport include their ability to enhance immunity, reduce

inflammation, and serve as a metabolic fuel source. However, these mechanisms remain to be fully validated in human studies.

Therefore, we describe these potential roles of SCFAs to influence/regulate cardiorespiratory health and immunity, exercise-induced muscle damage (EIMD) and immune responses, and direct energy-producing fuel availability.

A summary of the information included within the text on these beneficial roles is provided in Figure 1. This review includes brief discussions of the current literature which have performed investigations into the effect of SCFA administration in cellular, animal and human models, with the aim to provide a narrative review of the current data. Practical implications and future considerations for the potential use of SCFA-modulating behaviour in athletes are provided, alongside a conclusion on the basis for SCFAs to be used as a potential therapeutic/ergogenic aid to improve athletic health, performance, and recovery.

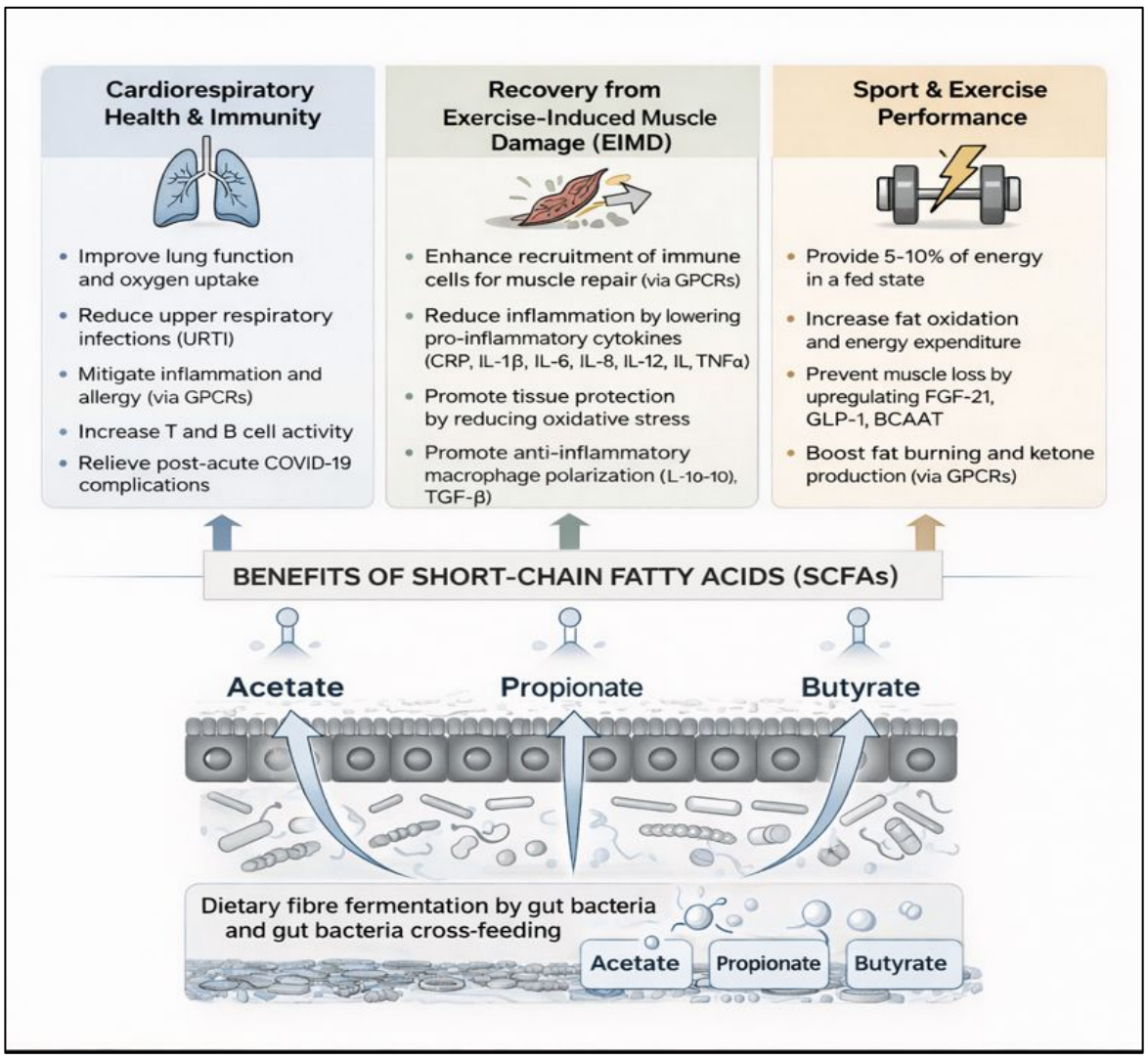

Figure 1. A summary of the potential roles for short-chain fatty acids in cardiorespiratory health, exercise recovery and inflammation, and fuelling performance in athletes.

Cardiorespiratory Health and Immunity

Considering the manipulation and/or supplementation of SCFAs to enhance cardiorespiratory performance and health in athletes aligns with the goal of optimising bacterial co-abundance groups within the gut microbial network. An optimal gut biodiversity consists of microbial species that act in a symbiotic nature to

carry out various roles in maintaining a healthy lung function. In contrast, a dysbiotic gut microbiome may cause impairment and dysregulation of lung function. Interestingly, recent studies have shown that gut dysbiosis may be improved through the enhancement of cardiorespiratory fitness.

Thus, a dynamic relationship between the gut microbiome and physical fitness has been suggested, with athletes possessing a more diverse gut microbial profile when compared to sedentary individuals[16,17] . Several species resident within the digestive tract has been shown to significantly correlate with lung function and cardiopulmonary exercise test parameters[16,17]. Among these, an increase in the butyrate-producing *Alistipes* and *Bacteroides* genus has been associated with an improvement in lung capacity, with *Granulicatella unclassified* and *G. sanguinis* contrastingly correlating negatively with oxygen uptake capacity (VO_2)[18]. It has also been reported that VO_2max associated changes by exercise training account for 22% of variance in bacterial species when considering the ratio of *Firmicutes* to *Bacteroidetes*[16]. Despite these findings identifying a relationship between butyrate-producing bacteria, exercise capacity, and microbial abundance, it remains a challenge to confidently confirm the optimal proportions of microbial species for cardiorespiratory health. Therefore, further investigation into tailored dietary strategies is warranted to increase specific microbial species and determine whether they mechanistically confer advantages in maintaining or improving cardiorespiratory capacity in athletic populations. Notably, despite superior lung capacity, a substantial proportion of athletes experience respiratory illnesses more frequently than non-athletes. This, in part, may be due to exercise-induced perturbations from heavy

training loads and competition, subsequently causing the loss of training days and potentially missed or substandard competitive appearances. Respiratory illnesses such as upper respiratory tract infections (URTI) are a common feature in athletes during an increased period of training, competition, disrupted sleep, and reduced energy or diet availability, all of which may bring about an influence and shift of gut microbial diversity and associated metabolites[19]. The modulation of athlete cardiorespiratory health by SCFA metabolites may be explained through the proposed gut-lung axis. The concept of gut metabolites remotely influencing immune responses in the respiratory system is well explained in an animal model by Sencio et al.[14]. The researchers induced a dysbiotic influenza A virus-conditioned microbiota that resulted in a lower production of acetate and an enhanced bacterial count in the lungs, subsequently increasing the susceptibility to secondary respiratory bacterial infections[14]. The same study also reported that a pre-infection loading of acetate (via drinking water) demonstrated bactericidal and/or bacteriostatic properties by diminishing bacterial count within the lungs and reducing the systemic spread of bacteria from the lungs[14]. Although acetate reduced bacterial count, it did not affect viral replication within the lungs nor gene expression associated with pulmonary barrier functions. For this reason, the authors reasoned that acetate played a direct influence on alveolar macrophages to confer bactericidal activities[14].

In another study, increased acetate levels were linked with a shorter duration of viral fever[20]. The study used a murine model to demonstrate that the gut-derived acetate protected the mice by reducing respiratory viral load through the secretion of interferon-β (IFN-β) and expression of interferon-stimulating genes (ISGs)[20]. In

addition to SCFA supplemented investigations, Trompette et al.[21], also noted that increased SCFA production in mice, via a high-fibre diet, enhanced the generation of lymphocyte antigen-6c (Ly6c$^-$) patrolling monocytes, increased activated macrophage numbers and reduced immune recruitment within the lungs. These actions led to reduced levels of respiratory immunopathology, alongside boosted T cell effector function. Hence, the supplementation/altered production of acetate, and perhaps other SCFAs, could potentially be used to induce a bactericidal/antiviral effect to confer respiratory protection in athletes. Conversely to direct SCFA supplementation, a fibre-rich diet was proposed to enhance the production of propionate for mediating the protection against allergic airway inflammation. In a mechanistic animal study, a high-fibre diet produced an increase in SCFA production and resulted in reduced lung tissue cytokine (IL-4, IL-5, IL-13 and IL-17A) response after 3 to 6 days following intranasal exposure to house dust mites[22]. Furthermore, the authors demonstrated a reduced cellular infiltration, goblet cell hyperplasia, mucus production, including lower serum levels of total IgE and house dust mite-specific IgG1, with direct treatment with propionate displaying similar mechanistic outcomes to those seen with a pectin-rich diet[22]. The authors noted that the protective mechanism of propionate is acquired via binding of the metabolite-specific G-coupled protein receptor (GPR)-41, GPR41, altering the bone marrow haematopoiesis to populate the lungs with the macrophage and dendritic cell precursors, consequently enhancing the phagocytic capacity of the lungs[22]. Although these SCFAs were shown to influence respiratory immune responses, they were undetected in the lung tissues. This implies that SCFAs mediate respiratory immune

function at the systemic level[22]. It has been suggested that the influence of gut-derived SCFAs on the respiratory system relies on repeated exposure to systemic antigen priming to reduce lung inflammation[23]. The dampening of IL4-producing $CD4^+$ T cells and IgE levels with SCFA exposure[23] demonstrates a potential immunomodulatory role for SCFAs to facilitate homeostasis, protective immunity, and tissue repair for asthma and allergy in athletes. Thus, athletes undergoing a high volume of training, who are highly susceptible to URTI, may benefit from immune priming by gut-mediated SCFAs or targeted SCFA supplementation. It is imperative to note that bacterial species residing in the lungs are vastly different from the nutrient-rich environment of the GI tract. The respiratory tract is low in nutrients for microbes due to its relatively low levels of mucus compared to the thick protective mucus layer within the gut[24]. Translation of these mechanistic effects into an athlete population, along with the exploration of associations between lung and gut microbiomes attributable to local and systemic responses, have yet to be determined and therefore provide a plausible approach for future research investigations.

Dietary intake in athletes varies with the type and intensity of training, and the amount and balance of nutrients play a key in supporting mucosal immunity. The presence of salivary IgA in the oral mucosa may be able to protect against URTI by neutralising bacteria and viruses before they are able to attach to respiratory mucosal surfaces[25]. Low levels of IgA antibodies, an immune marker found in saliva, place an athlete at a greater risk of contracting a URTI[26]. Conventional nutritional strategies by manipulation of dietary intake with high CHO have shown increases in salivary IgA in athletes[27] . However, with non-digestible CHO intake, the increase in

gut-derived SCFAs can lead to higher systemic levels of SCFAs and consequently increase salivary IgA concentration[28]. In a rat mechanistic study, it was reported that the increase in SCFAs through non-digestible CHO intake stimulated the SNS, subsequently upregulating polymeric immunoglobulin receptor (pIgR) messenger ribonucleic acid (mRNA) expression in the submandibular gland to mobilise IgA in the saliva[29]. In conditions of gut dysbiosis, enhanced IgA coating of intestinal bacteria, particularly *Lactobacillus*, suggests that follicular helper T cells (Tfh) cell proliferation modulates IgA production and response to the microbiota[30,31]. It is also interesting to note that IgA coated with *Lactobacillus*, and not IgA response alone, is able to alter microbiota composition and blunt the reduction of SCFA production associated with a dysbiotic environment[32,33]. On the contrary, only mild gut dysbiosis has been reported in IgA deficient human patients, however, other Ig subclasses (e.g., IgM) were found to compensate for reduced levels of IgA[34]. A high-fibre diet consisting of inulin and pectin, and thus increased gut-derived SCFA content, also enhanced the differentiation of naive B cells into B cells expressing Ig isotypes (IgG1, IgG2a, IgG2b, IgG3), including enhancing the levels of IgA and IgG in the circulation[35]. Therefore, fine-tuning the composition of the microbiota to increase SCFA production in the athletic population may regulate antibody responses to further protect against respiratory inflammation and, in turn, reduce the incidence of respiratory illnesses which lead to reduced capacity for training and competition.

The role of SCFAs in direct mucosal antimicrobial activity was also described in a Kiwi fruit consumption study. Antimicrobial peptides, such as β-defensins, were indirectly stimulated by SCFAs in colonic epithelial cells, therefore suggesting

SCFAs as a contributor to host immune regulation. SCFA regulation of intestinal immunity in maintaining gut homeostasis has been mainly observed through the inhibition of histone deacetylase (HDAC) and activation of G-protein coupled receptors GPR41, GPR43 and GPR109a[33,36]. However, the link between gut-derived SCFAs and respiratory health has yet to be fully elucidated in human clinical studies. Researchers have proposed that the link, bridged by the systemic circulatory system, is where “cross-talk” occurs between the gut and the lungs[37,38]. SCFAs released via the gut, along with other nutrients, are first drained into the portal venous system before being transported to the liver and other organs for regulatory processes at the systemic level[39]. SCFAs are released into the circulatory system at a rate of approximately 35 μmol per kg body weight per hour, with acetate and propionate being the most abundant[40]. In a recent stable isotope study, systemic availability of colonic-administered acetate, propionate and butyrate was shown to be 36%, 9% and 2%, respectively[41]. Since butyrate is the main fuel source for gut epithelial cells, it is expected that butyrate not utilised within the gut will be metabolised by the liver[40], therefore reducing its presence in the systemic circulation. These metabolic behaviours may influence the consideration of strategies to increase system SCFAs for translation to sports purposes, with the consideration of metabolites such as butyrate perhaps providing an increased influence via a postbiotic supplementation protocol (i.e., via chemical compounds such as sodium butyrate). This approach is likely to increase the resultant levels of SCFAs which are transferred into the systemic system (i.e., not utilised within the gut), and therefore may further manipulate the “cross-talk” mechanisms proposed via the gut-lung axis.

In light of the current global pandemic of coronavirus disease 2019 (COVID-19), athletes who regularly travel for competitive purposes could benefit from enhanced protection against respiratory infections via increased SCFA production and/or supplementation. In this light, SCFAs may potentially be used as therapeutic agents for the protection against or blunted severity and response of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). A novel butyric acid releaser, N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), was administered orally and was able to regulate several genes associated with antiviral pathways through the modulation of key molecules. This included a co-receptor that enhances the ability of SARS-CoV-2 to enter host cells and potentiates the infectivity of the virus[42]. FBA has physicochemical characteristics for easy oral administration without the unpleasant organoleptic properties of butyric acid and/or butyrate salts (e.g., sodium). This approach may provide a compensatory approach to impaired SCFA production found to associate with COVID-19 infection and beyond 30 days post-COVID-19 recovery[43]. However, butyric acid itself is rarely given orally due to its rancid odour and unpleasant taste[44], with metallic salts a preferred approach (i.e., sodium, calcium, magnesium). Comparably, acetate can be administered through the intake of table vinegar (4-7% acetic acid) but is an approach that could induce GI discomfort (e.g., nausea) in many individuals[45].

Nutritional strategies manipulating dietary fibre and/or using prebiotics/probiotics may offer improved alternatives to enhance gut microbial diversity and subsequently improve the availability of SCFA metabolites. Distinct gut microbiome profiles have been linked to post-acute COVID-19 syndrome (PACS), suggesting that the human

gut microbiome may play an important role in the recovery from COVID-19. A recent study found that butyrate-producing bacteria, including *Bifidobacterium pseudocatenulatum* and *Faecalibacterium prausnitzii*, have the largest inverse correlations with PACS at 6 months[46].

Taken together, respiratory protection against inflammation and infection appears to be influenced by a diverse gut microbial community through the generation of a more "proportionate" level of active metabolites such as SCFAs. Indeed, the mechanism of action of SCFAs for respiratory immune response demonstrates their presence in an immunoregulatory role. Findings to date on the impact of systemic SCFAs present an opportunity to investigate associations between gut microbial biodiversity and the blood metabolome, a technique that is increasingly utilised in exercise studies[47]. These approaches allow an increased understanding of the likely mechanisms responsible for the alteration of the gut microbiota, and its potential impact on host health and immunity, via the impact of exercise training and/or targeted nutritional strategies.

Exercise Recovery and Inflammation

Exercise-induced muscle damage (EIMD) is a common and often unavoidable consequence of training and competition experienced by exercisers of all abilities[48]. The symptoms of EIMD can be present immediately post-exercise and may last for

up to two weeks[49]. Common symptoms include reduced muscle function, increased muscle soreness/delayed onset of muscle soreness (DOMS), swelling, reduced range of motion and increased muscle cell permeability[50]. The length and severity of symptoms vary depending on multiple factors including exercise intensity and duration, type of muscular contractions (eccentric lead to greater damage than concentric or isometric), number of joints used and how accustomed the individual is to the exercise stimulus[48]. Prolonged symptoms can have detrimental effects on subsequent training and performance meaning a rapid recovery is a priority for many exercisers, specifically athletes[50]. The process of EIMD can be split into two different phases. The primary phase is associated directly with the damage caused by the mechanical work of exercise and high forces being applied on the muscle(s), whilst the secondary phase has a delayed occurrence and is related to the inflammatory response to the structural damage[50]. Nutritional supplements (e.g., polyphenols) have been proposed as a strategy to reduce the recovery periods following EIMD, with the main outcome shown through a blunting of the secondary, inflammatory phase (see Owens et al., (2019)[50] for a review of nutritional strategies and EIMD.

The highly sophisticated post-exercise inflammatory response is crucial for the clearing of debris and dead tissue, as well as promoting adaptation and remodelling of the muscle and other relevant tissues. However, dampening this response and transitioning to an anti-inflammatory cascade of events is essential to avoid damage to healthy tissues (via excess reactive oxygen species (ROS) production from immune cells) as well as to stop the progression toward a chronic inflammatory environment[51]. This transition is also likely to alleviate the symptoms of EIMD[50]. The

muscle recovery process is initiated by neutrophil recruitment in response to protein biomarkers, creatine kinase (CK), myoglobin and other inflammatory profile released as a result of muscle injury[5252,53]. Findings suggest SCFAs induce the recruitment of neutrophils to the inflammatory site by activating the GPR43 receptors[54], thereby accelerating the immune response to inflammation. An increase in SCFA levels may also be initiated following ROS-induced muscular secretion of interleukin (IL)-6 and subsequent upregulation of lipolysis. This, in turn, provides a release of SCFA molecules which can be used as an energy source for muscle function/repair[49]. Muscle contractions necessitate the increase in adenosine triphosphate (ATP) production via oxidative metabolism which further generates ROS, a response associated with exercise adaptation[55] . Although a release of ROS is one of the ways in which neutrophils execute their cellular killing and clearing functions, excess ROS production can lead to the damage of surrounding healthy tissues[56]. A human in vitro ulcerative colitis model demonstrated that sodium butyrate increased the amount of hydrogen peroxide produced by neutrophils but reduced the more destructive myeloperoxidase (MPO) mediated ROS production[57]. No specific mechanisms were identified but it was suggested that sodium butyrate may reduce MPO activity[57]. A reduction in MPO activity could offer protection to healthy tissues during EIMD, thereby providing a potential mechanism in which SCFAs, and specifically butyrate, may improve recovery following muscle-damaging exercise.

Previous research has demonstrated that SCFAs promote a predominantly anti-inflammatory environment via regulation of chemokine and cytokine release and altering immune cell activity[54]. In-vitro human research has shown butyrate lowers

the pro-fibrotic cytokine, transforming growth factor-1 beta (TGF-1β) production from kidney cells at baseline and after stimulation in a dose-dependent manner[58]. Animal models explore potential mechanisms with reduced activity of the pro-inflammatory transcription factor nuclear factor kappa B (NF-kB) observed in kidney cells treated with butyrate only[59], as well as the three major SCFAs of acetate, propionate and butyrate. In humans, a recent meta-analysis investigating the effects of SCFAs, prebiotics, and synbiotics on systemic immunity showed that out of the 68 studies included, approximately half observed a reduction in at least one pro-inflammatory biomarker[60]. Biomarkers exhibiting a blunted response included C-reactive protein (CRP), IL-1β, IL-6, IL-8 and tumour necrosis factor-alpha (TNF-α). However, this meta-analysis focused on chronic inflammation and not an acute inflammatory response associated with exercise output. All above mentioned pro-inflammatory biomarkers have been shown to be elevated post-exercise in an intensity-dependent manner[61] and are therefore key components of the inflammatory processes in the secondary phase of EIMD. Further to reducing pro-inflammatory biomarkers, SCFAs have also been shown to increase the production of anti-inflammatory mediators. For example, *in vitro* monocyte production of the pro-inflammatory cytokine IL-12 has been shown to decrease, whilst the anti-inflammatory cytokine IL-10 increase following treatment with sodium butyrate[62]. Elevated levels of IL-10 could be important in EIMD as it has been shown to change macrophage metabolic phenotype from pro-inflammatory M1 to anti-inflammatory M2 cells. This phenotypic change is a key step in the EIMD inflammatory response, as it signals the transition

towards regeneration and repair processes[51] by stimulating the release of growth factors such as TGF-1β and activation of myogenic precursor cells[49].

As previously mentioned, SCFAs are ligands of the GPRs and act as inhibitors of HDAC. Acetate, propionate and butyrate can all bind to both GPR41 and GPR43, whilst butyrate also interacts with GPR109a. These three receptors are present on the surface of many cells throughout the body including immune cells such as neutrophils, monocytes/macrophages and T lymphocytes. These are all known to be key immune cells involved in the secondary stage of EIMD[50]. Previous work has shown that intake of a probiotic yoghurt reduced levels of airway inflammation which showed links to a reduction in IL-8 production in neutrophils and macrophages via activation of GPR41 and GPR43. For *in vitro* experiments, the addition of butyrate to murine macrophages reduced the production of IL-6 and TNF-α via GPR41 activation. *In vitro* activation of GPR109a has also been linked to reduced production of IL-6 and TNF-α in human monocytes, however, this was shown via administration of nicotinic acid and not butyrate or other SCFA molecules[6]. Data assessing butyrate activation of GPR109A is scarce, although one study has shown increased IL-10 production from macrophages in a colonic inflammation model.

Propionate and butyrate are HDAC inhibitors in immune cells. Although HDAC inhibition increases gene transcription, the specific genes promoted or suppressed can be determined dependent on chromatin and promoter status. In neutrophils, a reduction in TNF-α production has been observed in rats following propionate and butyrate treatment via HDAC inhibition, whilst in mice macrophages, butyrate reduced the pro-inflammatory biomarkers IL-6, IL-12 and nitric oxide.

The majority of the evidence across animal and human experiments supports the notion that SCFAs induce anti-inflammatory effects in immune cells which are associated with important actions occurring during secondary phase of EIMD. However, the results have not been uniform in nature[63]. All of the major SCFAs have demonstrated the capability of stimulating anti-inflammatory cytokine release. However, acetate and propionate have also been shown to increase IL-6 production in mice colonic cells, although this was suggested to be a protective measure to recruit neutrophils to stimulate a significant immune response to intestinal infection. The specific HDAC subtypes affected could also promote or inhibit the nature of the inflammatory response. Specific SCFA molecules utilised may induce differing responses, with butyrate generally inhibiting immune cell function (reducing inflammation), contrasting to acetate and propionate which facilitate the effects on immune cell function (increasing inflammation)[6]. Research to date shows that, in theory, the increased presence of SCFAs has the potential to dampen the inflammatory response associated with EIMD. This effect would provide a reduction in symptoms linked to detrimental performance, however, research in this area is lacking. It is, therefore, warranted for future investigations to seek to establish the generalised and mechanistic effects of SCFA manipulation/administration on post-exercise inflammation[35]. Furthermore, research is required to assess the benefits of dampening the inflammatory response to exercise in this manner, as an inadequate pro-inflammatory phase or stimulating the anti-inflammatory phase too early may lead to maladaptation and longer-term negative effects on muscular performance/adaptation. Finally, GPR41 and GPR43 receptors have now been

confirmed on human myocytes (Frampton et al, 2020) which could introduce an alternative forward perspective when focussing on the effect of SCFAs on EIMD, as well as investigations into muscle function as a whole.

Fuelling Athletic Performance

Prolonged strenuous exercise in athletes is frequently associated by an energy deficit that may impair performance. Exercise reduces intracellular ATP levels and disrupts the ATP/adenosine monophosphate (AMP) ratio, leading to cellular energy imbalance. AMP-activated protein kinase (AMPK) is stimulated in response to low intracellular ATP levels during exercise, leading to a restoration of ATP through acute control of metabolic enzymes and inhibition of anabolic pathways. Furthermore, lactic acid build-up and muscle glycogen depletion initiate myokine secretion of IL-6 which upregulates lipolysis and gluconeogenesis during prolonged muscle activity, thus increasing SCFA uptake by the muscle. The amount of SCFAs produced in the gut may provide a substantial energy source across several metabolic pathways. In our previous review, we highlighted several metabolic pathways to produce SCFAs via the fermentation of non-digestible CHO, such as fibres and resistant starch, by multi-gut bacterial species. These SCFAs may eventually be absorbed into the systemic system to interact with metabolic pathways, regulating metabolic homeostasis by generating glucose to support low energy conditions and possibly reduce recovery time from intense exercise in athletes[6].

Colonic microbes are the major sources of SCFA production through fermentation of dietary fibres in an anaerobic environment. The energy originating from dietary non-

digestible CHO is released as 88.5% SCFAs, and up to 92.8% of the SCFAs produced in the colon are absorbed while 2% is excreted in faeces. Colonic fermentation of SCFAs contributes around 5-10% of energy in the non-fasted state, suggesting SCFAs could be harnessed as energy producers for athletes to balance low energy availability during physiologically challenging conditions such as prolonged or intense exercise. Earlier, we mentioned that SCFAs are able to stimulate the SNS and activate an immune response. The stimulation of the SNS is also activated via GPR41 during low energy conditions, acting as an energy-sensing receptor to maintain energy homeostasis. The SNS controls cardiovascular and neuromuscular responses and is a central mechanism to exercise performance. Under starvation or a fasted state, ketone bodies may inhibit the SNS, impacting energy expenditure and thus may impair exercise performance. The role of SCFAs in dietary caloric intake and appetite regulation to improve energy balance has been previously investigated. A recent study in humans showed that an acute oral supplementation of sodium propionate (6845 mg) raised resting energy expenditure following an overnight fast and was independent of systemic glucose and insulin levels. Accordingly, the use of propionate may impact energy metabolism in athletes during prolonged, intense exercise and postprandial conditions. Similarly, colonic infusion of a mixture of butyrate, acetate and propionate has resulted in an increase in fat oxidation and energy expenditure, with further evidence of SCFAs affecting energy metabolism shown through the increase of VO_2 following propionate supplementation[64]. In contrast, some animal and human studies have demonstrated no changes in energy intake when propionate is added to the diet, and no effect on

appetite regulation when butyrate was administered intravenously. A targeted approach of delivering SCFAs via the GI tract, specifically the colon where SCFAs are produced, is necessary for the binding of SCFAs to intestinal receptors to exert the intended therapeutic effect without causing GI distress. Moreover, to ensure that metabolism is maintained, gaseous fermentation by-products such as carbon dioxide and hydrogen are removed via cross-feeding between gut microbes, which further substantiates the importance of microbial diversity within the gut[65].

It has been previously hypothesised that SCFAs may support the utilisation of physiologically low concentrations of pyruvate and lactate for glucose generation via the gluconeogenesis metabolic pathway. Intermediates such as succinate, lactate, or acetyl-CoA, which are catabolised from pyruvate, do not typically reach high concentrations in the colon[66]. However, these intermediates are further metabolised by cross-feeding of gut microbes, producing SCFAs as a source of fuel. These processes could be beneficial for athletes through the additional delivery of metabolic products to fuel exercise. It has been previously identified that acetate and butyrate are lipogenic whereas propionate is gluconeogenic, and that as much as 69% of total glucose production may be a result of glucose synthesis from propionate. Profiling of gut microbes has shown that synergistic metabolic cross-feeding ensures the microbial gut community is more energetically efficient[37]. Athletes have been identified to harbor a distinct gut microbial community that is associated with training duration. This bidirectional relationship indicates that exercise influences gut microbial composition, while the structure of the gut microbiome may, in turn, affect exercise performance. Several studies have

confirmed gut microbial 'richness' in athletes. A study of competitive cyclists demonstrated an increased abundance of *Prevotella* which *w*as associated with amount of CHO, amino acid and branched-chain amino acid metabolism, as well as increased *Methanobrevibacter smithii* which upregulates the production of methane, a product of CHO metabolism. Supplementation with the dietary fibre dextran has also been shown to enhance *Prevotella* and *Bacteroides* cross-feeding *in vitro*, producing favourable acetate-propionate ratios which have been found to attenuate intracellular lipolysis in human adipocytes. Interestingly, a higher *Firmicutes* to *Bacteroidetes* (F/B) ratio was positively correlated with faecal SCFAs and elite athlete F/B ratios were found to be significantly higher than that of non-athletes. Furthermore, nine weeks of high-intensity exercise also significantly increased the F/B ratio in non-athletes. Herein, the cross-feeding between species is considered to play an important role to induce the optimal ratio of SCFAs to promote energy conversion.

Nutritional strategies that target fat metabolism have been claimed as the future ergogenic aid to benefit endurance athletes[67]. It has been reviewed that endurance exercise and CHO-feeding have anti-lipolytic effects on adipose tissue due to lactate metabolism and insulin secretions, respectively. An investigation using an *in vitro* human white adipocyte model showed that acetate was mainly responsible for the anti-lipolytic effects. Furthermore, the production of propionate by gut microbes has been shown to influence metabolism by altering gut hormone release, reducing caloric intake and thus preventing weight gain with oral sodium propionate increasing the rates of whole-body fat oxidation. The anti-lipolytic effect of SCFAs is considered

to be of benefit to metabolic health in the long term, particularly in obese and diabetic patients who generally have high circulating free fatty acids. Thus, the potential to use SCFAs to induce fat metabolism seems favourable for clinical applications[65]. However, theoretically the anti-lipolytic effect may cause a counterproductive process to increase fatty acid availability needed for energy metabolism in athletes. Data in professional cyclists suggest that certain microbes present in the gut may beneficially influence the upregulation of other metabolic pathways, such as CHO metabolism, to maintain metabolic efficiency in athletes. Moreover, an untargeted metagenomics analysis of faecal microbial species in elite marathon runners showed an abundance of a specific strain, *Veillonella atypica,* with a further animal model investigation to confirm that *Veillonella atypica* improves the running performance time via the metabolic conversion of exercise-induced lactate into propionate[5]. Outside of athletic performance, lean humans who were previously sedentary and underwent aerobic exercise training showed an increase in the abundance of BMI-dependant concentrations of acetate, propionate and butyrate. These changes were seen in response to exercise-induced shifts in SCFA-producing gut bacterial taxa such as *Faecalibacterium spp.* and *Lachnospira spp.* and the expression of butyryl-CoA:acetate CoA-transferase (BCoAT) gene. BCoAT works by transferring a co-enzyme A group from butyryl-CoA to acetate, resulting in the creation of acetyl-CoA and butyrate, two substrates that tissues use for macromolecule synthesis or energy production. However, the conversion of SCFAs to alternative substrates may differ according to the various dietary practices followed by athletes. This could include having an increased intake of fibre or probiotic supplements. However, the role of

SCFAs in energy metabolism seems to be attributed to pre-existing enzymes in the body such as hormone-sensitive lipase (HSL), which could dictate the release of fatty acids. The release of fatty acids from adipose tissue triglycerides into the circulation as a fuel for subsequent exercise requires the activation of the enzyme HSL which is decreased in the presence of sodium acetate. Although athletes possess physiological and metabolic adaptations from various types of training regimes, an early microarray study in mice showed that SCFAs play only a minor role in the expression of genes involved in metabolic regulation at hepatic levels.

The preliminary, but promising, data available for the association of SCFAs and energy availability offer a rationale to athletes for the development of nutritional plans to tailor the makeup of the gut microbiome to increase the production of SCFAs. The reasoning behind this approach would be to increase energy availability for increased exercise capacity and thus performance. Typically, athletes require an increased caloric intake to maintain energy balance from the completion of regular physical activity in training and competition. Elevated total energy intake has been shown to be a driver for increased gut microbial diversity in elite athletes. Interestingly, a study on rugby players showed that players consumed high calories, protein, fat (mono- and polyunsaturated), and CHO per day, as would be expected, but an increased fibre intake (up to as much as 38 g per day) was also found in players when compared to non-athletic controls. Further analysis revealed that protein intake, CK levels, and urea were positively correlated with microbiota diversity. High protein (peptides and amino acids) intake may be something for athletes to consider limiting if its benefits cannot be firmly identified for their

discipline. The reasoning for this is whilst fermentation of protein constituents can yield increased SCFA content via deamination and decarboxylation by certain gut microbe strains. toxic by-products such as amines and ammonia are also formed through these processes. For perspectives, these elite rugby players had 22 phyla, 68 families and 113 genera compared to just 11 phyla, 33 families and 65 genera in the low BMI non-athlete control, and 9 phyla, 33 families, and 61 genera in the high BMI non-athlete control[68]. In another recent study on elite athletes, a total of 182 microbial taxa were detected, which included 2 kingdoms, 11 phyla, 17 classes, 21 orders, 40 families and 81 genera. Further comparison between higher and lower-level athletes showed that *Parabacteriodes* and *Phascolarctobacterium* were the two most prominent genera, with a predicted microbial gene function associated with histidine and CHO metabolism in higher-level athletes. It has been reported that a high intake of calories may increase GI permeability and gut inflammation. This is related to bacterial lipopolysaccharide (LPS) translocation into the systemic circulation across the intestinal membrane. Increased GI permeability and LPS are linked to disruption of metabolic homeostasis in sedentary populations, a condition that may be alleviated by metabolic adaptation through exercise training modulation of the gut microbiota. In this regard, there is considerable interest in the use of SCFAs as anti-inflammatory stimulators in order to offset any potential GI permeability. For example, gut microbial-derived butyrate has been shown to downregulate NF-κB expression *in vitro*, including increasing AMPK to enhance intestinal tight junction protein formation. The knock-out of intestinal AMPK production was found to negatively alter intestinal colon integrity in gut microbiota

composed of a higher abundance of *Clostridiales* and *Desulfovibrionales*. It was said that SCFAs were responsible for the activation of AMPK via acetate. This demonstrates a possible role for SCFAs in the protection of intestinal membrane integrity and endotoxaemia. Enhancing the gut microbiota with probiotics was also shown to reduce LPS in novice long-distance triathletes. Further investigations using SCFAs are needed to determine the exact mechanism of reduced LPS by the gut bacteria-derived SCFAs in athletes.

Although SCFAs may not be considered to be the major source of energy for athletic performance, evidence suggests that they significantly affect energy metabolism on the whole. Findings that demonstrate energy-sensing receptors interacting with gut metabolites provides a rational for the use of SCFAs to maintain energy homeostasis in low energy conditions such as prolonged and intense exercise. The role of SCFAs in fuelling athletic performance appears to follow a two-pronged approach, with exercise training directly affecting the composition and diversity of the gut microbiome and the resultant presence of particular microbes/communities providing further potential to positively impact sports performance. Therefore in theory, the benefits of training on exercise performance may be in some part regulated by the metabolic behaviour of the gut microbiota, with SCFAs offering a credible route to enhance the training response. However, it must be considered that optimising energy availability for athletes would also rely on cross-feeding between microbes to favourably produce metabolites such as SCFAs. Taken all together, the evidence suggests that enhancing microbial diversity should be incorporated as an essential component of an athlete's fuelling strategy.

Practical Considerations

While it is straightforward to provide scientific rationale and dietary recommendations to enhance athletic performance and/or recovery, several important considerations and practical challenges must be addressed when attempting to optimise performance through dietary behaviours. Firstly, the notion that SCFAs could improve sports performance and/or recovery is one of high profile and excitement at this time, but it cannot be overlooked that the very generation of these metabolites within the body is directly affected by diet. This is true for the delivery of precursor molecules for bacterial metabolism (e.g., fibre), but also the impact that dietary patterns/behaviours have on the general composition of the gut microbiome itself. Practically, this means that providing a large shift in dietary content such as increasing fibre intake may or may not increase the production of SCFAs depending on the host microbiome, but also it may not pragmatically translate to increased SCFAs within the circulation. Moreover, a large shift in dietary patterns may reduce the intake of other macronutrients (i.e. CHO and protein) which may not ultimately be beneficial to the athlete. We, therefore, need to understand through research ways to increase systemic SCFAs through realistic and conservative approaches to athlete nutrition. Data in this area are currently lacking and thus studies into athlete dietary manipulations and the impact on basal SCFAs are warranted.

One approach which could be taken is the direct (chemical) supplementation of SCFAs as a postbiotic agent. This would effectively navigate the difficulties of natural dietary interventions on SCFA production by providing a ‘ready-made’ metabolite for immediate uptake into the circulation. Whilst this method may be practical in the

sense that only the addition of a supplement on top of diet is required, there is still much work which must be performed in order to better understand the optimum strategies to follow. For example, should certain SCFAs be targeted over others, or would a multi-metabolite approach be comprehensive in its delivery of beneficial actions? It is likely that the appropriate SCFAs to target would be those that are the most abundant within the body (i.e. acetate, propionate, butyrate), but this still does not address current unknowns around the content and frequency of dosing strategies. This also includes the consideration of whether a short-term or long, continuous, dose is most appropriate to provide an athletic benefit, and whether any potential benefits will be realised via a chronic effect of supplementation or via an acute spike of SCFAs within the circulation. If the latter, the timing of the dose would become highly important. It is therefore important that future research considers not only the impact on sports performance, but also the pharmacokinetics/dynamics of SCFA supplementation itself.

The general market availability of SCFA supplements will directly impact their capacity to be included as a dietary supplement for athletes. At this time, butyrate is available to purchase online in pill capsule format from a small number of manufacturers/suppliers as its metallic salt (e.g. sodium, calcium and magnesium). It is not currently possible to purchase acetate and propionate as specifically designed supplementation products, despite both sodium acetate (E262) and calcium propionate (E282) both being used widely as additives in food manufacturing processes. However, acetate supplementation can be achieved through the intake of vinegar products, with apple cider vinegar capsules readily available to purchase in

health stores. One study has previously demonstrated the ability to increase blood acetate levels, with a sharp spike (approx. 2.5-fold increase) seen 15 min after a drink containing 15 mL vinegar (750 mg acetate), and a smaller but moderately maintained spike (approx. 1.4-fold increase) seen from 1 hr post capsule ingestion (nine capsules totalling 750 mg acetate)[69]. The practicalities of this approach may not make them appropriate for athletes. For example, a drink containing vinegar may not taste pleasant and could cause GI discomfort (although no mention of side effects, or lack of, were included in the publication), and the intake of nine capsules to achieve a desired dose is not realistic. Nonetheless, these data suggest that the increase of circulating SCFAs following ingestion occurs quickly and is short-lived, owing to the consideration that chronic supplementation may not provide a direct impact of SCFAs on metabolic processes. Even so, a chronic supplementation may still provide additional benefits when considering the anti-inflammatory/immunoregulatory responses described in this review, and therefore additional work to enhance our understanding of these physiological interactions is needed. Lastly, we must also consider whether the direct supplementation of SCFAs is well-tolerated within both a general and athletic population. Human studies supplementing with oral acetate salts are not currently available within the literature, and only one publication has reported the supplementation of propionate (as sodium propionate). Neither of these studies reported side effects or discomfort, with the propionate dose considered to be approximately three-times the amount of expected daily endogenous production of propionate (a total of 6845 mg of sodium propionate split across five equal half-hourly tablet ingestions). A number of studies have

provided oral supplementation of sodium butyrate to human participants, with a wide range of individual and daily doses. For example, individual doses as low as 150 mg have been performed without any reported discomfort or side effects[70]. Although not expansive in nature, the current literature suggest that a relatively low dose (e.g. 1-2 g) taken at 1-3 occasions per day is tolerable and therefore may form the basis for future studies in sports focussed research, although more evidence for the supplementation of acetate and propionate salts is required.

Concluding Remarks

It is not a novel thought to suggest that the gut microbiome may play a direct impact on the physiology of its human host, especially when considering daily health and susceptibility/severity of disease. It is also not unknown that there is a bi-directional relationship between the gut microbiome and exercise, with each factor providing an influence on the shaping of the other. However, the consideration for the action of gut bacteria-derived metabolites in sport, with a focus on SCFAs, is of recent and developing interest and poses an thought-provoking avenue for future research surrounding possible links to improvements in exercise performance and recovery, as well as athlete health. The literature examined and included in this review provide a tangible rationale for the direct impact that SCFAs can have on human physiology to afford benefits to the athletic population. These advantages include evidence that SCFAs are able to improve respiratory immunity with the aim to combat the elevated levels/severity of URTIs often reported in athletes; the link between SCFA supplementation and the blunting of pro-inflammatory and pro-fibrotic pathways to

aid in exercise recovery; and the role that SCFAs can play as a direct and usable energy source to help further fuel exercise and, therefore, contribute to improved performance and/or endurance capacity.

All in all, we find ourselves at the beginning of an exciting journey of research into the potential supplementation of SCFAs for sporting purposes. There are many questions that remain unanswered, and it is therefore imperative that research groups from around the globe are engaged in building a body of evidence to understand the most optimal and appropriate approaches to harness the benefits of the gut microbiome for athletic benefit.

CHAPTER 3

A LACTOBACILLUS CONSORTIUM PROVIDES INSIGHTS INTO SLEEP-EXERCISE-MICROBIOME NEXUS IN PROOF OF CONCEPT STUDIES OF ELITE ATHLETES AND IN GENERAL POPULATION

Abstract

The complex relationship among sleep, exercise, and the gut microbiome presents a unique opportunity to improve health and wellness. Here, we conducted the first large-scale investigation into the influence of a novel elite athlete-derived probiotic, consisting of a multi-strain *Lactobacillus* consortium, on sleep quality, exercise recovery, and gut microbiome composition in both elite football (soccer) players (n=11) and the general population (n=257).

Our two-phase study design, which included an open-label study followed by a controlled longitudinal study in a professional soccer team, allowed us to identify key interactions between probiotics, the gut microbiome, and the host. In the placebo-controlled study, we observed significant improvements in self-reported sleep quality by 69%, energy levels by 31%, and bowel movements by 37% after probiotic intervention relative to after placebo. These improvements were associated with a significant decrease in D-ROMS (a marker of oxidative stress) and a significantly higher free-testosterone/cortisol ratio. Multi-omics analyses revealed specific changes in microbiome composition and function, potentially providing mechanistic insights into these observed effects.

This study provides novel insights into how a multi-strain *Lactobacillus* probiotic modulates sleep quality, exercise recovery, and gut microbiome composition in both the general population and elite athletes and introduces potential mechanisms through which this probiotic could be influencing overall health. Our results emphasize the untapped potential of tailored probiotic interventions derived from

extremely fit and healthy individuals in improving several aspects of health and performance directly in humans.

Introduction

Human health is defined by a complex interdependent network of factors that include intrinsic genetic features as well as acquired habits that include diet; alcohol, tobacco, and drug use; as well as exercise habits. Between intrinsic features and acquired characteristics lies the microbiome. The microbiome is shaped by both our genetics and environment, while simultaneously influencing and being influenced by the host's health as well[71]. The bidirectional nature of health / microbiome interactions makes studies of the microbiome complex[72–75]. Many cross-sectional studies suffer from the chicken and the egg problem: Are the microbiome changes observed in a diseased population the cause of the disease under study or the result of poor health caused by the disease. Therefore, a new paradigm for understanding the role of the microbiome in human health is needed. Instead of trying to understand the role of the microbiome in health by comparing the microbiomes of healthy and diseases populations, we identify specific populations with a unique set of stressors to the host that create selective pressure on the gut microbiome. That selective pressure will create a unique microbial ecosystem with specific phenotypes that may help the host respond to the stressors or have negative effects on the host. A longitudinal analysis of these populations can uncover novel connections between the microbiome and host phenotypes (both beneficial and detrimental). For example, the gut microbiome adapts to exercise, and the composition of the microbiome

changes when one changes from a more sedentary to a more active lifestyle[76,77]. Correlating these changes to host outcomes over time can improve our understanding of the bacterial strains promoting health.

The three *Lactobacillus* strains found in the probiotic under study here (*L. acidophilus* FB0012*, L. plantarum* FB0015*, L. rhamnosus* FB0047*)* were isolated from fecal samples of the elite athletes[5]. Elite athletes have a different microbiota composition than sedentary individuals, and in fact, elite endurance runners have been shown to have higher levels of *Veillonella* post-workout (a selective pressure)[78]. While *Veillonella* is particularly interesting in this context, as it has the ability to convert lactate, a byproduct of fatigue, into propionate, a potential energy source for host cells, other strains such as these lactobacilli may also promote health and performance through alternate mechanisms of action.

To understand the role of the microbiome in host health, it is vital to study them using controlled interventional studies. Even in small populations, by collecting baseline data and understanding what aspects of health change alongside important blood and urine biomarkers, we can begin to tease apart the role of specific strains in human health. We can understand pharmacokinetics - Does the microbe engraft? If so, where? We can understand pharmacodynamics - What effect does the microbe have on the host system? Which biomarkers change? And we can begin to hypothesize the mechanism of action - How does the microbe affect the host and cause the specific effects under study?

Physical and athletic performance is dependent on the microbiome[79]. *In vivo* rodent models have shown that voluntary exercise as well as run-to-exhaustion can be decreased by depleting the microbiota with antibiotics and restored through fecal microbiota transplant. Here, we describe the first set of studies reporting on safety and effects of a human athlete-derived probiotic, including an open label study in healthy human volunteers and a controlled longitudinal study in a professional soccer team, which validates the results of the initial open label study in terms of improvements in general health, bowel movements, sleep, and energy. We also collected blood and stool from the placebo controlled study, which has allowed us to perform a detailed multi-omics analysis that has led to some hypotheses and models by which this probiotic exerts its effect in these populations.

Materials and Methods

Origin of probiotic strains

Samples were collected as described previously[78]. In short, volunteers were provided with a 15 ml falcon tube with a 1 ml pipette tip inserted inside. Volunteers were instructed to dip the pipette tips into soiled toilet tissue, then place them back into the tubes and label the tubes with the date and time of collection. Samples were kept at 4 °C for short-term storage until sample pickup, at which point they were placed into a −80 °C freezer for long-term storage. Fecal samples were thawed on ice and resuspended in 2–5 ml of PBS. Serial dilutions were made of resuspended samples and then plated onto MRS agar petri dishes reduced to a pH 5.5 with acetic acid. Plates were incubated under anaerobic conditions at 37C for 48h. Individual

bacterial colonies were picked and transferred to 96 deep well plates and grown in MRS liquid culture for 48h. Bacterial cultures were pelleted for DNA extraction, genome sequencing, and annotation.

Open Label Study

Participants

Emails were sent to more than 1000 potential participants who had expressed interest in FitBiomics products and consented to receiving emails, to recruit participants for the study. Of these, 785 filled out the initial survey and were invited into the study. Those that expressed interest were sent a sample of probiotics and surveys to take before, during, and after 2 weeks of supplementation. 257 participants completed all surveys. Of these 257, the average age was 40.9 years (± 14.6 years). 53% were female, 46% were male, and 1% chose to not share their gender. The majority of survey respondents were White (80%), followed by Hispanic / Latino (10%), Asian (8%), American Indian / Alaskan Native (4%), Black (3%), Middle-Eastern / North African (2%), and Native Hawaiian / Pacific Islander (1%).On average, the participants exercised 5.6 days per week (± 1.7 days). The majority of respondents (58%) did not follow any particular diet, but many respondents followed common types of restrictive diets including intermittent fasting (13%), gluten free diet (12%), dairy free diet (12%), vegetarian diet (5%), vegan diet (5%), paleo diet (4%), pescetarian diet (3%), keto diet (2%), and Whole 30 diet (2%). Gastrointestinal disorders were present in this cohort. 12% had had a previous irritable bowel

syndrome (IBS) diagnosis, 2% had Celiac disease, 2% had inflammatory bowel disease (IBD), and 1% have regular gastroesophageal reflux disease (GERD).

Overview

Surveys, consent forms, and marketing materials were provided to participants. All respondents provided informed consent through online forms. The study was performed over two weeks and consisted of baseline survey data followed by two weeks of supplementation with the probiotic at a low dose (10 billion cfu per capsule) and a high dose (35 billion cfu per capsule). Simple randomization was used to allocate subjects to a group. One capsule was ingested orally per day over two weeks, total = 14 capsules. Participants filled out online survey questionnaires at the end of the first seven days and at the end of 14 days.

Supplementation

Participants were required to take one capsule per day for 14 days. Each capsule contained 10 billion or 35 billion cfu of lyophilized *L. acidophilus* FB0012, *L. plantarum* FB0015, and *L. rhamnosus* FB0047 with dehydrated potato starch. The probiotic strains are encapsulated in acid resistant capsules and packaged in Alu-Alu blister packs.

Surveys

The questionnaires were created using TypeForm (www.typeform.com). Four questionnaires in total were sent to participants. The first was an intake form

consisting of 23 demographics, habits, and lifestyle questions. The second was 18 questions focused on understanding the baseline symptom and habit status of that participant that week. The third survey (25 questions) was taken after one week of supplementation, and the fourth survey (41 questions) was taken after the second week. Both of these surveys asked questions related to benefits and side effects experienced by the participants. The fourth and final survey also included a variety of questions related to overall experience during the study, willingness to purchase the product, and likelihood of recommending the product.

Data processing and statistics

Survey data was exported from Typeform into an Excel spreadsheet. Survey included numerical, categorical, and open ended questions. Data was processed into a format compatible with analysis using Python 3.6 in a Jupyter Python notebook. Categorical data was converted to numerical data when possible, and open ended questions were analyzed manually or scanned automatically for key words / phrases and converted into categorical data. Statistically significant differences were calculated using Python's Numpy and Scipy libraries using the Mann-Whitney U Test and a significance level of < 0.05. Random Forest modeling was performed using the Python Sci-Kit Learn library.

There was no significant difference between arms in terms of whether the participants believed the treatment improved their overall health and wellness (assessed using a Chi-square test). However, significant differences in ratings of different benefits post-placebo and post-probiotic were assessed using an

independent paired T test, and four of the eight benefits were found to have significant differences. The choice of statistical tests was dictated by the distribution and nature of the data. Mann-Withney U was used for independent group comparisons where the data were not normally distributed or were ordinal in nature, Paired-t-test specifically used for longitudinal or matched-pair data.

Controlled Longitudinal Study

Participants

Eleven trained, male elite soccer players (body mass 81.5 ± 2.5 Kg, height 1.85 ± 0.2 m, age 26.5 ± 2.0 years), completed this pilot study. No players were taking non-steroidal anti-inflammatory drugs, or had taken antibiotics or probiotic supplements in the preceding 3 months. Soccer players did not have any gastrointestinal disorders, and did not report taking any purposeful ergogenic aids (aside from macronutrients) or supplements for 1 month prior to the study. As this is not a double-blind study, soccer players were blinded to the treatment, but not the researchers. Moreover, this is not a two-arm study but a single arm longitudinal study with a placebo period followed by a probiotic supplementation period. We used identical capsules for the “probiotics" and "placebo" with no difference in look, smell, or taste. As the capsules were identical, it was impossible for the players to discern which capsules were the probiotics and which ones the placebo. They were told they would be taking both placebo and probiotic but not told which order.

Overview

The Experimental protocol was approved by the Ethical Independent Committee for Clinical, not pharmacological investigation in Genoa (Italy) and following the ethical standards of the 1964 Declaration of Helsinki (Rif. 2020/12). The protocol was retrospectively registered on ClinicalTrials.gov (registration number: n.NCT06093139). All the volunteers signed an informed consent. All persons gave their informed consent prior to their inclusion in the study and details that might disclose the identity of the subjects under study have been omitted. The study was performed during an overall period of 25 weeks, starting the 8th November 2021 and completed the 6th May 2022. In detail, it was a first period of 12 weeks where athletes ingested 4 capsules of placebo per week, followed by 1 week of non-supplementation, and another 12-weeks period where athletes ingested 4 capsules of probiotics per week.

Supplementation

During the first 12 week period (from 8th November 2021 to 6th February 2022) soccer players were required to take one capsule per day for four consecutive days of the placebo capsule (placebo consisted of the same capsules filled with potato starch), then one week of non-supplementation, followed by the second period of supplementation (from 14th February 2021 to 6th May 2022) where players were required to take one capsule per day for four consecutive days of the probiotic capsule (composition same as described above - *L. acidophilus* FB0012, *L. plantarum* FB0015, and *L. rhamnosus* FB0047 encapsulated in acid-resistant

capsules alongside additional potato starch as a filler). The players received probiotics during the week, on the days of their training sessions during the training season. The Club would not change their training schedule, and every player would follow their nutritional periodization plan and a supplementation periodization plan as normal. The players were not aware of the content of the capsules.

Short Questionnaire

A 12-items short questionnaire was administered after the placebo-supplementation period and after the probiotics-supplementation period. The first nine of these questions consisted of symptoms and benefits experienced by the participants as measured using a numerical rating scale from 1 to 5, where 1 corresponded significantly worse compared to their personal normal, 3 corresponded to no change from personal normal, and 5 corresponded significantly better compared to their personal normal. Two of the final three questions were related to potential adverse effects experienced by the participants, and the final question was on the belief that these probiotics would affect the participant's overall health and athletic performance.

Blood collection and analysis

Venous blood samples were obtained by venipuncture one week before the start of this pilot study (baseline values), one week after the placebo-supplementation period, and one week after the probiotics-supplementation period, always at the same time of day. Blood samples were centrifuged at 1,000 x g for 15 min and cell-free plasma or serum was aliquoted and stored at -80°C until analysis. Plasma 25-

hydroxyvitamin D3 was analyzed by chemiluminescent immunoassay on a Roche Elecsys Analyzer 170. The ferritin concentration was measured using the immunoturbidimetric method, on a Roche Cobas Integra 400 biochemical analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Serum cortisol (C) was analyzed with competitive immunoassay using direct chemiluminescence technology (Advia Centaur, Siemens). Free testosterone (FT), was analyzed with ELISA enzyme immunoassay, REF-EIA-29294 (DRG instruments GmbH, Germany); thus the FT:C ratio was obtained. The reactive oxygen metabolites (ROMs) and the biological antioxidant potential (BAP) were measured using the derived ROMs (d-ROMs) and BAP kits, respectively, from Diacron (Grosseto, Italy), according to the manufacturer's instruction. TNF-alpha (TNF-) was measured by ELISA enzyme immunoassay. The study was completed from November and May of the same season (2021-2022) and all players began the study at the same time.

Fecal Metagenomics

Fecal samples were self-collected by each participant using Fe-Col (Alpha Laboratories Ltd, Eastleigh, United Kingdom), a disposable paper device to prevent sample contamination, and SMARTeNAT (Copan SpA, Brescia, Italy) for fecal sampling and preservation, as previously done[78]. Samples were stored at -80C within 72 hours of collection. One stool sample aliquot was stored raw, while 25 mg was used for DNA extraction. DNA was extracted using the DNeasy Powersoil kit and standard protocols. Shotgun metagenomics analysis of stool samples was performed at Diversigen. Samples underwent Diversigen's standard quality control

(PicoGreen ds DNA quantification) and library preparation. Samples were sequenced on an Illumina NovaSeq 6000 using a 2x150bp flow cell with a mean target depth of 20 million reads.

Fecal Metabolomics

Metabolomics was performed on ProDigest's MetaKey global polar metabolomics platform. Raw stool samples were subjected to a solid-liquid based extraction protocol, and subsequently lyophilized. 50.0 ± 1.0 mg of lyophilized material was weighed and dissolved in ultrapure water that contained internal standards. After shaking the resulting mixture and performing subsequent centrifugation, the supernatant was collected and filtered through a polyvinylidene fluoride filter (0.22 µm pore size). From the purified extract, a 10-µL aliquot was injected into the ultra high performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) system and performed as described previously[80,81]. Chromatographic separation was achieved on a Vanquish quaternary pumping system (Thermo Fisher Scientific, USA), equipped with an Acquity HSS T3 C18 column (1.8 µm, 150 x 2.1 mm) (Waters Corporation, UK). A binary solvent system consisting of ultrapure water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, was used at a constant flow rate and by applying a gradient profile. Detection was performed on a Q-Exactive™ standalone bench top quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, USA), which was preceded by heated electrospray ionization (HESI-II source) in polarity switching mode. The instrument was operated at a resolution of 140,000 full

width at half maximum and in full-scan mode (m/z scan range of 53.4 – 800 Da), meaning that all detected ions were registered whereby no fragmentation was applied. Metabolites were compared to an in-house library and the analysis targeted using an open scan profiling.

Metagenomics Data Processing and Analysis

DNA sequences were taxonomically classified using the MetaPhlAn2 analysis tool. Functional profiling was carried out using the HUMAnN2 analysis pipeline. The functional tables were filtered to the same subset of samples as the filtered taxa tables. Univariate statistics were used as well. Values below the limit of quantitation (LOQ) were replaced by half of the lowest detected relative abundance above the LOQ. Selection of statistical tests was based on a set of preliminary parametric tests, applied separately. Normality was assessed using Shapiro-wilk normality tests (cut-off for rejection of normality p-value ≤ 0.05) and by reviewing quantile-quantile (Q-Q) plots, homogeneity of variances was checked using Levene's tests (cut-off for rejection of homogeneity of variances p-value ≤ 0.05) and outliers based on Z-scores (cut-off of |z-score|> 3.5). As the large majority of metabolites were found to be non-normally distributed, we opted to use Wilcoxon tests to compare treatments, unless otherwise stated.

Metabolomics Data Processing and Analysis

Metabolites were identified and quantified using Xcalibur software version 4.0.27.21 (Thermo Fisher Scientific, USA) was used. In general, a metabolite was considered

below the LOD/LOQ if the metabolite's observed peak area was below 1000,000 arbitrary units. Following peak integration, the area ratio was determined for each metabolite by calculating the ratio between the area of the metabolite and that of the most suited internal standard. For the applied multivariate statistical analysis, unsupervised principal component analysis (PCA X) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) modeling were performed using SIMCA® version 17. We focused on metabolites that were significantly changed compared to baseline and / or compared to placebo but for where there was no significant change between baseline and placebo.

Multi-omics analyses

We used Qiime2[82] to calculate microbiome diversity measures, and chose Shannon entropy as the measure of alpha diversity. Differences in Shannon diversity were determined by the Kruskal-Wallis test (normalized p-value ≤ 0.05). Qiime2 longitudinal module, specifically volatility analysis, was used to calculate net average abundance changes of taxa over the course of the study. Correlations between taxa, functions and metabolites were calculated using Qiime2 SCNIC module (SparCC method)[83]. The significance threshold was set at $|R| > 0.1$. For every metabolite, a taxonomic-functional cluster was determined, composed of species and functions correlated with the metabolite and with each other. A correlation heatmap was created using Python *seaborn* package. The final correlation network was manually created based on connections of metabolites with similar clusters or depending on their contributions to pathways of interest based on literature sources.

Testosterone analysis

To investigate the importance of the 3β-hydroxysteroid dehydrogenase gene and whether its relative abundance was changed during the study, each sample was blasted against the known gene from *Mycolicibacterium neoaurum* (NZ_CP074376.1: 2036414-2037514), and the number of hits was compared between baseline, placebo and probiotic groups. Only hits with a similarity of at least 97% were considered, and the final comparison was made possible by normalizing the hits by the number of sequences in the forward read pair of each sample. A two-tailed t-test was performed to determine whether there were significant differences between the groups.

Results

Open label study identifies benefits in sleep and energy

The open label study was designed to help understand the benefits associated with supplementation of three athlete-derived probiotic strains (commercially known as Nella and provided by FitBiomics): *L. acidophilus, L. rhamnosus,* and *L. plantarum* encapsulated in acid-resistant delayed release capsules with potato starch as additional filler. During the study, the participants supplemented with a high dose (3.5×10^{10} cfu) or low dose (1×10^{10} cfu) probiotic, one oral capsule per day, for two weeks. At baseline, 1 week, and 2 weeks, participants were asked to fill out surveys. The baseline survey consisted of baseline habit and lifestyle questions, medical history, demographics, and other questions to help understand the types of participants who were interested in this probiotic. The 1 week and 2 week surveys

focused on tracking adverse events (AEs) as well as understanding the benefits that participants were experiencing. The final survey (at 2 weeks) also included final questions on overall experience during probiotic supplementation.

More than 1,000 participants were invited into the study, and while 756 completed the first survey, 257 completed all surveys (Figure 2a). Surveys from these 257 were used for analysis. Most AEs were mild, limited, and included symptoms generally associated with probiotic use such as gas, bloating, mild stomach upset, and changes in bowel movements. These generally subsided by the end of probiotic supplementation. There were no significant differences in AEs between high and low dose groups. Of the 257 participants who completed all surveys, 12 (4.7%) left the study due to the side effects that they experienced.

After 2 weeks of probiotic use, 94% of participants reported benefits in at least one of the areas assessed (Figure 2b). The top five most commonly reported benefits include improved sleep quality (45.1%), shorter recovery time post-exercise (38.5%), decreased frequency of fatigue (38.3%), decreased soreness after workouts (38.1%), and better / more regular bowel movements (34.6%). Participants were also asked to rate their overall experience with the probiotic at the end of the two weeks. We used these data to create a model to evaluate which characteristics those that experienced benefits had in common or were driving the reported positive experience. A random forest machine learning model was implemented to identify features that drove the best overall experience (AUC = 0.74). Of the features deemed most important, four features (General health/digestion/fitness, change in bowel movements, energy level, and current health/wellness) were benefits that had

statistically significant differences between high and low overall experience ratings (Figure 2c-f). These results show that those that had the highest rated overall experience tended to report better current health, higher energy, better bowel movements, and better general health / digestion / fitness than those that reported a low overall experience.

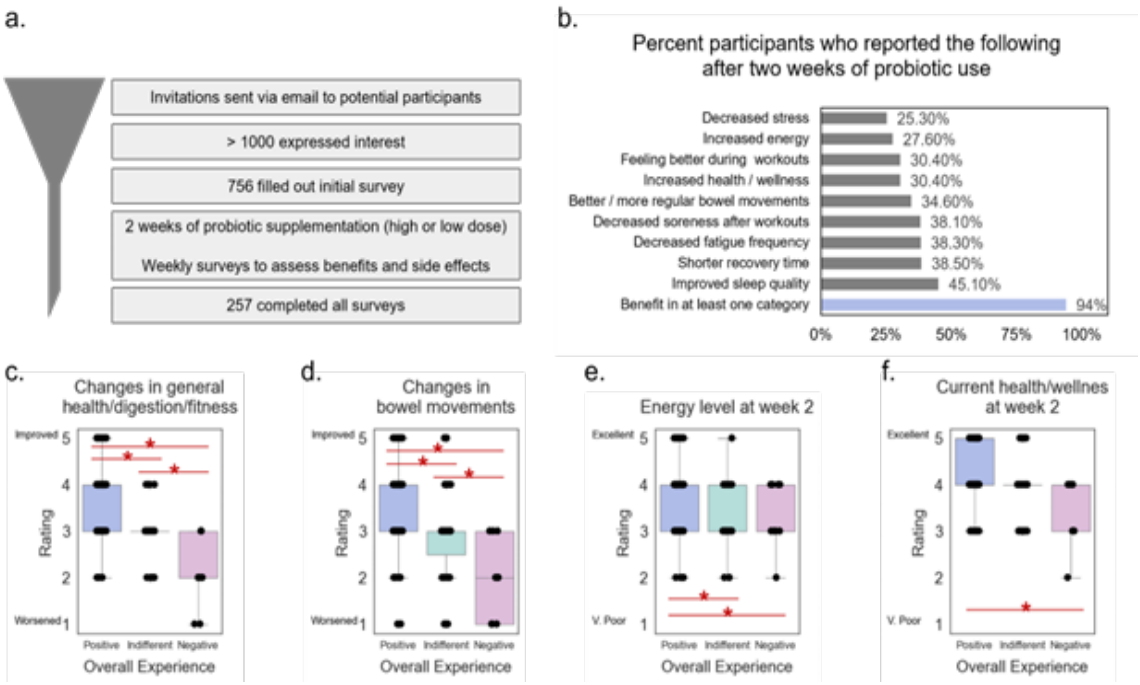

Figure 2: **Open Label Study was performed to better understand the product benefits.** a) Over 1000 participants were invited to the study. Participants took 1 capsule of probiotic (either a high or low dose) daily for 2 weeks, completing a baseline survey and additional weekly surveys. 257 participants completed the survey. b) 94% of participants reported an improvement in at least one of the benefits described in the survey. The three benefits most often reported included increased sleep quality, shorter recovery time, and decreased frequency of fatigue. c) Four features of the random forest model were benefits that had statistically significant differences between high and low overall experience ratings. Statistically significant changes are marked with red stars.

Controlled longitudinal study in soccer club used to validate open label study

To validate the results of the open label study and further explore the benefits associated with probiotic use, we designed a placebo-controlled longitudinal study and enrolled 15 elite Italian soccer players from a professional SERIE A team. Of the 15 who started the study, 11 completed all aspects over the 24 week study (Figure 3a). All participants were males (age: 28.7 ± 5.0 years), who perform regular intense exercise as part of the soccer training and competition season. As part of the study, participants took four capsules per week for 24 weeks and were blinded to whether they received placebo or probiotic. During the first 12 weeks, they consumed four placebo capsules per week, and during the second 12 weeks, they consumed four probiotic capsules per week containing 1 x 10^{10} cfu probiotic strains. There was a one week washout between 12 week periods Stool, urine, and blood were collected at baseline, post-placebo, and post-probiotic. No significant changes to diet and exercise load occurred during the study period.

A short survey was also administered post-placebo, and post-probiotic, which included 8 questions on potential benefits of the probiotic (on a 1 - 5 rating scale). The survey questions assessed many of the same benefits that the open label study did. No AEs were reported by participants, and no participant discontinued due to AEs. There was no significant difference between arms in terms of whether the participants believed the treatment improved their overall health and wellness (assessed using a Chi-square test). However, significant differences in ratings of different benefits post-placebo and post-probiotic were assessed using an independent paired T test, and four of the eight benefits were found to have

significant differences (Figure 3b). These benefits included a general health rating, improved sleep quality, improved energy level, and improved bowel movements - some of the same benefits that were most commonly reported by the participants in the open label study. In particular, these results validate the observed improvements in sleep quality and bowel movements, which seem to promote better general health.

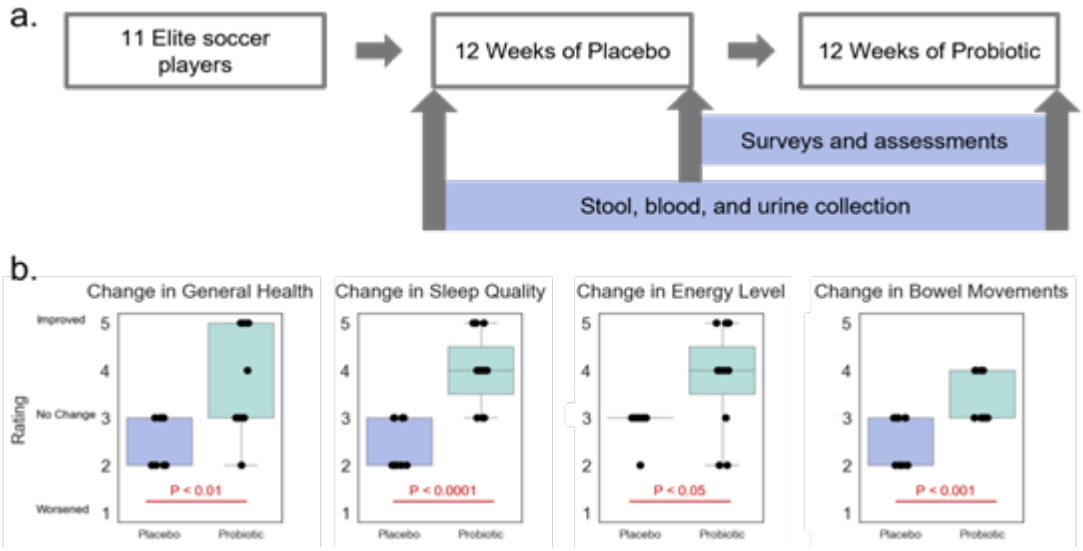

Figure 3: Results of the controlled longitudinal study in a professional sports team. a) Study participants were recruited from a professional Italian soccer team. Participants were provided with placebo for 12 weeks, followed by a short washout and then probiotic for 12 weeks. Stool, blood, and urine were collected at baseline, after placebo, and after probiotic use. Surveys and assessments were performed after the placebo and after the probiotic. b) Four benefits were found to be significantly improved after the probiotic compared to the placebo: General Health, Sleep Quality, Energy Level, and Bowel Movement Quality.

Samples were collected for understanding mechanism of action

While probiotics are well-known for their ability to promote better gut health, digestion, and regular bowel movements, and some small studies have even found correlations between gut microbiome composition and sleep quality[84–86], and that

probiotics may promote sleep quality under specific disease conditions[87,88], the results of interventional probiotics studies have varied enough that the benefits of probiotics for sleep in the general population and clues to the underlying mechanisms remain an open question[89,90]. Therefore, we were interested in using the biological samples collected from participants for developing hypotheses for a mechanism by which the probiotic bacteria could potentially improve sleep quality of the host. A panel of 32 plasma biomarkers were chosen for analysis. These biomarkers included general measures of health as well as commonly measured biomarkers associated with exercise and performance. An ANOVA analysis was performed to identify trends ($p < 0.1$) and significant ($p < 0.05$) differences associated with probiotic use as well as markers that simply tend to change over time (Table 1, Figure 4).

The majority of the biomarkers with significant changes seemed to be related to 1) oxidative stress / inflammation, 2) recovery from strain, and 3) red blood cell physiology. Markers from these three categories seem to be both changing over time and potentially with probiotic use. Starred biomarkers in Table 1 (and shown in Figure 4) correspond to those biomarkers with unique changes associated with probiotic use (changes from baseline to probiotic or from placebo to probiotic timepoints but no change or opposing change from baseline to placebo timepoints). Three of these markers are related to testosterone levels: free testosterone, free testosterone to cortisol ratio (FT:C), and plasma testosterone to cortisol ratio (T:C). These markers, in particular FT:C, are associated with improved recovery from physical strain and could contribute to the increased energy participants reported[91].

Markers of oxidative stress were also changed. Derivatives of reactive oxygen metabolites (D-ROMS) and biological antioxidant potential (BAP) decreased with probiotic use compared to baseline. The hematocrit decreased with probiotic compared to placebo but the values generally remain within the healthy range for adult males. TNF-alpha and IL-6 are two pro-inflammatory cytokines that also change over time. TNF-alpha increases with probiotics compared to baseline. However, IL-6 displays some remarkable changes. After the placebo period, IL-6 concentration dramatically increases, but after probiotic, it returns to baseline levels. However, IL-6 is a transient and acute marker of muscle work, and it is not possible to assign these changes only to the probiotic use, considering the activity load of previous days preceding the blood samples.

Vitamin D3 displayed a similar but opposite pattern - decreasing during placebo but increasing back to baseline levels with probiotic.

To determine whether any of these biomarker changes were associated with any of the benefits significantly associated with probiotic compared to placebo, we classified participants as responders (reported a benefit) or non-responders (no benefit or worsening of symptoms reported). Due to the small number of participants in the study and the generally high fraction of responders, there were no significant associations between these biomarkers and response.

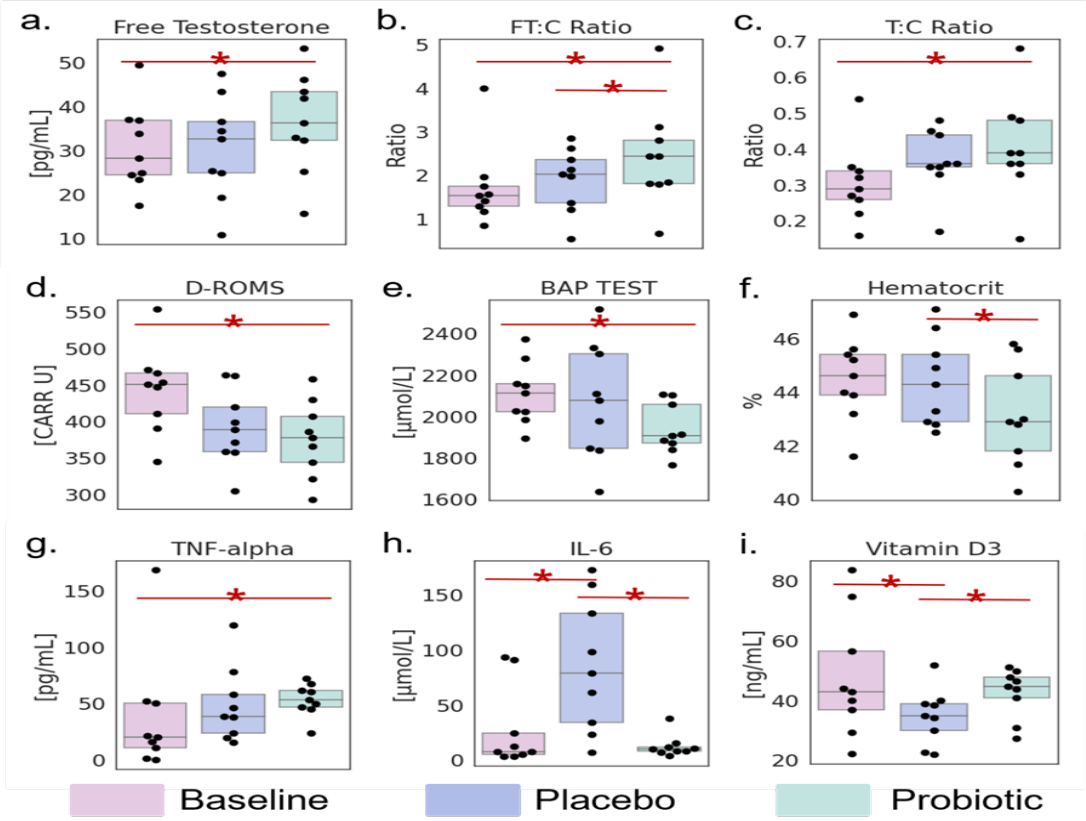

Figure 4. Blood biomarkers that change with probiotic use. Plasma biomarkers were assessed to understand which host biomarkers changed with probiotic use. a-c) Testosterone increases were associated with probiotic use. In particular, free testosterone and the free testosterone to cortisol (FT:C) ratio were increased with probiotic compared to baseline and placebo. Plasma testosterone to cortisol ratio (T:C) was only increased with probiotic compared to baseline. d-e) Markers of oxidative stress including derivatives of reactive oxygen metabolites (D-ROMS) and biological antioxidant potential (BAP) appeared to decrease with probiotic use compared to baseline. f) Hematocrit was decreased with probiotic compared to placebo. g-h) Important cytokines also changed with probiotic use. TNF-alpha was increased with probiotic compared to baseline. IL-6 significantly increased with placebo and decreased with probiotic back to baseline levels. i) Vitamin D3 also significantly changed at both timepoints, but was decreased with placebo and increased back to baseline levels with probiotic.

Table 1. Plasma biomarkers that changed (significant or trending) with probiotic use

Significant values ($p < 0.05$) are bolded, while those with only a trend ($p < 0.1$) are italicized. Starred biomarkers are those with unique changes with probiotic but not placebo. We can roughly classify all

these markers into 3 categories: 1) changes to testosterone / cortisol ratio (amount of strain on body / whether getting sufficient recovery); 2) Measures of inflammation and oxidative stress; 3) Changes to red blood cells (iron levels, etc).

Biomarker	**Change observed with placebo**	**Change observed with probiotic**	**Main effect p-value**	**p value (T0 - T1)**	**p value (T0 - T2)**	**p value (T1 - T2)**	**Category**
Vitamin D3*	Decrease from baseline	Increase from placebo	**0.011**	**0.017**	0.422	**<0.001**	Other
TNF-alpha*	No change	Increase from baseline	**0.045**	0.321	**0.012**	*0.091*	Oxidative stress / inflammation
IL-6*	Increase from baseline	Decrease from placebo	**0.035**	**0.014**	0.810	**0.024**	Oxidative stress / inflammation
Plasma testosterone	No change	No change	*0.087*	0.574	*0.074*	*0.085*	Recovery from strain
T:C ratio*	No change	Increase from baseline	*0.066*	*0.091*	**0.022**	0.485	Recovery from strain
FT:C ratio*	No change	Increase from baseline and placebo	**0.035**	0.810	**0.014**	**0.024**	Recovery from strain
Free testosterone*	No change	Increase from baseline and placebo	**0.003**	0.560	**0.017**	**<0.001**	Recovery from strain
Cortisol	Decrease from baseline	No change	*0.076*	**0.023**	0.174	0.303	Recovery from strain
Transferrin saturation	Decrease from	Decrease from baseline	**0.021**	**0.039**	**0.011**	0.875	RBC physiology

(TSAT)	baseline						
MCV	No change	No change	*0.086*	*0.051*	*0.051*	1.000	RBC physiology
Homocysteine	Decrease from baseline	Decrease from baseline	**0.018**	**0.007**	**0.009**	0.897	Other
Iron	Decrease from baseline	No change	**0.036**	**0.028**	*0.058*	0.560	RBC physiology
Hematocrit*	No change	Decrease from placebo	**0.045**	0.847	*0.077*	**0.036**	RBC physiology
Eosinophils	Increase from baseline	Increase from baseline	**0.013**	**0.005**	**0.005**	1.000	RBC physiology
D-ROMS*	No change	Decrease from baseline	**0.005**	*0.052*	**0.001**	0.301	Oxidative stress / inflammation
BAP test*	No change	Decrease from baseline	*0.095*	0.912	**0.015**	*0.054*	Oxidative stress / inflammation

Metagenomics and metabolomics analyses were combined to hypothesize mechanisms for survey and biomarker results

To understand how these biomarkers are being influenced by the gut microbiota we performed a multi-omics analysis consisting of deep (20 million reads) shotgun metagenomics sequencing and global polar metabolomics profiling of the stool samples provided by the participants. We performed an initial summary analysis of the metagenomics data and used Shannon entropy as a measure of alpha diversity

(Figure 5a). The alpha diversity did not change over time. However, a longitudinal analysis of the data using net average change in abundance calculated as a part of Qiime2's longitudinal module volatility analysis identified 11 taxa that significantly increased or decreased in relative abundance from baseline (Figure 5b, Table 2). Of these eleven, seven increased only with probiotic, not with placebo. However, with the exception of *Faecalibacterium prausnitzii*, after plotting these strains individually, the average change over time seems to be driven by a small subset of participants and is not seen generally.

To gain a better pharmacokinetic understanding of the probiotic product, we queried the metagenomics data for the species of lactobacilli present in the probiotic (*L. plantarum, L. acidophilus*, and *L. rhamnosus*). While there were no significant changes in abundance of these species over time, there was a significant increase in the Lactobacillaceae family in general (Figure 5d). The scientific consensus is that lactobacilli tend to be transient members of the gut community, so it is not surprising that the abundance of these specific species does not change over time with probiotic usage. However, they do promote a healthier gut environment overall, which may explain the slight increase in Lactobacillaceae abundance.

Next, because of the changes in testosterone observed during the study, we investigated whether the microbiome could be modulating testosterone levels. Other studies have found that features of the microbiome such as relative abundance of Firmicutes correlates with testosterone levels in males. Furthermore, 3βhydroxysteroid dehydrogenase is an enzyme known to be expressed by gut microbes that can degrade testosterone[92]. Abundance of genes encoding this

enzyme have been negatively correlated with testosterone levels. Here, we looked for sequences with similarity (97% cutoff) to this gene in the data at baseline, post-placebo, and post-probiotic. We found that there were no significant changes in the fraction of hits at the baseline, placebo, and probiotic timepoints (Figure 5d). However, in general, there were few hits similar to this sequence. This sequence was chosen due to previous reports suggesting a role of this gene in human testosterone levels, but there may be less similar gene sequences with similar functions that are currently unknown. Furthermore, we may be prevented from observing significant differences due to the small sample size of this study.

The global polar metabolomics analysis yielded 178 metabolites detected above the methodological limit of quantitation. Orthogonal partial least squares discriminant analysis modeling (OPLS-DA) was used to identify metabolites associated with the different timepoints in a supervised manner. Twenty-one metabolites were significantly associated with different timepoints in the study. We focused our analysis on those 8 metabolites that were found to be important in baseline vs post-probiotic and post-placebo vs post-probiotic but not between baseline and placebo (Table 3).

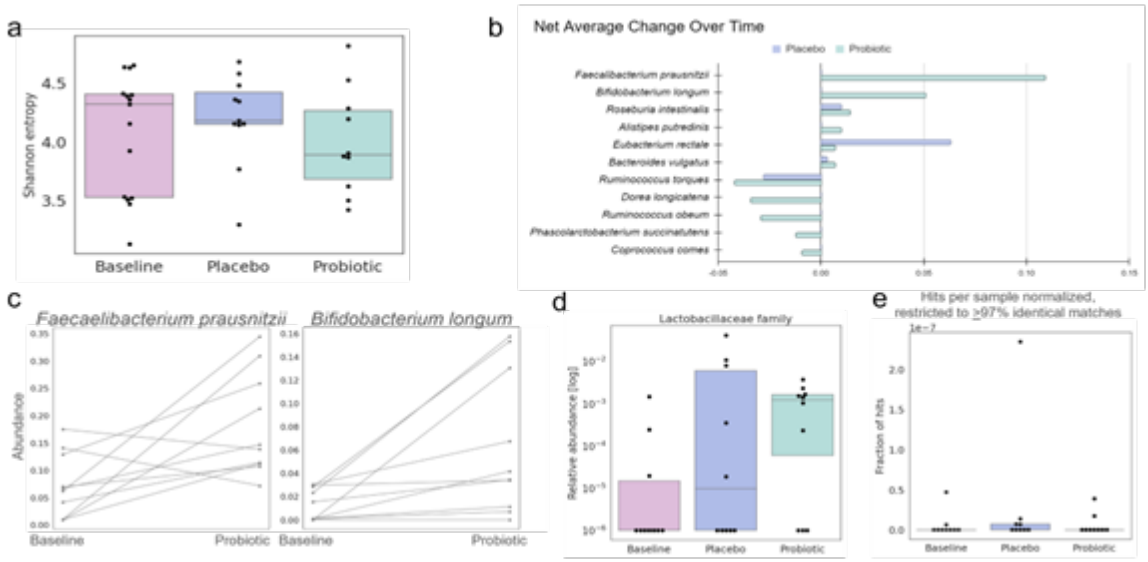

Figure 5. Microbiome community changes over time. a) There were no changes in alpha diversity over time. b) A longitudinal analysis of the microbiota community using the longitudinal module of Qiime2 found a list of bacterial species that change over time with placebo and / or with probiotic. c) These observed changes are generally driven by changes in a subset of participants. Example here shown with *F. prausnitzii*, which increases in most participants over time in contrast with *B. longum*, which increases in only a subset of participants. d) The abundance of the Lactobacillaceae family increases with probiotic use but not placebo. e) No significant differences were observed in terms of number of sequences that are similar to 3β-hydroxysteroid dehydrogenase, which has the ability to degrade testosterone.

Table 2. Taxa that significantly change over time

Bolded taxa change only with probiotic use. Net average change is in terms of relative abundance.

Species	**Net average change with Probiotics**	**Net average change in Placebo**
Faecalibacterium prausnitzii	**0.109**	**NA**
Bifidobacterium longum	**0.051**	**NA**

Roseburia intestinalis	0.014	0.010
Alistipes putredinis	**0.010**	**NA**
Eubacterium rectale	0.007	0.063
Bacteroides vulgatus	0.007	0.003
Ruminococcus torques	-0.042	-0.028
Dorea longicatena	**-0.034**	**NA**
Ruminococcus obeum	**-0.029**	**NA**
Phascolarctobacterium succinatutens	**-0.012**	**NA**
Coprococcus comes	**-0.009**	**NA**

Table 3. Metabolites that are associated with different timepoints in OPLS-DA modeling. The Variable Importance in Projection (VIP) score is used as the indicator of importance here. A VIP score > 1 indicates a metabolite that is relatively important. All metabolites were found to be decreased in the probiotic condition compared to baseline or to placebo.

Metabolite	**Baseline vs Probiotic**		**Baseline vs Placebo**		**Placebo vs Probiotic**	
2-hydroxyisocaproic acid	0.40	$p < 0.05$	0.48	$p < 0.05$	0.83	$p > 0.05$
Alanine / Sarcosine	0.71/0.72	$p < 0.05$	0.93/0.94	$p > 0.05$	0.76/0.76	$p > 0.05$
Betaine	0.58	$p > 0.05$	0.77	$p > 0.05$	0.75	$p > 0.05$
Citrulline	0.74	$p > 0.05$	1.19	$p > 0.05$	0.62	$p < 0.05$

Nicotinic / Picolinic acid	0.79	$p > 0.05$	1.16	$p > 0.05$	0.69	$p < 0.05$
Pantothenic acid	0.73	$p > 0.05$	1.01	$p > 0.05$	0.72	$p < 0.05$
Threonine	0.77	$p > 0.05$	1.16	$p > 0.05$	0.66	$p < 0.05$
Fructose / Galactose	0.58	$p > 0.05$	1.10	$p > 0.05$	0.52	$p > 0.05$

In order to understand how these metabolites might be related to changes in the microbiome in terms of both which taxa are represented in the sample as well as which functions and pathways are present, we used a SparCC based correlational approach (Figure 6a). First, the eight metabolites that likely changed due to probiotic consumption were correlated with species that changed in the metagenomics data. Similarly, those same eight metabolites were correlated with the functions and pathways present in the metagenomics data. Once we had that list of species as well as list of functions/pathways correlating with specific metabolomic changes, we assessed which of the taxa and functions correlated with each other.

This resulted in a list of metabolites, taxa, and functions/pathways that were potentially connected to changes due to probiotic use. A clustering-based approach was inconclusive, so a biosynthetic approach was utilized to understand the relationship between these features. This approach identified a cluster of metabolites and pathways related to oxidative stress (Figure 6b). Nicotinic acid / picolinic acid (difference could not be determined with the metabolomics methods used) was negatively correlated with arginine and ornithine degradation and menaquinol

biosynthesis. Both arginine and ornithine can be broken down to produce NO. Citrulline is another metabolite found to be significantly changed (decreased) with probiotic use. However, citrulline can be recycled back to ornithine. Menaquinol and two molecules of nitric oxide (NO) can be converted into menaquinone, nitrous oxide, and water.

Menaquinone (Vitamin K) is an important nutrient for the human host, with roles in bone, heart, blood, immune, and cognitive health, and absorption of microbially sourced vitamin K from the small intestine is vital for human health. Both nitric oxide and menaquinone are known antioxidant molecules, and microbially modulated nitric oxide has roles in promoting better exercise performance in the mouth microbiome. Nitric oxide may also inhibit cortisol production, which could help explain the testosterone:cortisol ratios observed in the biomarker analysis. Four taxa were correlated with these changes: *Bacteroides finegoldii*, *Eubacterium cylindroides*, *Eubacterium ventriosum,* and *Oscillibacter* unclassified. *Bacteroides* species and *Eubacterium lentum* are known biosynthetic producers of menaquinone. Three out of four of the species here are potential menaquinone producers. In the model we hypothesize here (Figure 7), the probiotic encourages growth of menaquinone producing bacteria. Increase in NO and menaquinone modulates oxidative stress, increasing energy and promoting better sleep for the host.

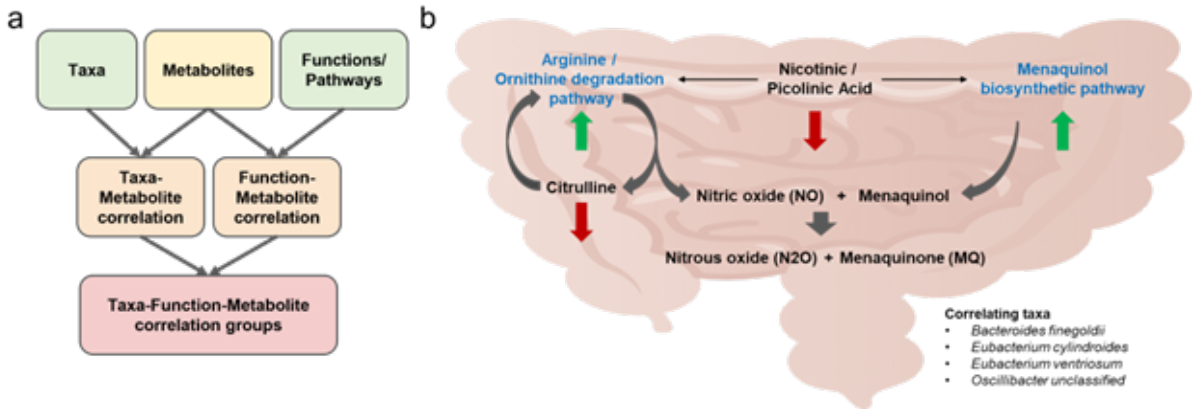

Figure 6. A multi-omics analysis was performed to understand microbiota and metabolite community changes in the athletes. Metabolites that changed with probiotic use but not placebo were identified. a) The SparCC algorithm was used in a correlational analysis of taxa, pathways, and metabolites that changed over time. In this way, we identified a list of taxa, pathways, and metabolites that correlate with each other. b) A biosynthetic pathway approach was used to understand how the different correlating taxa, pathways, and metabolites were related, and a cluster was identified that was associated with menaquinone production and reduced oxidative stress. Nicotinic / picolinic acid is a biosynthetic product of tryptophan. In our data we found significant correlations between nicotinic / picolinic acid and menaquinol biosynthesis and arginine / ornithine degradation. Arginine / ornithine is metabolized into menaquinol and nitric oxide with citrulline as a byproduct. Menaquinol is converted into menaquinone (Vitamin K) and nitrous oxide (N2O) Citrulline can be converted back to ornithine to continue this process. The *Bacteroides* genus and some *Eubacterium* species are known producers of menaquinones.

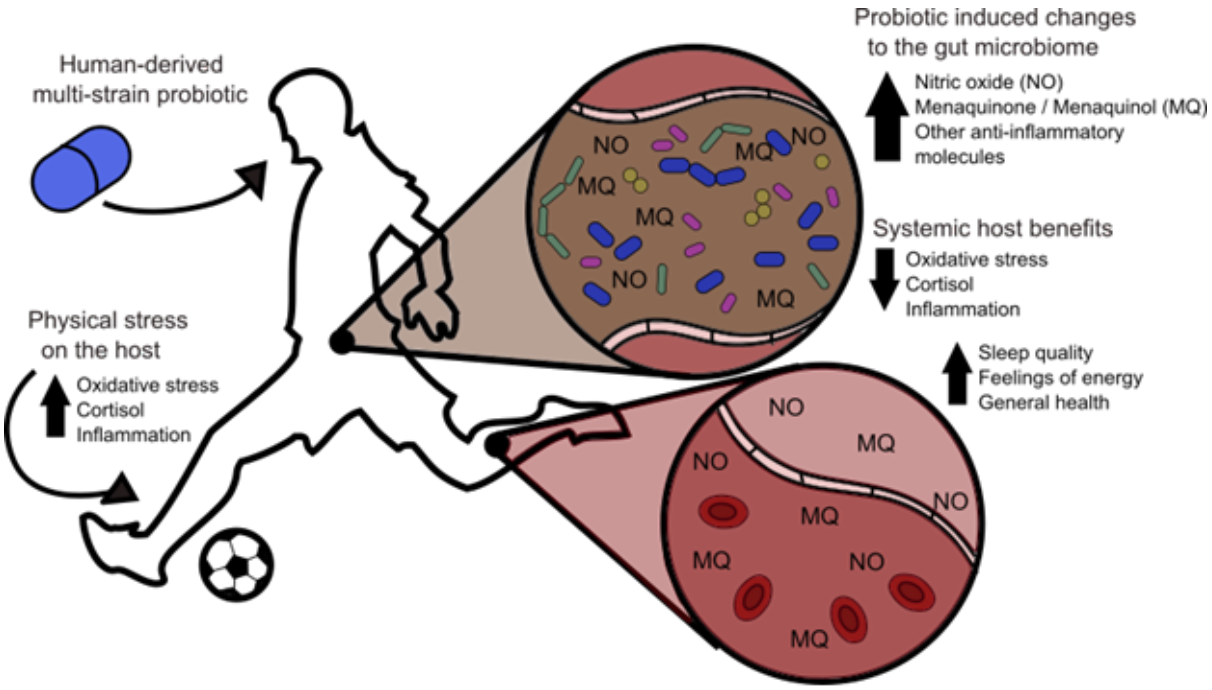

Figure 7. Probiotics provide anti-inflammatory and antioxidant benefits for the host This probiotic encourages growth or production of menaquinones. Increase in NO and menaquinone (MQ) reduces oxidative stress. Alternatively, outside stressors (exercise, physical strain) induce oxidative stress. The microbiome responds to these changes by changing the composition or regulation of pathways to promote increased production of menaquinones and NO.

Discussion

While the gastrointestinal benefits of lactobacilli probiotics are well-known, we observed more novel benefits in these studies in terms of participant reported improvements in sleep and energy. The gut microbiome's capacity to adapt to exercise has garnered considerable attention in recent years, with accumulating evidence suggesting that the health benefits of exercise may be mediated by the microbiome[68]. The probiotic intervention investigated in this study consists of three

lactobacilli strains isolated from elite athletes, whose gut microbiomes have adapted to strenuous exercise. We hypothesize that these exercise-adapted lactobacilli harbor genomic adaptations that contribute to the significant improvements observed in sleep quality, general health, and energy levels after intervention. Moreover, the exercise-adapted microbiome more generally may be responsible for the health improvements conferred by exercise, including sleep quality.

The intricate interplay among sleep, exercise, and the gut microbiome has gained considerable interest in recent years, with a growing body of research illuminating the bidirectional relationship between the gut microbiome and exercise. In this study, we investigated the impact of a novel probiotic intervention, developed through the analysis of the gut microbiomes of elite athletes, on sleep quality, exercise recovery, and gut microbiome composition in the general population and in professional athletes[76].

We observed significant improvements in sleep quality, energy levels, and bowel movement quality after the intervention. Sleep quality and exercise recovery are closely linked, as better sleep contributes to enhanced recovery from physical exertion[93]. The observed improvements in sleep quality and energy suggest that the probiotic intervention could be modulating the host's physiology and stress response, possibly through the modulation of testosterone, cortisol, and inflammatory markers. Further work is needed to validate these associations and to better understand the underlying mechanisms.

The multi-omics analysis conducted in this study revealed specific changes in blood biomarkers, microbiota, and metabolites associated with the probiotic intervention.

Among the observed changes, we found alterations in the levels of specific bacteria such as *F. prausnitzii* and *B. longum*, which have been linked to exercise performance and gut health in previous studies[94,95]. Additionally, we identified changes in oxidative stress markers, such as the BAP test and D-ROMS, which could be influenced by the gut microbiome[66]. These findings suggest a possible role of the gut microbiome in modulating host physiological responses to exercise and sleep.

Based on these findings and the literature on the intersection of sleep, exercise, and the gut microbiome, we propose a few models of how the exercise-adapted Lactobacilli strains in the probiotic intervention influence sleep quality and other health parameters. One possible model that emerges from our data revolves around the role of oxidative stress in sleep regulation. Sleep quality has been shown to be influenced by the balance between pro- and anti-oxidants in the body[96,97]. As physical exertion increases oxidative stress, the body responds by adjusting antioxidant levels to maintain homeostasis. In addition, the competitive season entails high intensity peaks, mental stress, and travel. The peaks of BAP test and IL-6, in particular, at placebo point to an external physical stressor, possibly a hard training session or a match within 3-4 days.

In our study, we observed changes in biomarkers of oxidative stress including BAP, D-ROMS, cortisol, TNF-a, and IL-6 following the probiotic intervention. However, while BAP and D-ROMS are expected to negatively correlate, we observe decreases in both markers. Because the observed changes are not far outside the normal range for these biomarkers and not particularly associated with an unhealthy state,

perhaps the decrease in BAP is a result of low D-ROMS. The human host may not require high antioxidant potential when healthy and the levels of reactive oxygen metabolites are low. Our results suggest that the probiotic may enhance sleep quality by modulating the oxidative stress response, ultimately promoting a more balanced redox state conducive to sleep.

Several studies on sleep and inflammation have predominantly measured IL-6 as a marker of systemic inflammation[97]. The pattern of values we have observed in IL-6 is consistent with the findings regarding improvement in sleep quality. Sleep disturbances, assessed by symptom reporting with single or multiple items or a questionnaire, correlated with elevated levels of pro-inflammatory cytokines (IL-1, IL-6, TNF-ﻯ) [31]. Although it cannot be stated with certainty that the probiotic supplement reduces the level of IL-6, the link found between reduced IL-6 level and improved sleep quality is an important signal for the efficacy of these Lactobacillus strains. Especially for *L. plantarum* species, in vivo studies report that it can block the expression of proinflammatory cytokines[98]. A recent study in mice also connected changes in IL-6 and TNF-ﻯ to oxidative stress in the brain induced by chronic sleep restriction[75]. The authors found that chronic sleep restriction increased TNF-ﻯ and reduced IL-6 alongside increased oxidative stress and altered gut-brain hormones. The probiotics used in this study were able to ameliorate the oxidative damage to the brain induced by sleep restriction, reduced neuroinflammation, and positively regulated gut-brain hormones. It remains to be seen whether these effects also

occur in humans, but the results of this study suggest that a similar mechanism may be relevant.

Another potential model involves the impact of the probiotic intervention on the production of neurotransmitters and other sleep-regulating molecules. Certain gut microbes are known to produce neuroactive compounds, such as gamma-aminobutyric acid (GABA) and seratonin, which can influence sleep quality[99]. The observed changes in the microbiome community, particularly the increase in taxa such as F. prausnitzii and B. longum, may lead to alterations in the production of these neuroactive compounds. This, in turn, could modulate sleep-wake cycles and improve sleep quality.

Furthermore, our study revealed changes in testosterone and cortisol levels following the probiotic intervention. The testosterone-to-cortisol (T:C) ratio is an important indicator of an athlete's recovery status and is influenced by the balance between anabolic and catabolic processes[100]. An increase in the T:C ratio may signify enhanced recovery and reduced stress, ultimately leading to better sleep quality. The probiotic intervention may impact the host's hormonal balance, possibly through the modulation of microbial metabolites and their effects on the endocrine system, which in turn influences sleep quality. On the other hand, cortisol and oxidative stress have been shown to be negatively correlated. Perhaps the increased FT:C and T:C ratios are another marker of improved oxidate status of the participants. In fact, testosterone, cortisol, oxidative stress, and even Vitamin D (another marker found to improve with probiotic compared to placebo in this study) have previously been shown to be connected in other studies of soccer players.

Lastly, the probiotic's impact on sleep quality could be related to its effects on the immune system. The gut microbiome is known to play a crucial role in the regulation of both innate and adaptive immunity[101]. The observed changes in inflammatory markers, such as TNF-ᵅ and IL-6, suggest that the probiotic intervention may modulate the immune response in a way that promotes a more restful sleep. In previous studies, while there tends to be negative correlation between proinflammatory cytokines and improved sleep (as we observe with IL-6), neither sleep disturbance nor sleep duration are generally associated with changes to TNF-ᵅ, which we observe to be increased with probiotic use. In the recent study connecting chronic sleep restriction to oxidative stress and neuroinflammation in mice, TNF-ᵅ levels are increased with sleep restriction, while IL-6 is decreased, which is the same pattern we observe in participants post-probiotic use, which is associated with improved sleep quality. More work is needed here to tease apart the relationship between sleep and changes in these inflammatory markers.

There are several limitations to these studies. In the longitudinal placebo-controlled trial, the greatest limitation is the sample size: Only 11 participants completed all aspects of the study. This has limited our ability to identify significant associations between changes to the microbiome, blood-based biomarkers, and participant reported outcomes. Future research should include larger randomized controlled studies to investigate these effects with greater statistical power. Another limitation was the study design. This study was initially designed as a randomized controlled crossover study, but the logistics of working with a professional soccer club during

competition season necessitated a simpler study design. The longitudinal aspect of the study introduces potential confounding variables such as changing seasons, which could explain the changes observed in Vitamin D3. Other unknown confounders may explain some of the observed biomarker and microbiome changes as well. While participants were instructed to maintain the same diet, exercise, and lifestyle habits throughout the study, it is impossible to retrospectively determine whether this was the case. Future randomized controlled parallel arm or crossover studies will limit these confounding variables and validate the effects observed here. A major limitation of the open-label study of 257 individuals was that it lacked a control arm, making it susceptible to bias from the placebo effect. However, the results from the open-label study were critical in the design of the placebo-controlled trial, without which we would have not identified the association with sleep quality. Another limitation of the manuscript is that there is no non-survey data related to effect on the general population, therefore we cannot confirm that the same effects are present in elite athletes and the general population

We believe that this work can serve as a model for future clinical investigations of probiotics and other supplements, beginning with a large but low-cost survey-based study exploring a large range of effects that may be conferred by the probiotic. Then any significant effects identified in the large study, serving as hypotheses, can be independently tested and validated in smaller but rigorous placebo-controlled trials.

Limitations

This study was originally designed as a Baseline-Treatment-Return to Baseline (ABA) design, a quasi-experimental single-case approach in which participants first receive a placebo (baseline phase), followed by the probiotic intervention (treatment phase), and finally a return to placebo condition. The inclusion of a second baseline phase was intended to strengthen causal inference by allowing observation of whether any changes observed during treatment would revert once the intervention was withdrawn.

However, due to logistical constraints- specifically the conclusion of the competitive championship- it was not possible to implement the planned return-to-baseline (placebo) phase. As a result, the study ultimately included only the initial baseline and treatment periods. The absence of the second baseline phase reduced the methodological robustness of the design, limiting our ability to fully attribute observed changes to the probiotic intervention and weakening internal validity. Without the withdrawal phase, it is not possible to determine whether the treatment effects would have been maintained, diminished, or reversed once the intervention was removed.

Furthermore, although single-cases designs are particularly valuable for their capacity to control for confounding variables through repeated measurement and within-subject comparison, the present study was constrained by its small sample. Specifically, we examined only one team of elite soccer players (n=11), which restricts both statistical power and external validity. The within-subject nature of the design partially mitigates inter-individual variability, the limited sample size and the absence of replication across teams reduce the generalizability of the findings.

Conclusion

In conclusion, we have used a two-phase study approach to identify potential benefits of a multi-strain *Lactobacillus* probiotic and validated those results using a controlled longitudinal study in an athlete population. This study suggests potential improvements in digestion and moderate increases in energy and improved sleep quality. However, the promising findings of the present study should be interpreted with caution. The absence of the planned return-to-baseline phase, together with the limited sample size and lack of replication, constrains the strength of causal inferences that can be drawn.

While the observed effects are suggestive and provide preliminary support for the potential impact of the probiotic intervention, further investigations should adopt fully implemented experimental designs and larger samples to strengthen internal validity. Multicenter studies involving multiple professional soccer teams would enhance both statistical power and generalizability. The use of randomized, placebo-controlled trial (RCT) designs, ideally with double-blinding procedures, would allow for more robust causal conclusions and reduce potential sources of bias. Such methodological improvements would provide a stronger evidence base regarding the efficacy of probiotic supplementation in competitive soccer settings.

CHAPTER 4

PROBIOTICS IN FOOTBALL (SOCCER): A SURVEY ON PRACTITIONER'S CURRENT PERCEPTIONS AND PRACTICES

Abstract

Probiotic supplementation is attracting attention of the sports community to promote good health, adaptation to training, recovery and exercise performance.

Sixty football practitioners (n=47 performance nutritionists, n=4 head of performance, n=1 physician, n=5 sport scientists, and n=3 strength & conditioning coach) from 5 different countries participated in a study consisting of a survey including 5 domains: demographic and professional characteristics (*Who*); perceptions about probiotics for health and performance (*Why*); methodological procedures about probiotics (*What*); mode of supplementation (*How*) and real-life practices about probiotics supplementation (*When*).

Data revealed 5 main findings: (1) overall agreement on the importance of probiotics for soccer players (80.0%) and on the consumption under the practitioner's supervision (76.8%); (2) lack of consensus about the effects on athletic performance and recovery; (3) strong agreement on the use of probiotics to treat diarrhea (61.0%) and gastrointestinal symptoms (66.1%); (4) choice of multi-strain and specie probiotics supplements (85.2%); and (5) frequency of probiotics supplementation of 1 dose every day (69.1%) and in capsules formulations (83.3%).

This study provides actionable insights into the implementation of probiotics supplementation in elite soccer players. While practitioners recognize the importance of probiotics for soccer players on gut health, further research and professional debate are warranted to develop empirical knowledge and provide pragmatic recommendations about the effects of probiotics and athletic performance, sleep and recovery.

Introduction

Probiotic supplementation has gained increasing interest among sports science and medical practitioners working in elite football[6,78]. This growing attention is largely driven by the potential benefits of probiotics on gut health, immune function, and overall athlete well-being; areas that are frequently challenged in the high-stress environment of professional sport. Soccer players are exposed to frequent travel[102,103], variable training loads, and psychological pressures, all of which may predispose them to gastrointestinal (GI) symptoms and immune suppression[104].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. West et al. and Clarke et al. showed that probiotics can reduce the risk and severity of gastrointestinal disturbances, enhancing mucosal immunity, and modulating inflammation[7,105]. These benefits are of clear relevance to football practitioners seeking to support consistent training, rapid recovery, and performance readiness. However, despite this growing interest, the evidence base remains limited and often lacks sport-specific application.

Currently, there is no established consensus regarding the most effective probiotic strains, dosing protocols, or supplementation in elite football[7]. This has created a practice-evidence gap, where applied use may be influenced more by practitioner belief, anecdotal experience, or commercial product availability than by scientific data. With this in mind, we aimed to investigate the real-world perceptions and practices of stakeholders supporting elite soccer teams regarding probiotic supplementation. By capturing data across professional roles and countries, this research seeks to identify current trends, highlight areas of uncertainty, and provide

a foundation for future work to inform evidence-based guidelines in professional football environments.

Materials and Methods

Subjects

A sample of practitioners working with elite soccer players was recruited *via* personal messaging applications (e.g. WhatsApp), e-mail and social media (e.g. Instagram, Facebook, Linkedin) through the professional networks of the research team. Eligibility criteria were: be > 18 years old; have adequate experience working in an elite sporting environment (>2 years); be a Physician, Nutritionist, Head of performance, Sport scientist, Strength & conditioning coach; currently work in elite professional level (First league or second league during season 24-25); currently work with the first team Men or Women or both. Participants provided informed consent, and the study received ethical approval by the Ethical Independent Committee for Clinical, not pharmacological investigation in Genova (Italy) and followed the ethical standards of the 1964 Declaration of Helsinki (Rif. 2020/12).

Study Design

A cross-sectional, survey study design was used to survey practitioners' beliefs and practices pertaining to probiotic supplementation in elite soccer. The survey was designed in English language using an online platform (Qualtrics$^{\text{CORE XM}}$).

Survey development

The first step to develop the survey was a literature review for two primary purposes: (1) to clearly define the construct and (2) to determine if measures of the construct already exist. Hereafter the first online meeting the survey designer group (T.B., S.M., A.P.) drafted fifty survey items that adequately represent the construct of interest in English, a language that respondents can easily understand and using the vocabulary of the target population. Once first survey draft items were written, we asked a panel of experts (G.C., R.M., G.M., J.C.) to evaluate the content of the survey to improve the overall quality, clarity and relevance of designed questions, resulting in the final fourth version. After the experts helped refine the questions, we performed a cognitive pre-testing to determine the content validity and to figure out how potential respondents interpret the items and if the interpretation matched what the survey designer had in mind. In this qualitative technique, analysis does not rely on statistical tests of numeric data but rather on coding and interpretation of written notes from the interview. Thus, the sample sizes used for cognitive interviewing involved 10 participants. Then we conducted pilot testing, 10 subjects of the target population complete the survey in the planned delivery mode (e.g. web-based format). The data obtained from the pilot test were reviewed to evaluate item range and variance, assess score reliability and review item to design the final version that was sent to participants. Data collection took place between September 2024 and May 2025 using an anonymous questionnaire after obtaining the authors' permission.

Survey structure

As illustrated in figure 8, the survey consisted of 40 questions. Items included multiple-choice questions (*i.e.*, choose from the following list), free text and an array of 5-point Likert scales (from *"strongly disagree"* to *"strongly agree"*) questions surrounding the nature of probiotics supplementation in elite soccer players. The survey was organized into five thematic sections:

Who - Participants characteristics, including demographic data and professional background (i.e. country, level of league, gender, age, years of experience, job role, level of education).

Why - Beliefs and perceived benefits of probiotic use, specifically regarding recovery, sleep, physical performance, and gut health.

What - Methodological aspects of probiotic supplementation, such as the type and formulation of supplements used.

How - Mode of supplementation, including routes or methods of administration.

When - Real-life practices such as recommended duration, frequency and types of probiotic supplements.

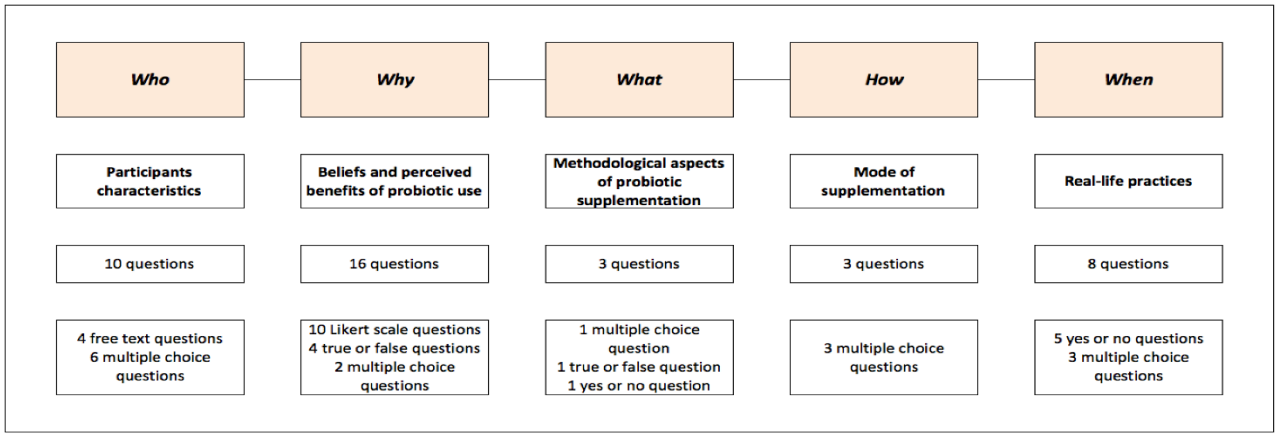

Figure 8. An overview of the structure of the survey organized in five thematic sections.

Data analysis

Descriptive statistics were computed to summarize participants' responses, including means and standard deviations for continuous variables and counts and percentages for categorical items. Response distributions were examined and reported through frequency analyses and tabulations, with figures provided where informative. All analyses were conducted in Python (v3.9). Unless otherwise noted, summary estimates are presented alongside appropriate measures of dispersion, and reporting follows conventional guidelines for descriptive studies.

Results

A 100% response rate was achieved. Practitioners from "the big five" leagues (Spanish La Liga, English Premier League, Italian Serie A, German Bundesliga and French Ligue 1) and second leagues of the same countries, participated in this study.

The "Who"

Sixty practitioners (male: n=50; female: n=10), 35.2 (8.0) years old, with 8.6 (6.1) years of experience in elite soccer teams volunteered participate in this study. The majority of them are performance nutritionists (78.3%). Respondents' characteristics are presented in figure 9.

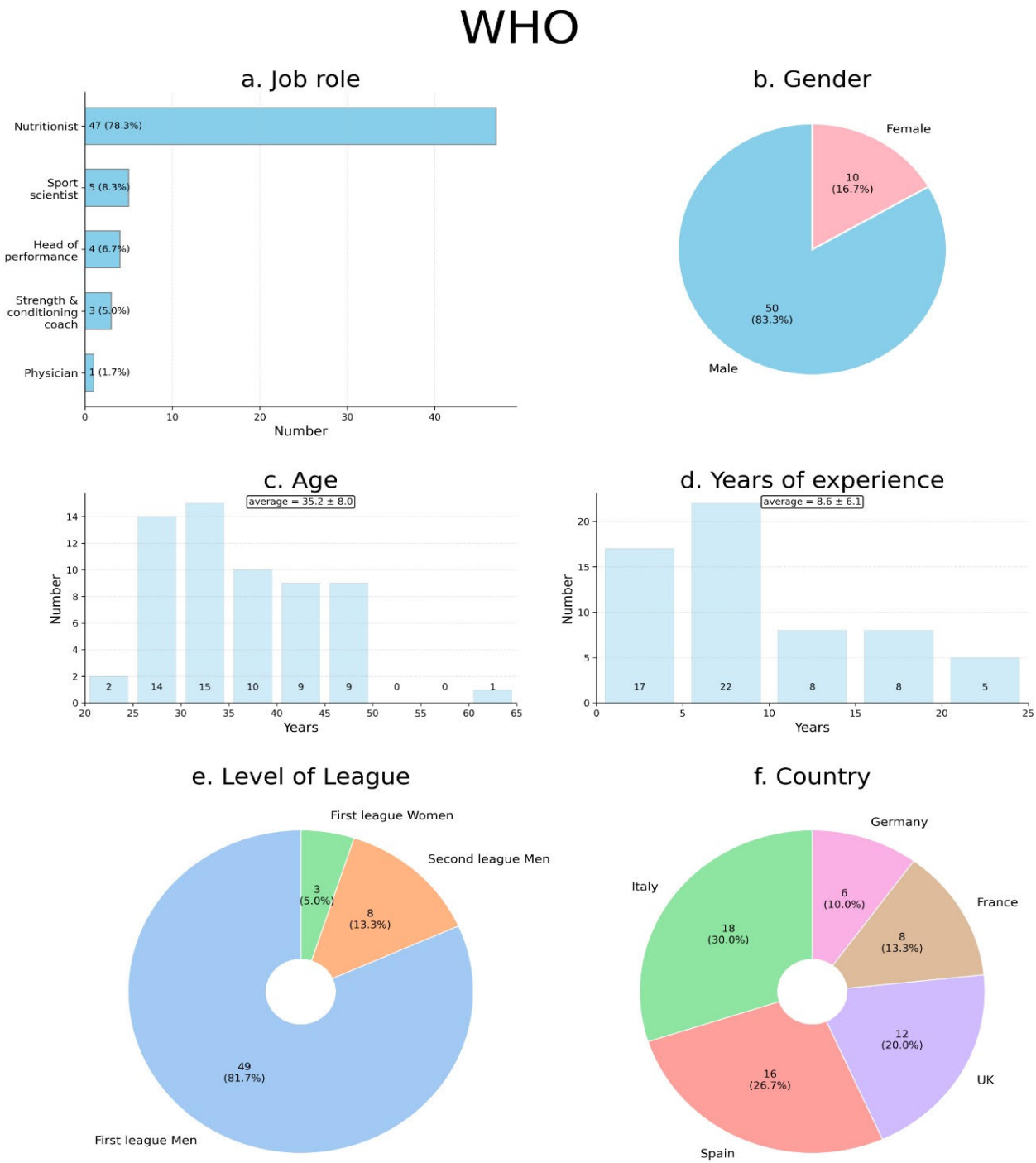

Figure 9. Frequency distribution of the participants' characteristics, including demographic data (a. job role, b. gender, and c. Age) and professional background (d. year of experience, e. Level of League, and f. country of work).

The "Why"

To better understand beliefs and perceptions regarding probiotics, participants were asked to indicate their level of agreement with statements about the perceived effects of probiotic supplementation on physical performance, gastrointestinal symptoms and immune function.

A substantial proportion of practitioners (48 out of 60; 80.0%) strongly recommend the use of probiotics. The two most frequently recognized benefits were: (1) modulation of the immune response and (2) improvement of gut health. In contrast, there was a notable level of disagreement concerning the potential benefits of probiotics for enhancing recovery and sleep quality. A moderate level of agreement (55.0%) was reported regarding the positive effects of probiotics on athletic performance, particularly with the respect to mental performance and endurance capacity.

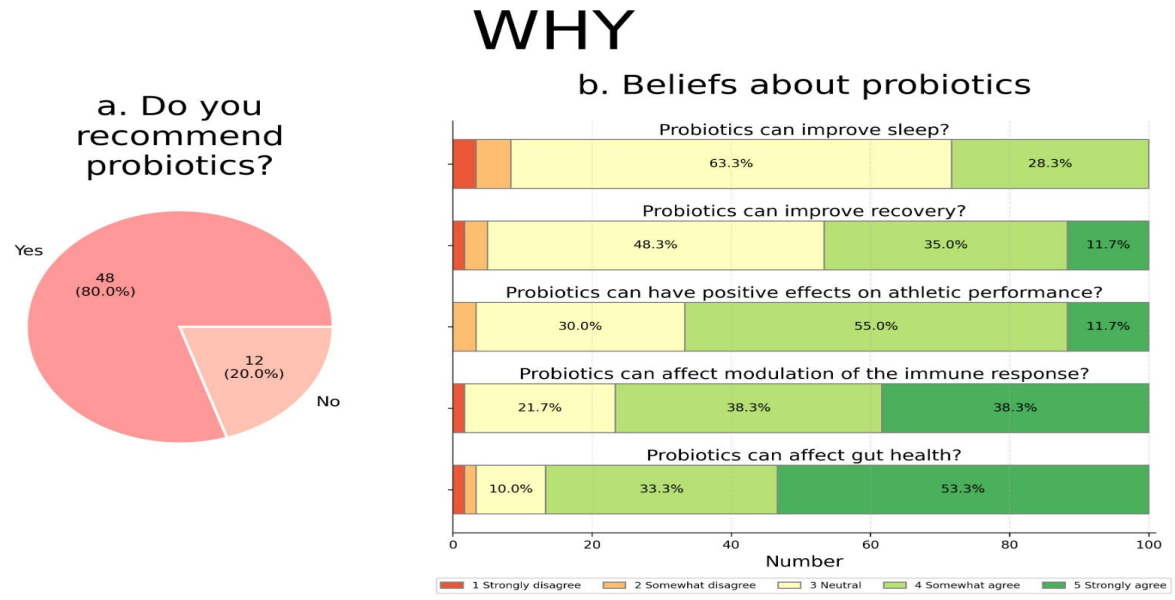

Figure 10. Frequency distribution of the participants' probiotics knowledge. (a): agreement on probiotics recommendation. (b): beliefs about potential effect of probiotics.

The "What"

To assess the level of awareness regarding the safety of probiotic use, we asked practitioners whether they believe it is safe to take probiotics without consulting a physician, whether soccer players are informed about the potential risks of consuming prohibited substances, whether they are concerned about anti-doping regulations, and which probiotic formulations are preferred. Over one third of respondents (39.0%) believed it is safe to take probiotics without medical advice. A majority (64.4%) reported that soccer players are generally unconcerned about the use of supplements, including probiotics, in relation to anti-doping laws. Regarding formulation preferences, three-quarters of the practitioners (75.0%) indicated that capsules are the most preferred probiotic form among soccer players.

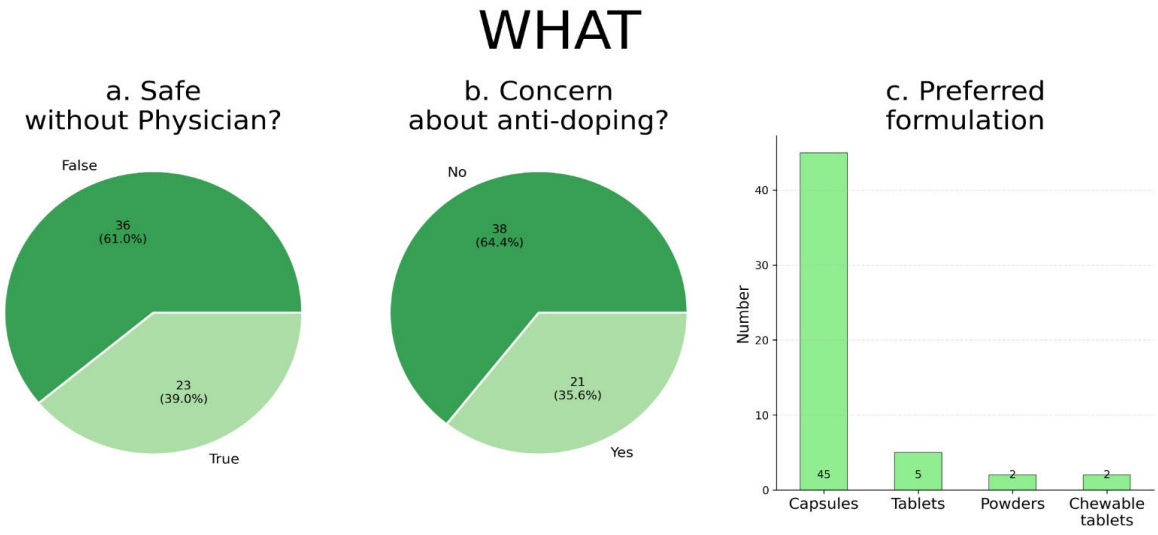

Figure 11. Frequency distribution of the participants' probiotics usage. (a): safety of probiotics uses without medical advice. (b): concern about anti-doping laws; (c): preferred probiotic formulations.

The "How"

Figure 12 summarizes the timing of probiotic supplementation and the extent of practitioner supervision. The majority of practitioners (76.8%, 43 out of 60) reported overseeing their athlete's probiotic intake, whereas a smaller proportion (23.2%) relied on the athlete's self-administration. Regarding the timing of supplementation, there appears to be no clear consensus among practitioners. Specifically, 39.6% recommended taking probiotics on an empty stomach, 37.7% advised ingestion with food, and 18.9% suggested consumption prior to meals without specifying the time of the day (e.g. breakfast, lunch or dinner). Only a small minority (3.8%) advised probiotic supplementation post-exercise.

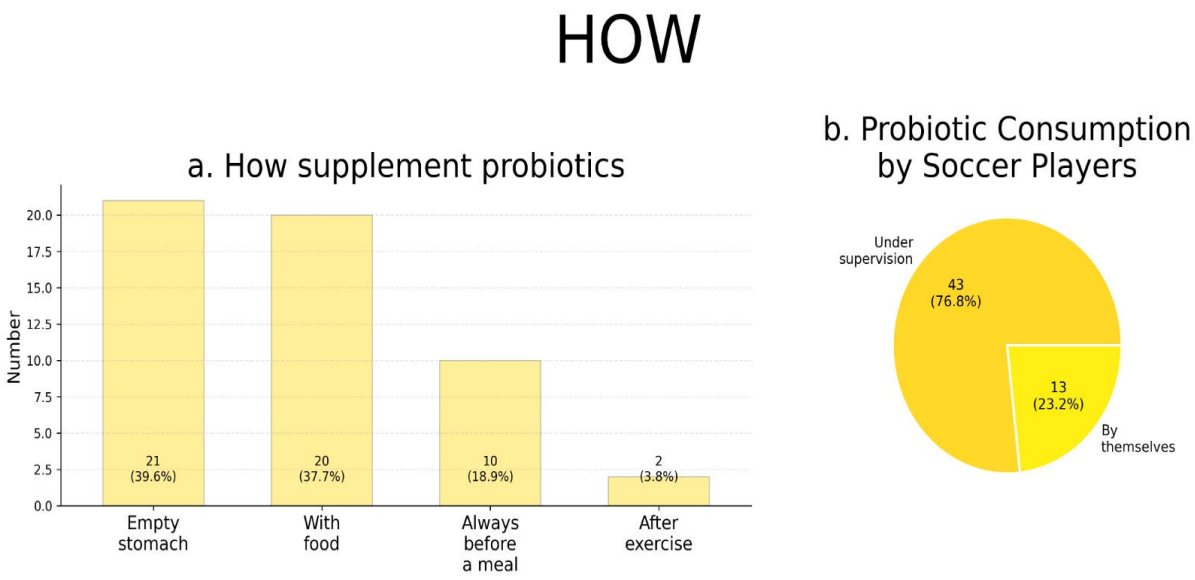

Figure 12. Frequency distribution of the participants' methodology of probiotics usage. (a): timing of probiotics recommendation. (b): mode of supplementation.

The "When"

Figure 13 illustrates the lack of a standardized protocol among practitioners regarding probiotic supplementation. Specifically, 17 out of 60 respondents (30.9%) recommended a supplementation period of 4 weeks, 12 (21.8%) suggested 12 weeks, and 6 (10.9%) indicated 8 weeks. Notably, a substantial proportion (20 out of 60; 36.4%) reported alternative durations without specifying the exact length of supplementation.

In terms of dosing frequency, 38 practitioners (69.1%) advised a single daily dose, whereas 12 (21.8%) recommended two doses per day. Although there is no consensus on the optimal duration of supplementation, the majority (46 out of 60; 85.2%) favored multi-strain and multi-species probiotic formulations over single-strain products, which were recommended by only 8 practitioners (14.8%).

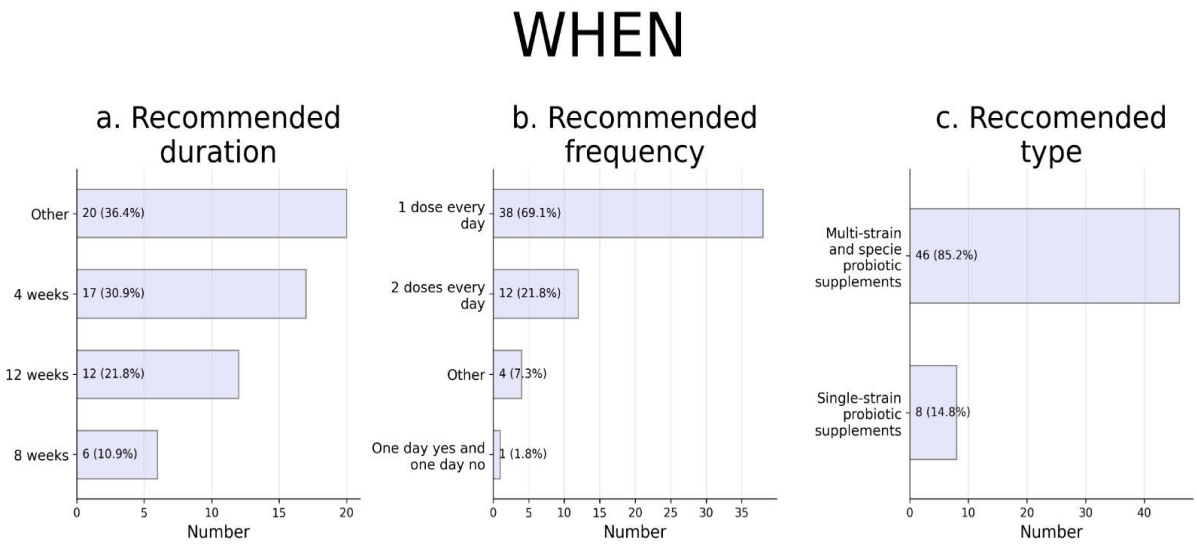

Figure 13. Frequency distribution of the participants' timing of probiotics usage. (a): window of supplementation. (b): frequency of supplementation. (c): type of probiotics supplements used.

Discussion

The aim of the present study was to investigate beliefs and practices of football practitioners regarding probiotic supplements. Using a specifically designed questionnaire, we surveyed 60 elite practitioners working in elite football clubs. The main findings were the following: (1) overall agreement on the importance of probiotics for soccer players (80.0%) and on the consumption under the practitioner's supervision (76.8%); (2) lack of consensus about a standardized protocol of supplementation and around effects on athletic performance and recovery; (3) strong agreement on the use of probiotics to treat diarrhea (61.0%) and gastrointestinal symptoms (65%); (4) choice of multi-strain and specie probiotics supplements (85.2%); and (5) frequency of probiotics supplementation of 1 dose every day (69.1%) and in capsules formulations (83.3%).

To the best of our knowledge, this is the first time that the practices and perceptions of practitioners working in elite context of soccer teams regarding probiotic supplements have been explored.

This study involved mainly sport nutritionists (78.3%), followed by sport scientists (8.3%), head of performance (6.7%), strength and conditioning coaches (5.0%) and only 1.7% of physicians. Participants are representative of the big five leagues (30% Serie A, 26.7% La Liga, 20.0% Premier League, 13.3% Ligue 1 and 10.0% of Bundesliga) with an average of 8.6 years of experience in elite soccer. The results of this work clearly show that practitioners working with major leagues clubs are interested in and recommend probiotics (80.0%), in particular to modulate the

immune response and to improve the gut health. It is well known that several well-characterized strains of *Lactobacillus* and *Bifidobacterium* showed the ability to regulate the mucosal immune response[101], to enhance the activity of macrophages[106], stimulating the production of secretory IgA, promoting regulatory T cell differentiation and modulating key signaling pathways such as Toll-like receptors (TLRs). These effects contribute to the attenuation of pro-inflammatory cytokines like IL-6, TNF-α, while enhancing anti-inflammatory mediators like IL-10[54,98]. One common characteristic of single-strain *Lactobacillus* or multi-strain *Lactobacillus* and *Bifidobacterium* is that they are lactic acid bacteria, capable of producing lactic acid, improving lactose tolerance and enhancing both innate and acquired immunity.

A recent systematic review and meta-analyses revealed that probiotics could improve gut health, upregulating tight junction proteins such as claudins and zonulin, reducing intestinal permeability, and preventing microbial translocation. However, the impact of probiotics on gut health in athletes is an emerging area of research with current gaps that include focused research on subjects prone to gastrointestinal issues during exercise, specific gastrointestinal symptoms, dietary controls and inter-individual variability in colonization of probiotics[107].

In this survey, practitioners recognized the benefits of probiotics to treat diarrhea (36 out 60, 60.0%) and to manage common gastrointestinal symptoms such as cramping, bloating, abdominal pain and heartburn (39 out 60, 65.0%). These beliefs are based on the current evidence that supports the moderate benefit of multi-strain probiotic supplementation for reducing frequency and severity of gastrointestinal symptoms during training in athletes, especially with supplementation periods

varying from 2-13 weeks[108,109]. Schreiber *et al*. (2021) reported in elite male cyclists, lower incidence of nausea, belching and vomiting at rest, and decreased incidence of cramping and bloating during training, after 90 day of supplementation with a probiotic supplement consisting of 5 multi-strains of *Lactobacillus* and *Bifidobacterium,* when compared to placebo[109]. Moreover, recreationally runners, supplemented with *bacillus coagulans* for 4 weeks reported significantly fewer and less severe gastrointestinal symptoms during training and in a marathon race than those received a placebo[110]. Similarly, Pugh et al. (2019) showed beneficial effects on gastrointestinal symptoms during marathon running, of a blend of *Lactobacillus* and *Bifidobacterium* ingested for 28 days prior to a marathon, when compared to a placebo[111]. Most recently, we reported improved gastrointestinal symptoms in 11 trained elite soccer players, after a 12 weeks of multi-strain probiotic supplementation, when compared to 12 weeks of placebo. Albeit, emerging evidence from athletes indicates the potential of probiotics for managing gastrointestinal symptoms, further research must investigate the impact of different probiotic strains and the duration of intervention in real-world sporting scenarios[112].

A moderate level of agreement (55.0%) was reported by practitioners involved in this survey regarding a beneficial effect of probiotics on athletic performance, particularly with respect to mental performance and endurance capacity, while only 35% of our sample believe that probiotics can improve recovery from exercise. Adikari et al. (2020) reported that soccer players who received *Lactobacillus Casei Shirota* for 8 weeks, compared to placebo, improved their reaction time during a cognitive test and showed a significant improvement in theta (relaxation) and delta (attention) waves,

findings that suggest the potential of probiotics to modulate brain function and psychological improvement to exercise[113]. In a study involving highly trained female swimmers, *Bifidobacterium longum 35624* supplementation, improved cognitive functions during a period of 6 weeks of intense training[114]. A recent study conducted in amateur runners showed that a blend of *Lactobacillus acidophilus* and Bifidobacterium longum, supplemented for 5-weeks, improved the total distance traveled in the 12-min Cooper Running/walking test. The authors also found significantly greater post-exercise muscle microperfusion as measured by functional magnetic resonance (fMRI) indicating that probiotic intervention may improve aerobic exercise efficiency through improved vascular function[115]. In another study conducted in elite road cyclists, 4 months of supplementation with a blend of *Lactobacillus* and *Bifidobacterium* strains, increased the VO_{2max} of 5.6%, and of 2% the duration of training until failure and the degree of decrease in heart rate[116]. Despite there are some promising effects of probiotics supplementation on endurance performance, limited studies have investigated the impact of probiotic intervention on power-specific sporting performance in athletes. In addition, most of these investigations have assessed the impact of probiotics in co-ingestion with another macronutrient. For example, Tarik et al. (2022) combined 20 g of whey protein powder with 2 billion CFU *Bacillus coagulans* Unique IS-2, once daily for 60 days, showing an improvement of vertical jump power by 7.86% and an increased one repetition maximum on leg press by 16.61% in resistance-trained males[117]. Recently, a combination of 8 bacterial strains of *Lactobacillus* and *Bifidobacterium* plus *Streptococcus thermophilus* with 1 g of omega 3 EPA and DHA, for a period of

eight-week, showed a synergistic effect in sprint swimming performance, enhancing anaerobic power and recovery in male sprint swimmers[118].

Only one study evaluating the effects of probiotic *Lactobacillus plantarum* PS128 in isolation, for a period of 8 weeks in triathletes[119].

Even though emerging evidence suggests improvements in athletic performance following probiotic intervention, mechanisms underpinning the ergogenic benefits are still to be fully elucidated.

The majority of practitioners maintained a *"neutral"* position regarding the ability of probiotics to modulate recovery post exercise and only the 35% of practitioners (21 out of 60) reported *"somewhat agreement"* about probiotics supplementation as a tool to improve exercise recovery. These findings are not surprising and are in line with the literature. In fact, the evidence is limited to a few studies that, despite showed potential effects of probiotics to reduce associated markers of exercise-induced muscle damage in healthy athletes and elite rugby players, and don't describe optimal dosages, timings, and strains of probiotics to enhance training adaptations and performance.

To better understand the real-world knowledges about safety and methodological use of probiotics, we asked if "It is safe to take probiotics without consulting a physician?"; the majority of the practitioners (37 out 60; 61.0%) believe that before starting a period of probiotics supplementation it is good practice to consult a physician, while 23 out 60 (39%) answered that they advise probiotics without consulting the team doctor. Regarding probiotics' safety, 68.4% of participants reported not perceiving anti-doping concerns to use probiotic supplements by soccer

players, concerns about anti-doping laws were reported by one third (31.6%). Previous data revealed that 55.5% of young athletes had familiarity with anti-doping laws, and recently, only the 23.1% of nutritionists working in elite soccer teams agreed or strongly agreed that dietary supplements are safe. Although practitioners recognize that probiotic supplements have an excellent safety profile, with several clinical trials showing very few side effects or adverse events following probiotic supplementation, there is still necessity for a comprehensive education of all team members about sports supplements and careful supervision involving the team's physician.

In terms of formulations, practitioners of this study reported that in their context, soccer players prefer capsules (45 out 60; 75.0%). Capsules are convenient solid dosage forms to deliver probiotics. These formulations allow the application of various functional excipients to improve shelf-life, gastrointestinal stability, cell viability and to control the release rates and target sites of probiotics.

We also investigated how supplementation of probiotics is organized in daily practice, and we found that most practitioners (76.8%) oversee probiotic dispensing, whereas a smaller proportion (23.2%) relied on the athlete's self-administration. Regarding the timing of supplementation, there is an increased confusion and appears to be no clear consensus among practitioners. Specifically, 39.6% recommended taking probiotics on an empty stomach, 37.7% advised ingestion with food, and 18.9% suggested consumption prior to meals without specifying the exact time and neither the meal (e.g. breakfast, lunch or dinner). Only a small minority (3.8%) advised probiotic supplementation post-exercise. These answers suggest

that, to date, practitioners have not a clear and unanimous knowledge about the proper timing of supplementation. Most scientific evidence and expert guidance support taking many oral non-enteric coated bacterial probiotic products with or 30 minutes before a meal, rather than on an empty stomach, because food (e.g. milk containing some fats or cooked oatmeal) buffers gastric acid and increases survival of many strains. While, timing is less critical when surface coating technologies are adopted to enhance the stability of probiotics in the gastrointestinal tract.

Similarly, there is no consensus regarding the duration of supplementation. Among the 60 professionals surveyed, 17 reported using a 4-week period, 12 reported extending supplementations to 12 weeks, and 6 reported following an 8-week protocol[7,107,120].

The optimal duration of probiotic supplementation remains a topic of considerable heterogeneity in clinical and experimental research. Evidence spans from short-term interventions of approximately 4 weeks, commonly employed in studies targeting immune of inflammatory outcomes, to longer protocols lasting 8, 11 or 12 weeks, frequently adopted in trials focused on metabolic, neurological, or quality of life endpoints[7]. For instance, in athletes, randomized controlled trials evaluating immune and inflammatory markers have utilized durations ranging from 28 to 90 days, with multiple studies clustered around the 4-week, 8-week and 12-week period. Beyond duration differences, for example a 4-week daily probiotic regimen in rugby union players to prevent upper respiratory and gastrointestinal infections, or 5-weeks in endurance athletes, methodological inconsistencies add further complexity. Many trials have small sample sizes, diverse strain compositions, variable dosages, and

inconsistent reporting of training loads or other confounding factors such as dietary intake, hurdles that impair cross-study comparisons and generalizability. The International Society of Sports Nutrition (ISSN) position stands emphasize that probiotics are like strain-and dose-dependent, and require at least 14 days before major training or competition to allow adaptation, but they do not prescribe fixed durations or protocol for athletes[7]. This highlights that the notion of a standardized protocol doesn't align with the current knowledge base. In addition, athletes themselves are a heterogeneous group with varied training intensity, competition schedules, nutritional status, and baseline microbiota. The outcomes, ranging from immune health and gastrointestinal symptoms to performance and recovery, also differ widely, further complicating any harmonized approach.

Overall, the heterogeneity in supplementation parameters, methodological variability, and the individualized nature of athletic populations collectively underscore the difficulty in stating that a "standard protocol" exists. Instead, probiotic use in athletes should be considered context-specific, with protocol customization based on strain, athlete profile, intended outcomes, and timing relative to training or competition.

Limitations

As with all survey reports, our data is based on self-reported responses, with the potential for social desirability bias where the respondents may attempt to "look good" with their responses.

In addition, this study has other limitations worth considering. Using a convenience sample, the respondents' roles and professional levels, mainly sports nutritionist, and

their working environment features were not fully and equally represented. Therefore, it is possible that perceptions of other stakeholders involved in decision making (e.g. coaches and players) were not considered. Second, the degree to which the data represents other clubs from different leagues or athletes of different competition levels and age groups remain uncertain.

Conclusions

This study provides the first systematic overview of practitioners' perceptions and practices regarding probiotic supplementation in elite football. The findings highlight a strong consensus on the use of probiotics to support gut health and immune function in line with the strength of the scientific evidence, alongside recognition of their role in managing gastrointestinal symptoms. However, uncertainty persists regarding their effectiveness for enhancing recovery, sleep and athletic performance, reflecting the limited and heterogeneous evidence currently available. In fact, research with elite athletes is typically scarce, either because there are fewer athletes, or because their availability to participate in studies is limited.

Practitioners predominantly recommend multi-strain formulations in capsule form, administered daily and under professional supervision, yet there is no agreement on optimal timing and duration of supplementation. This lack of standardized practice underscores the pressing need for robust, sport-specific research to define effective strains, doses and protocols tailored to the unique demands of professional football.

In conclusion, while probiotics are widely perceived as beneficial for soccer players' health and are increasingly integrated into elite practice, further high-quality

investigations are required to provide evidence on timing and duration of supplementation in order to guide practice.

Only 55% of participants advise probiotics to enhance physical performance and this highlights the lack of strong evidence in favor of the potential ergogenic effect of these supplements. However, there is an interest in knowing more about their applications in the specific context of elite soccer, in particular to explore which strains are effective, what type of physical performance can be improved and or protocols including doses and duration of the intervention.

CHAPTER 5

GENERAL DISCUSSION

This doctoral research explored how gut microbiome modulation through short-chain fatty acids (SCFAs) and probiotic supplementation may influence the health, recovery, and performance of athletes.

La conclusione di tutto è che abbiamo un utilizzo limitato per essere sicuri di dire a practitioners di usare i probiotici (non sono sicuro che i cambiamenti visti siano dovuti ai probiotici)

Across three interconnected investigations—a narrative review (**Chapter 2**) and two empirical studies (**Chapter 3** and **Chapter 4**)—this thesis examined (i) the mechanistic role of SCFAs as microbial metabolites capable of influencing immune and energy systems, (ii) the effects of a novel athlete-derived probiotic on sleep and recovery, and (iii) the real-world perspectives of practitioners in elite football regarding probiotic use. Together, these studies advance our understanding of the gut–muscle–brain axis in sport and suggest that microbial interventions can serve as promising adjuncts to traditional nutrition and recovery strategies.

MAIN FINDINGS

SCFAs as postbiotic ergogenic aids

The review in **Chapter 2** synthesized current evidence linking SCFAs—particularly acetate, propionate, and butyrate—to anti-inflammatory, immunoregulatory, and metabolic processes relevant to sport[5,22]. SCFAs, produced via microbial fermentation of dietary fibers, were shown in preclinical models to blunt pro-inflammatory cytokines (IL-6, TNF-α), enhance epithelial barrier integrity, and

provide additional energy substrates. These mechanisms could theoretically enhance exercise recovery, immune defense, and endurance performance. However, translation from mechanistic studies to human athletic contexts remains limited[39]

Probiotic supplementation and the sleep-exercise-microbiome nexus

The studies reported in **Chapter 3** provided the first experimental evidence that targeted multi-strain Lactobacillus supplementation, derived from elite athletes, can modulate recovery-related parameters. Both the open-label study (n = 257) and the controlled longitudinal trial in professional soccer players (n = 11) revealed significant improvements in sleep quality, energy levels, and bowel function, along with reductions in oxidative stress and improved free-testosterone/cortisol ratios[94,121]. Multi-omics analyses demonstrated shifts in gut microbial composition, including increases in Faecalibacterium prausnitzii. Collectively, these results support the hypothesis that gut-derived metabolites influence systemic physiological networks.

Practitioner perspectives in elite football

Chapter 4 extended these findings to applied settings by capturing current practices and perceptions of sports scientists, physicians, and nutritionists working in professional football. The survey revealed that while probiotic supplementation is widely recognized for immune and gastrointestinal health, there remains uncertainty

regarding strain selection, dosing, and timing[7]. Although over half of practitioners acknowledged a potential link between probiotics and recovery or performance, reliance on anecdotal evidence remains common.

Conceptual advances and future perspectives

Across the three studies, a central theme emerges: **the gut microbiome acts as a dynamic regulator of athlete physiology,** mediating the interface between metabolism, adaptation and recovery[94]. Together, the molecular, physiological, and applied findings converge to propose that performance capacity is not solely determined by host genetics or training load, but also by the adaptive plasticity of the microbial ecosystem within the gut. Integration of these studies allows three conceptual advances to be articulated:

1. **From Substrate to Symbiosis** – Athletic Performance is contingent not only on nutrient availability but on the metabolic capacity of the microbiota to process, recycle and signal through metabolites as short-chain fatty acids (SCFAs) and tryptophan derivatives[122].
2. **The Microbial Athlete Paradigm** – Elite athletes exhibit distinctive microbial signatures shaped by training; these profiles may act as both biomarkers and mediators of physiological adaptation[5].
3. **Systemic Influence of Microbial Modulators** – Targeted microbial or prebiotic interventions exert measurable effects on neuroendocrine and

inflammatory pathways, thereby extending microbiome influence beyond the gut to systems governing motivation, stress resilience, and recovery.[12,95]

Future research directions

Building on these insights, future research should pursue: (1) Controlled human SCFA trials, to isolate causal links between microbial metabolites and performance adaptations; (2) Mechanistic multi-omics studies, integrating metagenomics, metabolomics, and transcriptomics to delineate microbial-host signaling pathways; (3) Personalized microbiome profiling, to stratify responders versus non-responders to microbiome-targeted interventions; (4) Microbiome–sleep–recovery interface, exploring the circadian and neuroendocrine dimensions of microbial modulation; and (5) Applied sports guidelines, translating mechanistic insights into practical, evidence-based nutrition strategies that account for microbial ecology and individual variability.

These will clarify causal pathways and support the development of microbiome-informed performance nutrition.

Concluding remarks

This thesis lays the groundwork for a mechanistic and translational research program in microbiome-informed performance nutrition. Future studies should move beyond the associative evidence and implement controlled, longitudinal intervention trials in elite and sub-elite athletes to determine casual relationships between specific microbial taxa, SCFA production, and performance-related outcomes. For

instance, randomized supplementation studies using defined probiotic consortia could be combined with multi-omics profiling (metagenomics, metabolomics, and transcriptomics) to identify microbial signatures predictive of recovery kinetics, inflammatory modulation, and training adaptation.

In parallel, future research should investigate dose-response relationships and temporal dynamics of SCFA production in relation to training load, dietary periodization, and competition stress. Integrating continuous physiological monitoring (e.g. HRV, inflammatory biomarkers, muscle damage markers) with microbiome profiling would allow the development of predictive models linking microbial composition to readiness and resilience.

A further step involves testing personalized microbiome-targeted nutritional strategies. Machine learning approaches could be employed to stratify athletes based on microbial configurations and predict individual responses to probiotic or postbiotic interventions, moving the field toward precision performance nutrition.

From a more exploratory perspective, future work could examine whether long-term modulation of the gut microbiome enhances training adaptation at the mitochondrial and neuromuscular level, potentially influencing metabolic flexibility and fatigue resistance. Ultimately, this line of research may enable the development of microbiome-based biomarkers and intervention protocols integrated into routine sports performance monitoring systems.

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