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Special Issue Reprint

Dietary Habit, Gut Microbiome and Human Health

Edited by
Xi Wang

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Guest Editor

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Editorial

Unveiling the Gut Microbiota: How Dietary Habits Shape Health Through Microbiome Modulation

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This Editorial provides an overview of the Special Issue "Dietary Habit, Gut Microbiome, and Human Health" which was recently published in *Nutrients*.

The intricate relationship between dietary habits and human health, mediated by the gut microbiome, has become a focal point of research in recent years [1,2]. As a complex and dynamically evolving ecosystem, the gut microbiome plays a critical role in host metabolism, immunity, and neurological function [3,4]. Diet acts as a primary modulator of gut microbiota composition, which subsequently influences the development and progression of various diseases [2]. This Special Issue, "Dietary Habit, Gut Microbiome, and Human Health", compiles a series of cutting-edge articles that explore the interplay between diet, the gut microbiome, and human health. The nine included studies cover a broad spectrum of topics, ranging from the impact of specific dietary components on the gut microbiota to the therapeutic potential of dietary interventions in the prevention and management of diseases such as obesity, diabetes, autoimmune thyroid disease, cognitive impairments and endemic osteoarthritis. These articles not only provide novel insights into the mechanisms underlying diet–microbiome interactions but also underscore the potential of targeting the gut microbiome to facilitate disease prevention and treatment, thereby offering valuable guidance for future research and clinical practice in this domain.

Hernandez et al. investigate the effects of time-restricted feeding on cognitive function and gut microbiome composition in aging rats. Comparing time-restricted feeding with a ketogenic diet and a calorically matched control diet, they demonstrate that time-restricted feeding enhances cognitive performance irrespective of macronutrient composition. Furthermore, they identify significant differences in gut microbiome diversity and composition among the diet groups, and reveal that an abundance of Allobaculum is linked to cognitive task performance (Contributor 1). These findings suggest that time-restricted feeding may be a viable alternative to caloric restriction for improving cognitive and physical health in later life. This study also highlights the gut–brain axis as a promising therapeutic target for addressing cognitive dysfunction.

Wu et al. investigate alterations in the gut microbiota in rats resulting from selenium (Se) deficiency and exposure to T-2 toxin. Their study seeks to elucidate the potential implications of these factors with regard to Kashin–Beck disease (KBD). Their findings indicate that Se deficiency and T-2 toxin exposure induce significant changes in the gut microbiota of rats. In their research, at the phylum level, Firmicutes predominated in both the Se-deficient (SD) group and the T-2 toxin exposure group, whereas the proportion of Bacteroidetes was reduced in the SD group compared to the T-2 group. At the genus level, Ruminococcus_1 and Ruminococcaceae_UCG-005 exhibited higher relative abundance in the SD group, while Lactobacillus and Ruminococcaceae_UCG-005 were more prevalent in the T-2 group. This study underscores the distinct modifications in gut microbiota induced

by Se deficiency and T-2 toxin, offering valuable insights into the potential role of gut microbiota in the pathogenesis of KBD (Contributor 2). It highlights the need for further investigation into the complex interactions between environmental factors, gut microbiota, and joint health.

Fagunwa et al. explore the effects of dietary sodium on gut microbiota, revealing that a high-sodium diet (HSD) significantly alters the microbiota by decreasing the presence of Bacteroides and increasing the prevalence of Prevotella compared to a low-sodium diet (LSD). Their study suggests that the Bacteroides/Prevotella (B/P) ratio is a more precise marker for gut microbiota changes than the Firmicutes/Bacteroidetes (F/B) ratio, due to its clearer differentiation between sodium intake levels. They also identify sodium reabsorption genes in the epithelial sodium channel of the guts of people who eat an HSD as opposed to an LSD (Contributor 3). These findings underscore the B/P ratio's potential as an indicator of dietary sodium intake and its effects on gut health, offering new insights and directions for future research on the gut microbiome's role in health.

Duysburgh et al. investigate the prebiotic effects of baobab fiber (BF) and Arabic gum (AG), both individually and in combination, and demonstrate that BF and AG significantly influence the composition and metabolic activity of the gut microbiota in both the proximal and distal colon. Notable findings within this work include an increase in Bifidobacteriaceae and Faecalibacterium prausnitzii populations, enhanced production of short-chain fatty acids (SCFAs), and a reduction in branched-chain fatty acids (BCFAs) and ammonium levels. The co-supplementation of BF and AG at a lower dosage exerts synergistic prebiotic effects, evidenced by biological activity throughout the entire colon, increased abundance of Akkermansiaceae and Christensenellaceae in the distal colon, and elevated levels of spermidine and other metabolites of interest (Contributor 4). This research underscores the potential of BF and AG co-supplementation as a promising prebiotic strategy for enhancing gut health.

The review conducted by Chen et al. provides an in-depth analysis of the complex interplay between dietary patterns, gut microbiota, and athletic performance. It elucidates the pivotal role of gut microbiota in human health, particularly its influence on metabolism and immune function. The authors underscore the significant impact of both training and nutritional strategies on athletic performance, examining how various dietary patterns modulate the gut microbiota, which subsequently affects sports performance (Contributor 5). The review details the primary functions of the gut microbiota and its association with exercise, indicating that athletes possess distinct gut microbiota compositions compared to sedentary individuals, characterized by greater diversity and an increased abundance of beneficial species. Ultimately, the review posits that specific dietary patterns can enhance sports performance by modulating the gut microbiota.

Wang et al. examine the effects of integrating exercise with konjac glucomannan (KGM) on antibiotic-induced dysbiosis of the gut microbiota in mice. Their findings indicate that this combined intervention is more effective than individual interventions in restoring alterations in gut microbiota composition and diversity caused by antibiotic treatment. Furthermore, in their work, a combination of exercise and KGM significantly enhances microbial purine metabolic pathways. This study underscores the potential of employing a combination of exercise and KGM as a promising strategy to mitigate the adverse effects of antibiotics on the gut microbiome (Contributor 6). Nonetheless, further research is warranted to determine the optimal timing and intensity of exercise interventions. This study offers valuable insights for the development of strategies aimed at preventing and managing gut microbiota dysbiosis.

Yun et al. investigate the impact of Lactobacillus plantarum P111 and Bifidobacterium longum P121 on obesity and depression or cognitive impairment-like behavior induced by

a high-fat diet (HFD) in mice. Their findings indicate that these probiotics, whether administered individually or in combination, can mitigate HFD-induced body weight gain, liver steatosis, and depression or cognitive impairment-like behavior. The results demonstrate that oral administration of P111 or P121 significantly reduces HFD-induced weight gain and enhances lipid profiles in both the blood and liver. Moreover, these probiotics downregulate NF-κB activation and TNF-α expression in the liver and colon while concurrently upregulating AMPK activation. They also attenuate HFD-induced depression/cognitive impairment-like behavior and hippocampal NF-κB activation, leading to an increase in the hippocampal BDNF-positive cell population and BDNF levels (Contributor 7). Notably, the combined administration of P111 and P121 (LpBl) exhibits a more pronounced effect in ameliorating body weight gain, liver steatosis, and depression or cognitive impairment-like behavior. This study underscores the potential of P111 and P121 as promising candidates for the prevention and treatment of these conditions.

Zhang et al. investigate the causal relationship between gut microbiota and autoimmune thyroid disease (AITD) through the application of a Mendelian randomization approach. Following an examination of 119 gut microbiota and 9 metabolites, their research identifies a causal association between 3-indoleglyoxylic acid levels and the risk of AITD, implying that metabolites derived from gut microbiota may play a role in the pathogenesis of AITD. Additionally, this study underscores the potential causal effects of Ruminococcus torques on Graves' disease and taurocholic acid on Hashimoto's thyroiditis (Contributor 8). In summary, this research offers novel insights into the influence of gut microbiota on AITD, thereby laying the groundwork for future investigations and potential therapeutic interventions.

Type 2 diabetes (T2D), a chronic metabolic disorder with extensive health implications, has been increasingly associated with gut microbiota dysbiosis. The review article "Targeting the Gut Microbiota for Prevention and Management of Type 2 Diabetes" provides valuable insights into this intricate relationship. The authors conduct a comprehensive analysis of how alterations in the gut microbiota, including imbalances in bacterial species and changes in metabolite production, contribute to the pathogenesis of T2D (Contributor 9). They emphasize the potential of modulating the gut microbiota through nutritional interventions, such as specific diets and supplements, as well as physical exercise, to restore microbial homeostasis and enhance glycemic control. This review improved our understanding of the gut microbiota–T2D connection and its implications for the development of novel therapeutic strategies for T2D management.

This Editorial summarizes the nine articles that comprise this Special Issue, emphasizing the crucial link between dietary patterns, gut microbiota, and human health. The findings deepen our understanding of the mechanisms involved in these processes and suggest promising strategies for disease prevention and treatment, paving the way for future research and practical applications in this dynamic field.

Conflicts of Interest: The authors declare no conflicts of interest.

List of Contributions:

1. Hernandez, A.R.; Watson, C.; Federico, Q.P.; Fletcher, R.; Brotgandel, A.; Buford, T.W.; Carter, C.S.; Burke, S.N. Twelve Months of Time-Restricted Feeding Improves Cognition and Alters Microbiome Composition Independent of Macronutrient Composition. *Nutrients* **2022**, *14*, 3977.
2. Wu, Y.F.; Gong, Y.; Zhang, Y.; Li, S.J.; Wang, C.W.; Yuan, Y.Q.; Lv, X.; Liu, Y.L.; Chen, F.H.; Chen, S.J.; et al. Comparative Analysis of Gut Microbiota from Rats Induced by Se Deficiency and T-2 Toxin. *Nutrients* **2023**, *15*, 5027.
3. Fagunwa, O.; Davies, K.; Bradbury, J. The Human Gut and Dietary Salt: The Ratio as a Potential Marker of Sodium Intake and Beyond. *Nutrients* **2024**, *16*, 942.

4. Duysburgh, C.; Govaert, M.; Guillemet, D.; Marzorati, M. Co-Supplementation of Baobab Fiber and Arabic Gum Synergistically Modulates the In Vitro Human Gut Microbiome Revealing Complementary and Promising Prebiotic Properties. *Nutrients* **2024**, *16*, 1570.
5. Chen, Y.L.; Yang, K.; Xu, M.X.; Zhang, Y.S.; Weng, X.Q.; Luo, J.J.; Li, Y.S.; Mao, Y.H. Dietary Patterns, Gut Microbiota and Sports Performance in Athletes: A Narrative Review. *Nutrients* **2024**, *16*, 1634.
6. Wang, M.H.; Chen, Y.L.; Song, A.X.; Weng, X.Q.; Meng, Y.; Lin, J.R.; Mao, Y.H. The Combination of Exercise and Konjac Glucomannan More Effectively Prevents Antibiotics-Induced Dysbiosis in Mice Compared with Singular Intervention. *Nutrients* **2024**, *16*, 2942.
7. Yun, S.W.; Shin, Y.J.; Ma, X.Y.; Kim, D.H. *Lactobacillus plantarum* and *Bifidobacterium longum* Alleviate High-Fat Diet-Induced Obesity and Depression/Cognitive Impairment-like Behavior in Mice by Upregulating AMPK Activation and Downregulating Adipogenesis and Gut Dysbiosis. *Nutrients* **2024**, *16*, 3810.
8. Zhang, C.Y.; Teng, W.P.; Wang, C.Y.; Shan, Z.Y. The Gut Microbiota and Its Metabolites and Their Association with the Risk of Autoimmune Thyroid Disease: A Mendelian Randomization Study. *Nutrients* **2024**, *16*, 3898.
9. Zeppa, S.D.; Gervasi, M.; Bartolacci, A.; Ferrini, F.; Patti, A.; Sestili, P.; Stocchi, V.; Agostini, D. Targeting the Gut Microbiota for Prevention and Management of Type 2 Diabetes. *Nutrients* **2024**, *16*, 3951.

References

1. Sonnenburg, J.L.; Bäckhed, F. Diet-microbiota interactions as moderators of human metabolism. *Nature* **2016**, *535*, 56–64. [CrossRef] [PubMed]
2. Zmora, N.; Suez, J.; Elinav, E. You are what you eat: Diet, health and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 35–56. [CrossRef] [PubMed]
3. Sorboni, S.G.; Moghaddam, H.S.; Jafarzadeh-Esfehani, R.; Soleimanpour, S. A Comprehensive Review on the Role of the Gut Microbiome in Human Neurological Disorders. *Clin. Microbiol. Rev.* **2022**, *35*, e0033820. [CrossRef] [PubMed]
4. Manor, O.; Dai, C.Z.L.; Kornilov, S.A.; Smith, B.; Price, N.D.; Lovejoy, J.C.; Gibbons, S.M.; Magis, A.T. Health and disease markers correlate with gut microbiome composition across thousands of people. *Nat. Commun.* **2020**, *11*, 5206. [CrossRef] [PubMed]

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Review

Dietary Patterns, Gut Microbiota and Sports Performance in Athletes: A Narrative Review

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Abstract: The intestinal tract of humans harbors a dynamic and complex bacterial community known as the gut microbiota, which plays a crucial role in regulating functions such as metabolism and immunity in the human body. Numerous studies conducted in recent decades have also highlighted the significant potential of the gut microbiota in promoting human health. It is widely recognized that training and nutrition strategies are pivotal factors that allow athletes to achieve optimal performance. Consequently, there has been an increasing focus on whether training and dietary patterns influence sports performance through their impact on the gut microbiota. In this review, we aim to present the concept and primary functions of the gut microbiota, explore the relationship between exercise and the gut microbiota, and specifically examine the popular dietary patterns associated with athletes' sports performance while considering their interaction with the gut microbiota. Finally, we discuss the potential mechanisms by which dietary patterns affect sports performance from a nutritional perspective, aiming to elucidate the intricate interplay among dietary patterns, the gut microbiota, and sports performance. We have found that the precise application of specific dietary patterns (ketogenic diet, plant-based diet, high-protein diet, Mediterranean diet, and high intake of carbohydrate) can improve vascular function and reduce the risk of illness in health promotion, etc., as well as promoting recovery and controlling weight with regard to improving sports performance, etc. In conclusion, although it can be inferred that certain aspects of an athlete's ability may benefit from specific dietary patterns mediated by the gut microbiota to some extent, further high-quality clinical studies are warranted to substantiate these claims and elucidate the underlying mechanisms.

Keywords: gut microbiome; dietary pattern; sports performance; athlete

1. Introduction

In recent decades, with the rapid development of competitive sports worldwide, there has been an increasing demand for greater sports performance. Factors such as training strategies, dietary patterns and training environments have garnered significant attention in improving sports performance. Among these factors, dietary patterns are particularly crucial alongside training strategies. It is imperative for athletes to consume adequate nutrition to optimize their condition during training and facilitate proper recovery afterwards [1,2]. Different dietary patterns may yield varying effects on athletes' sports performance and be suitable for different athletic specialties [3–5]. However, there is a paucity of comprehensive reviews examining the potential mechanisms by which dietary patterns influence sports performance.

The human intestinal tract harbors a dynamic and complex bacterial community known as the gut microbiota, which emerging evidence suggests has beneficial effects on

human health, including strengthening the gastrointestinal barrier, improving immune function, and regulating glucose and fat metabolism [6]. Consequently, there is growing interest in investigating whether the gut microbiota acts as a mediator for various diseases such as obesity, diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD) [7]. Furthermore, recent attention has focused on exploring the potential role of the gut microbiota as a mediator between dietary patterns, especially for specific micronutrients such as dietary fiber and anthocyanins (ACNs) that are abundant in dietary patterns, and sports performance in athletes [8–10]. However, most studies have primarily evaluated the effects of supplements, like probiotics, on athletic performance rather than deeply investigating the relationship between dietary patterns and sports performance through their impact on the gut microbiota. This may be attributed to the complex interaction among different nutrients within dietary patterns and a limited scientific understanding of their specific influence on sports performance. Moreover, the application of dietary patterns on animal models may pose challenges, while using human models may impede the exploration of the potential mechanisms. Therefore, this review aims to summarize recent studies examining how some primary dietary patterns affect sports performance in athletes while also proposing some possible mechanisms involving nutrient-mediated interactions with the gut microbiota to provide practitioners with insights into enhancing sports performance through targeted dietary patterns.

2. The Overview of Gut Microbiota

2.1. Gut Microbiota

The human microbiota is defined as the microorganisms that exist in symbiosis with the human body, encompassing approximately 10^{14}–10^{15} bacteria [11]. It comprises bacteria, archaea, fungi, viruses, bacteriophages, and protozoa [12]. These microorganisms colonize various regions of the human body from birth onwards and are predominantly concentrated in the oral and nasal cavities, skin, the urogenital tract and the gastrointestinal tract [11]. Notably, within the gastrointestinal tract, recent studies have revealed a microbial cell count comparable to that of host cells [13]. Among the vast array of bacterial cells constituting the gut microbiota, which comprises around 2000 identified species [14–16], the microbial concentration gradually increases along the gastrointestinal tract, with an abundance of particular anaerobic taxa [17,18]. In the stomach, the acidic pH limits the existence of bacteria, so it presents the lowest number of bacteria, which are primary represented by *Lactobacillus*, *Candida*, *Streptococcus*, and *Helicobacter pylori*. However, in the colon, the favorable pH creates a more suitable habitat for bacteria such as *Bacteroides*, *Clostridium*, *Bifidobacterium*, and *Enterobacteriaceae*, and most of these species are obligate anaerobic bacteria, which participate in the decomposition of polysaccharides and the production of short-chain fatty acids (SCFAs) [19].

The gut microbial composition and diversity undergo changes with aging and are influenced by various factors. For instance, the mode of delivery significantly impacts the initial colonization of bacteria. It has been suggested that infants born through natural delivery predominantly harbor *Lactobacillus* and *Prevotella* species in their gut microbiota, while those born via Cesarean section tend to possess microbiota dominated by *Streptococcus*, *Propionobacterium*, and *Corynebacterium* bacteria. In adulthood, the gut microbiota forms a relatively stable community, but it might vary among individuals. This microbiota community is mainly represented by the *Bacteroidota* and *bacillota* phyla, as well as *Escherichia* and *Lactobacillus* to a lesser extent, but *Bifidobacterium* species remains constant. Among the elderly, Bifidobacterium species decrease in quantity, but Escherichia and *Lactobacillus* tend to increase [20,21]. Apart from the delivery mode, numerous other factors can also influence the diversity of the gut microbiota, including dietary habits, antibiotic usage, host genetics, lifestyle choices, surgical interventions, substance abuse disorders, mental health conditions, and physical exercise [6,7,19,22].

2.2. The Main Function of Gut Microbiota on Health

For a considerable duration, extensive research has focused on the perspective that bacteria are pathogenic to humans, exemplified by *Streptococcus pyogenes*, *Bordetella pertussis*, *Corynebacterium diphtheriae*, *Clostridium tetani*, *Salmonella typhimurium*, *Vibrio cholera*, and numerous others [22–25]. However, the majority of the microbiota are non-pathogenic and even crucial for human health. Substantial evidence now suggests that the gut microbiota plays a pivotal role in human well-being. It participates in metabolic functions by processing indigestible dietary residues and producing SCFAs, which contribute to host metabolic homeostasis [26]. SCFAs subsequently influence mucosal or systemic circulation to impact peripheral organs and tissues. Apart from SCFAs, numerous other microbial metabolites also play crucial roles in various physiological functions. These include bile acids, which promote lipid uptake and maintain gastrointestinal function; lipids such as Lipopolysaccharide (LPS) and Peptidoglycan, which enhance immune system function and regulate glucose homeostasis through the activation of the brain–enteric–liver axis; and choline, which regulates lipid metabolism and glucose homeostasis [27–29]. The bacteria species of the gut microbiota also participate in the synthesis of glycans, amino acids, vitamins and other essential components of the human metabolism [14,30]. Furthermore, the gut microbiota actively contributes to fortifying the gastrointestinal barrier by promoting the proliferation and turnover of epithelial cells, thereby enhancing its physiological function. Toll-like receptors (TLRs) play a key role in this process [30–33]. Within the small intestine's epithelium cells, Paneth cells recognize the enteric bacteria and subsequently initiate the expression of diverse antimicrobial factors through TLR activation, effectively safeguarding against pathogenic bacterial infiltration [31,33,34]. Additionally, the microbiota stimulates immunoglobulin (IgA) secretion and the production of antimicrobial molecules that inhibit the proliferation and colonization of pathogenic bacteria, thus facilitating the development of gut-associated lymphatic tissue (GALT) and bolstering the host immune system [34,35]. The immune system detects pathogen-associated molecular patterns (PAMPs), which are TLR ligands, enabling it to identify potentially pathogenic bacteria, and consequently leading to increased cytokine levels and the enhanced activation of T cells against these pathogens as a response. Although the gut microbiota has many benefits for the human body, dysbiosis characterized by a quantitative and qualitative imbalance in the microbial composition, along with reduced diversity among species, can give rise to various disorders, including diabetes, cardiovascular diseases, inflammatory bowel diseases (IBD), NAFLD, and obesity. Notably, the presence of *Akkermansia muciniphila*, which represents 3–5% of the typical intestinal microbial members, is decreased in obese people, and *Alistipes putredinis*, which belongs to the phylum Bacteroidota, seems to be represented in people with type 2 diabetes and obesity [19,36–39]. In this context, several studies have demonstrated that dietary interventions as well as exercise interventions hold promise as effective strategies for modifying the composition and diversity of the gut microbiota towards a more favorable community structure [19,40–43].

3. The Relation between Gut Microbiota and Exercise

Exercise is widely acknowledged to have a positive impact on human health, and recent studies have increasingly focused on its relationship with the gut microbiota (Figure 1). In contract to sedentary subjects, athletes and physically active individuals exhibit a greater diversity of fecal bacteria, an abundance of beneficial species [44–46], and a heightened microbial metabolism, as evidenced by increased activity in the carbohydrate and amino acid metabolic pathway [45–47]. Moreover, regular endurance exercise modulates the composition of the gut microbiota and reduces the presence of inflammation-associated proteobacteria [19].

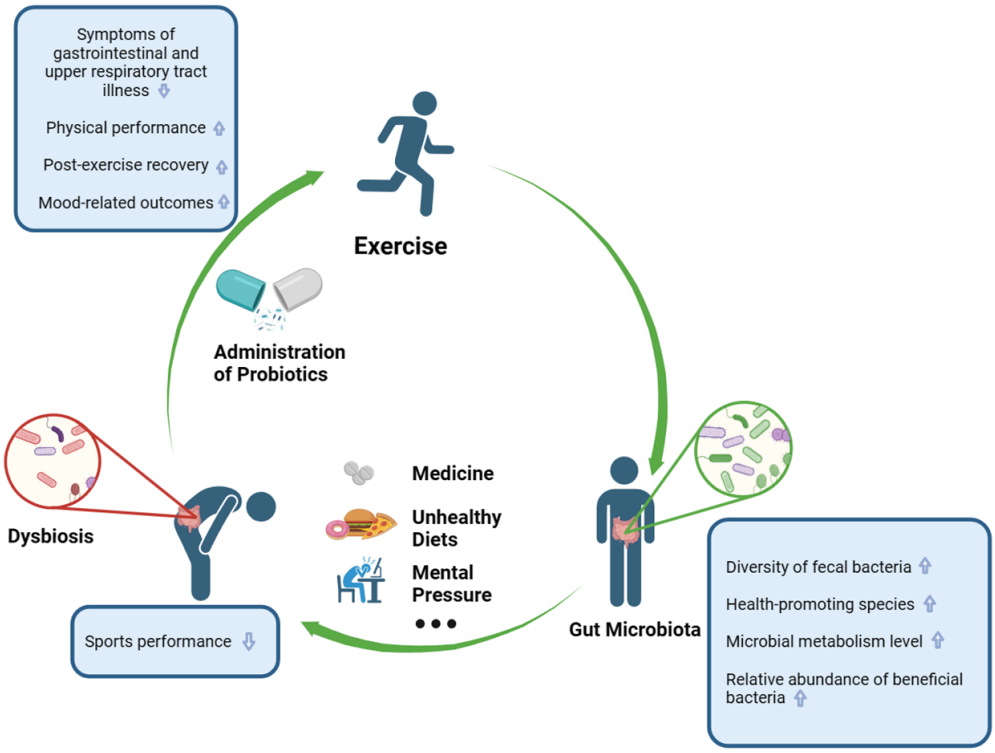

Figure 1. The interaction between exercise and the gut microbiota. Exercise can lead to changes in the gut microbiota [44,47–51]. Unhealthy lifestyles can lead to dysbiosis [36,39]. The administration of probiotics can affect the condition of the gut microbiota, which can subsequently affect sport performance [6,52]. The upward arrows indicate a rise or improvement, the down arrows indicate a drop.

3.1. Gut Microbiota in Athletes

A growing body of research has demonstrated that exercise exerts a modulatory effect on the gut microbiota, leading to a distinction in the microbial composition between athletes or physically active individuals and sedentary counterparts. As depicted in Table 1, there were significant differences in the major taxa at various levels between the two population groups. It is worth noting that the trend observed in the Bacteroidetes to Firmicutes ratio between the two groups across different studies was inconsistent [53,54], which may be attributed to several factors including substantial individual variance, human species, and enterotypes [55].

But generally, it is widely accepted that athletes exhibit an enrichment of health-promoting species within their gut microbiota, such as a higher abundance of *Akkermansia* spp. and *Prevotella* spp. [44,47–49,53–55]. The study conducted by Clarke et al. on male international rugby players from Ireland investigated the dietary intake and physical activity of these athletes, revealing a higher α-diversity in the gut microbiota compared to sedentary controls [44]. The study also included two sedentary control groups consisting of healthy non-professional athletes with different a body mass index (BMI), including a high BMI (BMI > 28) and low BMI (BMI < 25). According to the findings, the professional athletes exhibited greater diversity in their fecal microbiota compared to both control groups. The gut microbiota of elite athletes consisted of 22 phyla of bacteria, while only 11 and 9 phyla were found in the low and high BMI groups, respectively. Notably, increased *Akkermansia muciniphila*, associated with the lean phenotype, was observed in professional athletes and the low BMI group compared with the high BMI group. *Akkermansia muciniphila*, associated with positive metabolic function, is a mucin-degrading bacterium that inhabits the nutrient-rich mucus layer of the gut [56]. Furthermore, this study suggested that the microbial metabolism levels differed between professional athletes and sedentary groups,

as indicated by the increased activity in the carbohydrate and amino acid metabolism pathways in athletes. However, it is important to note that the differences in dietary patterns, which refer to a higher total energy, macronutrient (especially protein), and fiber intake in professional athletes compared with the control group, may also influence the gut microbial composition [44].

Table 1. Comparison of the gut microbial composition between athletes/physically active population and non-athletes/sedentary population.

Author, Year	Country	Sample Size, Sex and Age	Main Findings on Gut Microbial Composition	
			Athletes/Physically Active Population	**Non-Athletes/Sedentary Population**
Xu et al., 2022 [53]	China	n = 66 (males = 36, females = 30), Age: 18–25 years	Bacteroidetes (52.53%) Firmicutes (43.99%) Prevotella (20.88%) Bacteroides (24.96%) Faecalibacterium (6.86%) Megamonas (11.67%)	Bacteroidetes (62.81%) Firmicutes (32.14%) Prevotella (26.81%) Bacteroides (25.01%) Faecalibacterium (10.57%) Megamonas (5.15%)
Humińska-Lisowska et al., 2024 [55]	Poland	n = 52, males Age: 19–24 years	Enterotype: Endurance group: Bacteroides-driven (46.70%) Strength group: Prevotella-driven (50.00%)	Enterotype: Control group: Bacteroides-driven (40.90%) Ruminococcus-driven (40.90%)
Hintikka et al., 2022 [54]	Finland	n = 54 (males = 28, females = 26) Age: Athlete group: 27.1 ± 5.1 years Control group: 27.4 ± 5.6 years	Bacteroidetes (50.40%) Firmicutes (46.00%) Proteobacteria (2.30%) Actinobacteria (0.79%)	Firmicutes (48.30%) Bacteroidetes (46.20%) Proteobacteria (3.36%) Actinobacteria (1.57%)

Additionally, a study revealed that competitive cyclists exhibited a decreased relative abundance of *Bacteroides* spp. Furthermore, the relative abundance of *Prevotella* spp. was found to be higher in cyclists who engaged in training for more than 11 h per week compared with those who trained less frequently [45]. These findings provide evidence supporting the notion that physical exercise can induce alterations in the composition of the gut microbiota.

3.2. Impact of Exercise Interventions on Gut Microbiota

To further substantiate the impact of physical exercise intervention on the gut microbiota, several studies have been conducted to explore the causal relationship between exercise and alterations in the gut microbial composition (Figure 1). One study demonstrated that an endurance exercise intervention induced modifications in the gut microbial composition of sedentary, non-trained Finnish women, while controlling for factors such as dietary habits, weight, and body composition [50]. Notably, there were no significant changes observed in the total energy intake or macronutrient and dietary fiber consumption following training. Moreover, no discernible differences were found in the α-diversity of the gut microbiota or the phylum-level relative abundance between pre-intervention and post-intervention samples. However, endurance exercise did lead to an increase in the relative abundance of members of the genera *Verrucomicrobia* and *Akkermansia*, while reducing the levels of inflammation-associated Proteobacteria within the gut.

In addition to endurance exercise, resistance training also exerts an influence on the gut microbiota. Smith et al. demonstrated that 10 weeks of resistance training can improve the alpha diversity in younger untrained adults [57]. Another study conducted by Dupuit et al. explored the impact of a combination of high-intensity interval training (HIIT) and resistance training on the gut microbiota of postmenopausal women [58]; the authors indicated that the training intervention did not significantly change the alpha diversity

and overall taxonomy of the fecal microbiota but modified the beta diversity, which is inconsistent with the previous study, showing that more research about resistance training is needed.

However, several other factors may also impact the effectiveness of exercise on the gut microbiota. For instance, one study has indicated that BMI could potentially influence the response of the gut microbiota to physical exercise. According to this particular study [59], individuals with different body compositions (lean and obese) exhibit distinct baseline gut microbiota profiles. However, after a 6-week aerobic exercise intervention, no significant difference in the microbiota community composition was observed between lean and obese subjects.

3.3. The Influence of Gut Microbiota on Sports Performance

Exercise exerts a significant impact on the composition of the gut microbiota, while it is reciprocally influenced by the gut microbiota. Determining the precise effects of the gut microbiota on sports performance in human clinical studies poses a challenge due to the intricate interplay of nutritional, genetic and environmental factors [6]. However, germ-free animal models provide a novel approach and have already been employed to elucidate the impact of the gut microbiota on sports performance [60].

A cross-sectional study conducted by Hsu et al. investigated the swimming capacity of specific pathogen-free (SPF), germ-free (GF), and *Bacteroides fragilis* gnotobiotic mice. The results revealed that the swim-to-exhaustion time was the longest for SPF mice and the shortest for GF mice, indicating a compromised sports performance in the absence of a gut microbiota [60]. Although the effects of probiotics supplementation have been studied in athletes and physically active populations, the small number of participants, the different exercise intervention programs implemented, and the different training histories of the participants may have influenced the outcomes [61]; therefore, the results remain controversial. However, a review conducted by Marttinen et al. as summarized several benefits of probiotics for the athlete. The authors demonstrated that the administration of probiotics might reduce symptoms of gastrointestinal and upper respiratory tract illnesses, enhance physical performance, improve post-exercise recovery, and improve mood-related outcomes [6,62–65]. Therefore, there exists a significant association between the composition of the gut microbiota and sports performance (Figure 1).

4. The Influence of Several Typical Dietary Patterns on the Gut Microbiota

Personal dietary habits play important roles in shaping the composition of the gut microbiota in humans. Although further research is needed to fully understand the intricate relationship between diet and the gut microbiota, numerous studies have highlighted the significant impact of different types of dietary patterns on the composition of the gut microbiota within 24 h [66,67]. The dietary patterns of individuals can be broadly categorized into vegetarians, meat eaters and balanced eaters, each exhibiting a distinct profile in the gut microbiota. Different types of dietary patterns elicit distinct alterations in the proportions of Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Changes in the gut microbiota induced by dietary interventions are observed within 24 h and return to baseline levels within 48 h after discontinuation [66]. These changes encompass alterations in carbohydrate and protein fermentation, intestinal inflammation, fat oxidation, as well as an increase in amino acid availability, potentially promoting protein anabolism [46,68–71]. Furthermore, the quality, quantity and molecular characterization of carbohydrates, protein, and fat are key factors influencing both the composition and metabolism of the gut microbiota. Unhealthy dietary patterns can stimulate the proliferation of detrimental gut bacteria that pose risks to human health. However, a healthy dietary pattern has restorative effects on beneficial gut bacteria [72]. The maintenance and modulation of beneficial gut microbiota are vital for host health. In addition to general dietary patterns, probiotics supplementation and wholefood supplementation are also common nutrition strategies. Notably, probiotics are defined as living organisms with beneficial effects on health. Most

probiotic supplementations contain high concentrations of *Lactobacillus* or *Bifidobacterium* spp., which can support the immune system of the host, regulate gut permeability, and produce sanatory metabolites [73]. Unlike synthetic supplements, wholefood supplements are based on the core idea of supplying the body with nutrients in their pure, unaltered state. This implies that these supplements are rich in a broad spectrum of vitamins, minerals, antioxidants, and other crucial nutrients that are inherently found in the foods from which they are sourced [74,75]. In this section, the influence of several typical dietary patterns on the gut microbiota will be discussed in detail (Figure 2).

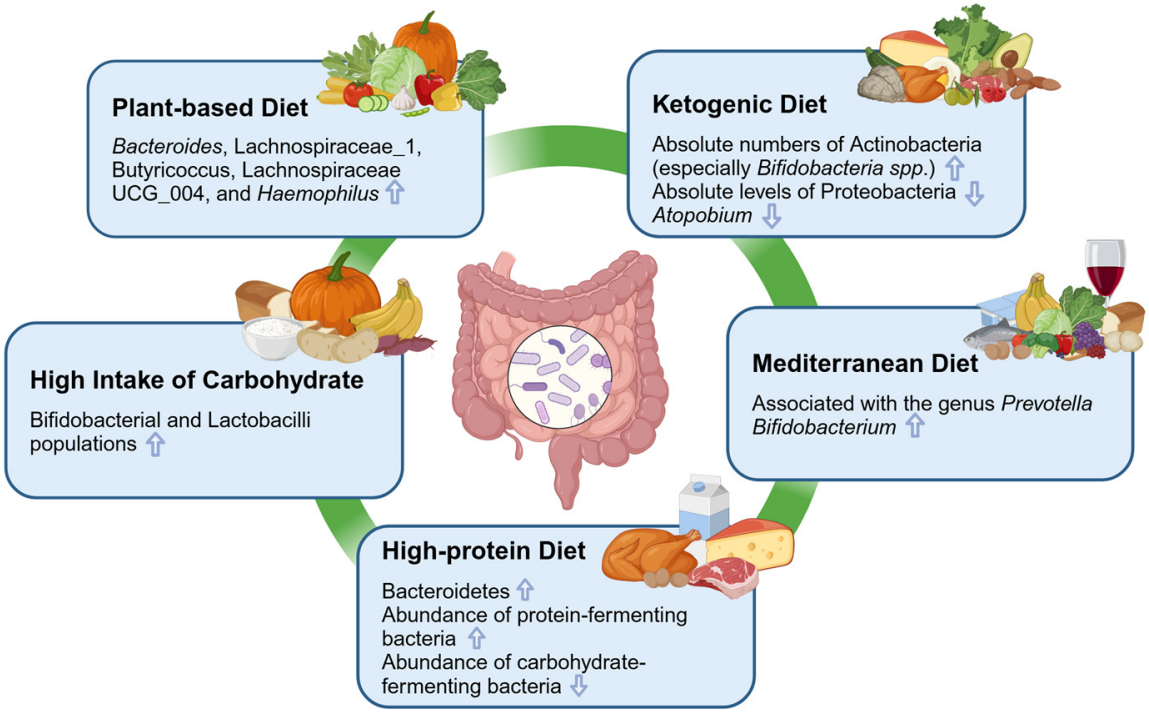

Figure 2. The effects of dietary patterns on the gut microbiota. Different dietary patterns will lead to different changes in the abundance of the gut microbiota. Reference: ketogenic diet [52], plant-based diet [76], high-protein diet [48,77], Mediterranean diet [78–80], high intake of carbohydrates [81]. The upward arrows indicate a rise or improvement, the down arrows indicate a drop.

4.1. Ketogenic Diet

The ketogenic diet (KD) is characterized by a high fat content, a low carbohydrate intake, and an appropriate proportion of protein and other essential nutrients. There are four main types of ketogenic diets: (1) the classical KD with a macronutrient ratio of 4% carbohydrate, 90% fat and 6% protein, (2) medium-chain triglyceride with a macronutrient ratio of 20% carbohydrate, 10% long-chain triglycerides fat, 60% medium-chain triglycerides fat and 10% protein, (3) modified Atkins with a macronutrient ratio of 10% carbohydrate, 65% fat and 25% protein, (4) low-glycemic-index diet with a macronutrient ratio of 10% carbohydrate, 60% fat and 30% protein [82]. It can be seen that the KD does not have a fixed nutrient ratio, but a high proportion of fat and a low proportion of carbohydrates should be guaranteed. The primary objective of this dietary pattern is to shift the glucose metabolism towards fat metabolism through the restriction of carbohydrate intake. Consequently, the KD can effectively lower blood sugar levels and increase free fatty acid and ketone production, thereby influencing neuronal excitability [83]. Notably, the KD is characterized by the production of ketone bodies (3-hydroxybutyrate, acetate and acetoacetate). The elevation in ketones contributes to an increase in anti-inflammatory and antioxidant activity, immune regulation, intestinal mobility and barrier function, cellular growth and differentiation, ionic absorption, as well as the prevention of distal ulcers, Crohn's disease, and colon cancers. Additionally, the KD was initially employed for manag-

ing refractory epilepsy and has progressively extended its application to encompass other neurological disorders [84], such as Parkinson's disease and Alzheimer's diseases. With the advancement of medical technology and sports science, there are several studies that have demonstrated the potential of this dietary pattern in enhancing sports performance in some ways [85–87]. Nevertheless, this diet still has some limitations; for example, the ability of muscle to use glycogen for oxidation is impaired after long-term ketoadaptation, leading to an inability to utilize the available glycogen, which provides a more effective energy source when the oxygen supply becomes limiting. Therefore, the performance of higher-intensity endurance exercise will be limited, which might increase the risk of injury for athletes [88].

A study conducted by Ang et al. in both mice and humans demonstrated that the ketogenic diet resulted in decreased levels of *Bifidobacterium*, which was mediated by the increased production of ketone bodies, especially beta-hydroxy butyrate. The decrease in *Bifidobacterium* reduced the levels of intestinal and visceral fat pro-inflammatory Th17 cells, which might be a potential mechanism contributing to the ketogenic diet's ability to reduce body fat because of the relationships between obesity and chronic low-grade inflammation. Furthermore, the ketogenic diet also decreased *Lactobacilli* and increased *Fusobacteria* and *Escherichia* [89].

Several studies have indicated that variations in the quantity and source of dietary fat can exert distinct effects on the host, with some of these effects potentially mediated by the gut microbiota. The consumption of saturated fat has been shown to increase the abundance of bacteria expressing LPS, leading to elevated levels of LPS and a pro-inflammatory state known as metabolic endotoxemia. Furthermore, excessive fat intake is also associated with reduced levels of butyric acid and retinoic acid [90], both crucial for maintaining gut homeostasis. Furthermore, the consumption of saturated fat can enhance the relative abundance of *Bilophila wadsworthia* by facilitating the conjunction of taurine with host LPS, which serves as a terminal electron acceptor and subsequently leads to the production of hydrogen sulfide and secondary bile acids. This cascade may ultimately result in intestinal barrier disruption and immune cell infiltration [91]. Hence, it implies that a KD characterized by a high saturated fat intake could potentially elevate the inflammatory level of the host. Conversely, polyunsaturated fat acid (w-3) can increase SCFAs and promote gastrointestinal integrity and inflammation. Furthermore, polyunsaturated fat increases the abundance of *Bifidobacterium*, *Lactobacilli*, and *Akkermansia muciniphila*, which are also increased by exercise. Thus, polyunsaturated fat might contribute to health and sports performance by mimicking the effects of exercise, but the dose remains controversial; more research is needed to investigate this [92,93].

Notably, with the advancement of research on diet and nutrition, the classical KD has undergone certain variations; for instance, the very-low-calorie KD (VLCKD) is characterized by a caloric intake of below 800 kcal/day. One study revealed that the VLCKD results in a more substantial weight reduction, rendering it an excellent option for weight loss [94,95]. Regarding the gut microbiota, a review has summarized the effects of the VLCKD on the gut microbiota [96]; in this study, the authors demonstrated that the abundance of Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobiota in people who undertook the VLCKD increased and that the abundance of Firmicutes, Firmicutes/Bacteroidetes ratio, Proteobacteria, and Actinobacteria decreased. These seemingly contradictory results suggest that further research is warranted to explore the impact of the VLCKD on the gut microbiota. Furthermore, the application of the VLCKD still remains controversial, especially for athletes, because this diet is used more in obese patients. However, the VLCKD could be used to control weight acutely in special sports during a special period with strict medical supervision, such as in gymnastics [97–99].

In conclusion, the impacts of the KD on the gut microbiota remains inconclusive and controversial, necessitating further studies to comprehensively understand its effects.

4.2. Plant-Based Diet

The plant-based diet is a dietary pattern primarily based on a diverse range of plants, encompassing seeds, fruits, and plant tissues that provide energy for human consumption. This includes cereals, tubers, legumes and their derivatives, as well as fruit and vegetable products. The distinguishing features of the plant-based diet are its high carbohydrate content, low energy density, low fat content, and absence of cholesterol, antibiotics or hormones [100]. Long-term adherence to a plant-based diet not only reduces the risk of many chronic diseases, but also contributes to the lower emission of greenhouse gases such as carbon dioxide during food processing compared to other methods [101]. Consequently, it plays an integral role in promoting human health and environmental preservation [102].

One study explored the microbial composition of 258 participants who adhered to one of four dietary patterns: the Western diet group, flexitarian group, vegetarian group, and vegan group [76]. Notably, the Western food group is characterized by a high intake of energy, salt, saturated fat, simple or added sugar, and a low intake of fruits and vegetables [103]. The vegetarian group is characterized by omitting defined food groups such as meat, sausage, fish, etc., and the vegan group is characterized by additionally omitting dairy products and honey [104–106]. Flexitarians generally consume meat or sausage once or twice per week [107]. The western diet group exhibited the lowest abundance of *Bacteroides*, Lachnospiraceae_1, Butyricoccus, Lachnospiraceae UCG_004, and *Haemophilus*; whereas the vegan group showed the highest abundance. For *Dorea*, the *Ruminococcus torques* group, *Eubacterium ruminantium* group, Ruminococcaceae, Lachnospiraceae_2, *Lactobacillus*, and *Senegalimassilia*, the lowest abundance was observed in the vegan group, while the highest abundance was observed in the Western diet group [76]. Notably, a high abundance of Lachnospiraceae in the vegan group indicates the extensive fermentation of plant-based polysaccharides into SCFAs like butyrate, which is beneficial for human health. For example, it serves as a crucial energy source for colonic epithelial cells, regulates intestinal inflammation, and confers protection against colon cancer in humans [76,108,109].

Furthermore, the vegan diet might also contribute to improving performance and promoting recovery in endurance sport by affecting body composition, blood flow, antioxidant capacity, systemic inflammation, and glycogen storage [110].

4.3. High-Protein Diet

People who stick to a high-protein diet can take in higher-quality protein and provide the body with amino acids. Protein is a macronutrient, as well as the main component of skeletal muscle. The uptake and catabolism of specific proteins by the liver and skeletal muscle are different, as is their ability to regulate the muscle protein synthetic response [111]. Amino acids can be metabolized into branched-chain fatty acids and SCFAs, ammonia, sulfides, indole, and phenolic compounds via the gut microbiota [112]. Some of these (e.g., SCFAs and indole) may be beneficial for the health of the gut, while other metabolites (e.g., ammonia and p-cresol) may decrease gut epithelium integrity [113].

The high-protein diet is widely popular and frequently adopted by fitness enthusiasts and athletes, particularly for the latter who engage in intense exercise routines that necessitate strict dietary practices to support optimal performance [114]. In contrast to the general population, athletes often consume significantly higher amounts of protein; however, if this excess protein remains unabsorbed, according to a study conducted by Moreno-Pérez et al. [48], it can enter the colon and promote the growth and selection of specific bacteria. In this study, a 10-week supplementation with protein, commonly used to meet the elevated protein requirements among individuals undergoing training, resulted in an increased abundance of Bacteroidetes while decreasing the taxa associated with overall health, including *Roseburia* spp., *Blautia* spp., and *Bifidobacterium longum*, among runners. Another study has compared the gut microbiota of bodybuilders consuming a high-protein diet with sedentary controls [77], and found that excessive protein intake increased the abundance of protein-fermenting bacteria such as *Clostridium*, *Bacillus*, *Staphylococcus*, and other species belonging to the Proteobacteria family. Moreover, the high-protein diet might

lead to a reduction in carbohydrate-fermenting bacteria, such as *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Ruminococcus, Roseburia*, and *Faecalibacterium*. The fermentation of incompletely digested protein in the colon might lead to the production of toxic metabolites such as ammonia, biogenic amines, indole compounds, and phenols. However, there was no significant difference in the abundance of selected bacteria (*Bifidobacterium* spp., *Bacteroides* spp., *Faecalibacterium prausnitzii*, *Akkermansia Muciniphila*) between the bodybuilder group and control group; the possible reason for this is that both of the two groups met the criteria for the recommended fiber intake, and the effect of high protein intake on the gut microbiota might have been attenuated by the appropriate intake of carbohydrate and fiber. Therefore, it is imperative to strictly control not only the types of protein consumed, but also the quantity ingested by athletes.

4.4. Mediterranean Diet

The Mediterranean diet (MD) originates from the Mediterranean region, including Greece, Spain, France, and Italy. It is based on the traditional dietary habits of the countries bordering the Mediterranean Sea. This dietary habit is characterized by a high intake of fruits, vegetables, cereals, olive oil, legumes and tree nuts, a moderate intake of seafood, and a low intake of sugar sweetened foods, red and proceed meat, and carbonated beverages [4]. However, there remains controversy surrounding its precise definition. In a recent study [115], the authors attempted to establish a unified definition of the MD by considering daily servings of key foods and their nutrient content: Vegetables: 3 to 9 servings; Fruit: 0.5 to 2 servings; Cereals: 1 to 13 servings; Olive oil: up to 8 servings.

Considering its energy intake and macronutrient composition, the MD can be classified as a predominantly plant-based dietary pattern, encompassing vegetables, fruits, cereals, and olive oil [116–118]. It is notable that the MD exhibits a relatively high fat content, with monounsaturated fats comprising twice the amount of saturated fat. The primary source of monounsaturated fats in the MD is olive oil, which is closely associated with the traditional olive cultivation in the Mediterranean region. Additionally, the MD allows for the moderate consumption of white and red meat. Extensive evidence supports that adherence to the MD promotes longevity while reducing the metabolic risks associated with diabetes mellitus, obesity, and other metabolic syndromes [119,120]. Moreover, it demonstrates a reduced risk of malignancy and cardiovascular disease while enhancing cognitive function [116].

Numerous studies have consistently demonstrated that the gut microbiota plays a crucial role as a potential mediator in the association between the MD and human health. A study has indicated that nearly 60% of the overall composition of the gut microbiota is responsive to dietary changes [121]. The MD can not only modulate the diversity and composition of the gut microbiota, but also improve the generation of SCFAs due to its high proportion of plant-based food [78]. Previous research has shown an association between the MD and *Prevotella* [78,79], while another study suggests that the MD contributes to reducing dysbiosis and increasing *Bifidobacterium* among patients with metabolic syndrome [78,80]. However, it should be noted that not all studies support the positive influence of the MD on the gut microbiota. Some investigations found no significant difference in the gut microbiota composition between individuals adhering to either the MD or Western diet interventions for 6 months. Therefore, further research is warranted to comprehensively explore the impact of the MD on the gut microbiota [78,122]. Furthermore, according to a narrative review conducted by Griffiths et al., the application of MD or individual foods and compounds in this dietary pattern might have potential positive effects on oxidative stress, inflammation, injury, illness risk, and cognitive and vascular function in competitive athletes [4].

4.5. High Intake of Carbohydrate

Limited research has been conducted on the high-carbohydrate diet, probably due to the fact that a high intake of carbohydrate is not typically considered as an indepen-

dent dietary pattern but rather as a supplementary measure in other dietary patterns to meet the energy requirements of athletes, given its role as a primary fuel source during exercise [123]. It is recommended that athletes consume ample amounts of simple carbohydrates to maintain glucose homeostasis and limit their fiber intake prior to exercise in order to minimize gastrointestinal discomfort. Non-digestible carbohydrates will be discussed later. Adequate carbohydrate consumption is crucial for athletes. The ingestion of simple carbohydrates before and during exercise (e.g., glucose, fructose, sucrose) can alleviate fatigue, facilitate rehydration and the maintenance of optimal fluid balance, and enhance sports performance [124–128]. For example, lactose may serve as an effective fuel source before, during and after exercise, thereby enhancing sports performance and aiding recovery while also potentially exerting beneficial effects on the gut microbiota, such as increasing Bifidobacteria and Lactobacilli populations [81]. According to a study conducted by Faits et al., which discusses the different effects of simple, refined, and unrefined carbohydrate-containing foods on the gut microbiota, after the consumption of an unrefined carbohydrate diet, the abundance of *Roseburia* was higher and fecal secondary bile acid concentrations were lower relative to the simple carbohydrate diet, whereas the abundance of *Anaerostipes* was higher after the consumption of a simple carbohydrate diet relative to the refined carbohydrate diet [129].

Notably, athletes in many sports often consume a high amount of fast-absorbed carbohydrates to maximize glycogen storage. However, they also aim to avoid non-digestible carbohydrates in order to prevent intestinal issues and other unfavorable syndromes that can negatively impact sports performance, such as bloating and diarrhea [130]. While a high intake of fast-absorbed carbohydrates can increase energy storage during training or competition, a low intake of dietary fiber may lead to the reduced production of short-chain fatty acids (SCFAs), altered intestinal transit times, and a loss of bacterial diversity [3], all of which have negative implications for long-term health [131]. Therefore, it is important for athletes to consume a certain amount of fiber to generate less gas after fermentation by the gut microbiota in order to gain health benefits and avoid gastrointestinal issues.

5. Different Dietary Patterns and Sports Performance—Gut Microbiota as the Mediator

5.1. Gut Microbiota as the Mediator

With the advancement of competitive sports, whether it pertains to athlete-to-athlete competition or the audience's heightened expectations for sporting event enjoyment, both lead to elevated demands on athletes' capabilities. Numerous factors, such as exercise intensity, dietary patterns, lifestyle choices and genetic inheritance, among others, can influence the sports performance of athletes or physically active individuals. The gut microbiota—an integral component of human beings since birth—has emerged as a prominent area of research interest due to its intricate composition and structure. Several studies have indicated disparities between the gut microbiota profiles of athletes and those of normal individuals. Numerous investigations have attempted to establish whether the gut microbiota is a mediator linking dietary patterns and sports performance. Here, we present a concise overview of the current primary evidence pertaining to the aforementioned dietary patterns and the discuss probable mechanisms by which dietary patterns affect sports performance (Table 2).

Table 2. The probable mechanism of dietary patterns affecting sports performance.

Author, Year	Dietary Pattern	Substance	Subjects	Pathway	Most Important Findings
(Caesar et al., 2015 [115])	Ketogenic diet	Saturated fat	Male mice	LPS/TLR4 pathway	Increases inflammatory indices in WAT
(Minevich et al., 2015 [119])	High-protein diet	*Bacillus coagulans GBI-30, 6086* Protein	Males (n = 11)	Promote the absorb and utilize of protein	Produces proteases which can increase amino acid absorption in humans

Table 2. *Cont.*

Author, Year	Dietary Pattern	Substance	Subjects	Pathway	Most Important Findings
(Zhu et al., 2017 [121])	High-protein diet	Animal protein	Male rats (n = 32)	Decrease the binding of CD14 and LPS-binding protein	Higher abundance of Lactobacilli Higher ratio of Firmicutes to Bacteroidetes Lower butyrate Lower SCFAs-producing bacteria Lower LPS-binding protein Lower transcription factor CD14 receptor Lower inflammation
(Jäger et al., 2007 [81])	Plant-based diet/ Mediterranean diet	Dietary fiber	C2C12 myotubes Female mice	AMPK/PGC-1α pathway	Enhances fatty acid oxidation of muscle
(Yang et al., 2023 [132])	Plant-based diet/ Mediterranean diet	Anthocyanins	C2C12 myotubes Male mice (n = 60)	AMPK signaling pathway	Reduces oxidative stress Promotes mitochondrial biogenesis Converse skeletal muscle fiber

This table shows the mechanisms of the effects of nutrients in different dietary patterns on the gut microbiota. AMPK: adenosine 5-monophosphate-activated protein kinase; CD14: cluster of differentiation 14; LPS: lipopolysaccharide; PGC-1α: proliferator-activated receptor gamma coactivator; SCFAs: short-chain fatty acids; TLR4: toll-like receptors 4; WAT: white adipose tissue.

To date, the impact of the KD on sports performance remains controversial. As mentioned earlier, the consumption of saturated fat increases the LPS level in the host, which activates toll-like receptors 4 (TLR4) and cluster of differentiation 14 (CD14), leading to obesity, increased inflammatory indices in white adipose tissue (WAT), and insulin resistance [133]. Interestingly, this effect was observed only in subjects consuming saturated fat. These findings suggest that athletes implementing a KD can increase their intake of unsaturated fats to avoid inflammation and insulin resistance. Additionally, the VLCKD may have a beneficial effect on obesity by regulating the gut microbiota and restoring homeostasis [96]. The study by Gutierrez-Repiso et al., 2019, discussed the association between the VLCKD and weight loss through the gut microbiota [134]. On one hand, the authors reported that the abundance of *Butyricimonas* and *Oscillospira* increased at the genus level. Notably, *Oscillospira* is positively associated with high-density lipoprotein, butyrate and leanness, while *Butyricimonas* is positively associated with energy metabolism and homeostasis between the microbiota and host. Both of these gut microbiota are beneficial for weight loss. On the other hand, the proportion of *Serratia* and *Citrobacter,* whose abundance

has been positively correlated to obesity, decreased. Therefore, the VLCKD can positively regulate the gut microbiota after obesity-relative dysbiosis. This dietary pattern enables rapid short-term weight reduction, making it suitable for athletes who need to quickly regain an optimal weight.

In terms of the high-protein diet, research has primarily focused on the impact of protein. Evidence suggests that the gut microbiota contributes to the absorption and utilization of protein, as well as the anabolism and functionality of skeletal muscle by providing fuel and storage and modulating inflammation. For example, the co-administration of the probiotic *Bacillus coagulans (GBI-30,6086)* with protein has been shown to reduce the inflammation of epithelial cells, enhance nutrient absorption and stimulate protease production for increased amino acid uptake in humans [135,136]. These effects have the potential to mitigate muscle damage and facilitate muscle recovery, thereby promoting sports performance [137]. In addition, animal studies have been conducted to investigate the effects of different protein types on the gut microbiota, with a particular focus on comparing animal-based proteins to plant-based proteins [138–141]. These studies have demonstrated that the consumption of meat protein leads to a higher abundance of Lactobacilli and an increased ratio of Firmicutes to Bacteroidetes, while also reducing levels of butyrate-producing bacteria (e.g., *Bacteroides* and *Prevotella*), LPS-binding protein, and transcription factor CD14 receptor when compared to non-meat protein intake. Furthermore, dairy proteins appear to have an intermediate effect between meat and non-meat proteins. It is worth noting that LPS-binding protein binds to CD14 in order to activate macrophages, which can subsequently produce inflammatory cytokines, leading to inflammation. Based on these findings, it can be hypothesized that athletes may benefit from consuming more meat protein rather than non-meat protein in order to mitigate muscle inflammation and maintain optimal sports performance. However, the studies mentioned above have primarily focused on rodents, with limited exploration of their effects on humans; one reason for this may be that it is difficult to intervene individually with different types of proteins in humans, and that other nutrients might interfere with the experimental results. A human study investigating the impact of various protein types on gut the microbiota and incorporating a high- or low-saturated fat component into the study design indicated that the intake of saturated fat may cover up the effects of protein types [142]. Another study conducted by Losasso et al. that compared the influence of vegan, vegetarian and omnivore-oriented Westernized dietary styles on the gut microbiota indicated that vegans and vegetarians show higher α-diversity than those who consume animal protein, the main operational taxonomic units associated with the phylum Bacteroidetes, and the genus *Prevotella*, which can improve glycogen storage, was more prevalent among individuals that consume more fiber and vegetable protein. However, the subjects in this study also consumed different nutrients, which may have influenced the results [143]. Consequently, it can be inferred that enhancing the protein bioavailability and absorption, as well as muscle protein synthesis, serves as an important mechanism through which the gut microbiota influences muscle mass and function. This mechanism is likely regulated by SCFA production, thereby affecting insulin sensitivity, inflammation, and insulin growth factor I (IGF-I) release to maintain anabolic–catabolic balance. Furthermore, more studies elucidating the effects of different protein types in humans that consider other dietary components beyond just protein consumption are needed [144,145].

It is noteworthy that dietary fiber plays an essential role in both the plant-based diet and MD, as it constitutes their main component. Dietary fiber is composed of complex carbohydrates, including fermentable (mainly soluble) and non- or poorly fermentable (mainly insoluble) fibers, as well as oligosaccharide. Dietary fiber influences the composition of the gut microbiota, contributing to the establishment and maintenance of a healthy and diverse gut microbiota while improving intestinal immunity [146]. However, the insufficient intake of dietary fiber may have adverse effects on human health. The dietary fiber in the aforementioned dietary patterns includes "Microbiota-accessible carbohydrate (MACs)", which are complex carbohydrates found in fruits, vegetables, legumes, and whole grains [116].

A study conducted by Xu et al. has shown that a high intake of MACs promotes lipid profile improvement, glycemic control, body weight reduction, and an inflammatory maker decrease compared with low MAC intake [147]. Furthermore, MACs can influence the gut microbiota and modulate the growth of species that produce SCFAs, which are the end products of dietary fiber fermentation in the intestines. SCFAs play an essential role in human metabolism. A study has indicated that SCFAs can directly activate Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) by increasing the AMP/ATP ratio in skeletal muscle and liver or indirectly activate it via the Ffar2-leptin pathway [148–150]. The activation of AMPK triggers the expression of proliferator-activated receptor gamma coactivator PGC-1α, which is known to regulate the transcriptional activity of key factors including peroxisome proliferator-activated receptors PPARα, PPARδ, PPARγ, liver X receptor (LXR), and farnesoid X receptor (FXR). These factors are crucial to regulate the metabolism of cholesterol, lipid, and glucose. The fatty acid oxidation of muscle and liver is ultimately enhanced, while de novo fatty acid synthesis in the liver is reduced [151,152]. In addition, SCFAs have been demonstrated to enhance the protein expression of PGC-1α and uncoupling protein-1 (UCP-1) in brown adipose tissue, subsequently promoting thermogenesis and fatty acid oxidation. These results suggest that the plant-based diet and MD, which are rich in dietary fibers, could be considered for dietary planning among weight-conscious athletes such as marathon runners. However, it is still crucial for endurance athletes to maintain an adequate intake of simple carbohydrates. For instance, in an international marathon competition that typically lasts for a minimum duration of approximately two hours, athletes require sufficient glycogen reserves to optimize their sports performance. Therefore, carbohydrate loading is commonly employed by endurance athletes as a strategy to enhance glycogen concentrations prior to competitions. However, it is crucial to avoid consuming carbohydrates that are indigestible and unabsorbable in the small intestine, such as fiber and resistant starch [88]. Nevertheless, scientific evidence suggests that adopting a high-carbohydrate, low-fiber dietary pattern can have detrimental effects on the gut microbiota and overall health. These effects include disruptions in intestinal transit times, the loss of bacterial diversity, and reduced SCFA production [153–155]. Thus, athletes should judiciously manage both the timing and quantity of their intake of simple carbohydrates and nondigestible carbohydrates to optimize their sports performance while minimizing gastrointestinal distress.

Notably, a clinical study conducted by Jang et al. in Korea revealed an inverse correlation between total protein intake and the diversity of the gut microbiota, showing that the athletes in resistance sport who have a high protein diet showed a decrease in SCFs-producing commensal bacteria [49]. However, another study demonstrated a positive correlation between a high protein intake and microbial diversity; the gut microbiota of athletes consisted of 22 phyla of bacteria, while only 11 and 9 phyla were found in the low and high BMI groups [44]. It is worth noting that Korean athletes did not meet the recommended dietary fiber intake ($\geq$25 g/day; median intake in bodybuilders 19 g/day, endurance athletes 17 g/day) [49]. In contrast, Irish rugby players' dietary fiber intake met the recommendation level (median intake 39 g/day) [44]. Undigested dietary fiber serves as an essential energy and carbon source of gut microbiota, contributing to its diversity and acting as a substate for SCFA synthesis. Therefore, it can be inferred that combining a high-protein diet with low-dietary-fiber diet may have detrimental effects on the gut microbiota composition. This finding suggests that dietary fiber also plays an important role in the high-protein diet. Further investigations are warranted to ascertain whether alterations in SCFA levels serve as a pivotal mediator of the favorable physiological effects associated with a high dietary fiber intake.

In addition to dietary fiber, anthocyanins (ACN) have recently attracted the attention of many researchers. Amongst some of the dietary patterns mentioned above, fruits and vegetables are important components, particularly certain fruits that are abundant in ACN, a subclass of polyphenols responsible for the red–blue–purple pigmentation observed in fruits [156,157]. These bioactive compounds possess potent antioxidant and

anti-inflammatory properties that can effectively modulate the secondary cascade associated with exercise-induced muscle damage (EIMD) [10,158–161]. Delphinidin and cyanidin are the most extensively investigated anthocyanins, which also encompass malvidin, peonidin, petunidin and pelargonidin. These compounds exhibit favorable physiological effects in humans [162]. The bioavailability of ACN in the human intestinal tract is limited, with only a fraction of the dietary intake being digested and absorbed in the small intestine. However, this bioavailability can be enhanced through interactions with the gut microbiota [163]. The sugar moieties of ACN undergo hydrolysis by bacterial enzymes in the colon, leading to the transformation of aglycone forms into a variety of compounds, including protocatechuic acid, vanillic acid and gallic acid [164]. According to a study [132], cyanidin consistently converts into protocatechuic acid, which exhibits multiple protective functions for muscle health, such as reducing oxidative stress, promoting mitochondrial biogenesis, and converting skeletal muscle fibers from type II to type I. These effects on oxidative stress reduction and mitochondrial biogenesis may have potential benefits for athlete recovery. Notably, the conversion of skeletal muscle fiber emerges as a promising research domain, deserving significant attention. In the past, the selection of athletes across various sports has heavily relied on hereditary factors due to the perception that one's skeletal muscle type is genetically predetermined and difficult to change through training. With advancements in our understanding of the skeletal muscle fiber conversion, as well as potential nutritional strategies, the process of athlete selection may become more adaptable. However, humans still cannot make genetic changes. This means that while a certain genetic hereditary factor, such as alpha actinin-3 gene (ACTN3), plays a decisive role in skeletal muscle fiber conversion, dietary patterns could be utilized as a helpful strategy to improve it [165].

However, the specific bacterial taxa responsible for the transformation of anthocyanins into protocatechuic or gallic acid remain unknown. The bacterial enzymes involved in ACN hydrolysis may be present in several taxa of the genera, such as *Bacteroides*, *Clostridium* and *Eubacterium* [26,163]. Furthermore, different microbiota compositions may be associated with distinct pathways of ACN biotransformation, potentially leading to diverse effects ranging from beneficial to unknown outcomes [166,167], implying that the interaction between ACN and the gut microbiota could vary among individuals. Therefore, further research is needed to investigate individual differences in ACN metabolism and its potential health-promoting effects.

5.2. Practical Application

As mentioned earlier, different dietary patterns affect sports performance in different ways. Athletes should choose the appropriate dietary pattern on the basis of their actual situation during training. Athletes who need to control their weight strictly during competition in heavy sports, athletics and gymnastics may consider a ketogenic diet, which would enable them to lose weight in a short time, but this dietary pattern also has limitations; it is not suitable for enhancing strength in weight lifters or high-intensity cyclists, for example [168]. In terms of the plant-based diet, current evidence supports that this diet does not have a significant impact on sports performance, but as mentioned before, the special micronutrients in the plant-based diet have anti-inflammation and antioxidant effects to a certain extent, and it would be friendly to vegan athletes [169]. For athletes who seek to gain muscle mass and strength, such as bodybuilders, the high-protein diet is a good choice, because it is necessary to generate more muscle protein and prevent lean mass losses during the periods that restrict energy intake to promote fat loss [170]. Compared with other dietary patterns, the Mediterranean diet may be more suitable for most athletes; both aerobic and anaerobic athletes can select this dietary pattern, whose strengths are that it is rich in foods that can support high energy demands and that it can provide the antioxidants, essential vitamins and minerals that promote recovery [3]. In practice, these dietary patterns are used alternately or in a certain period of time, because any special dietary patterns used for a long time will cause adverse reactions [3,98].

6. Conclusions

In recent decades, it has been increasingly acknowledged that the gut microbiota plays an important role in human health and sports performance. As mentioned earlier, the impact of various dietary patterns on the gut microbiota and their subsequent effects on sports performance may vary. Therefore, further evidence is required to substantiate the relationship between different dietary patterns and their components with the gut microbiota and sports performance. In addition, it should be noted that diet is inseparable from the host; it is challenging to strictly disentangle exercise from daily diet during an experiment, as the individual contributions of each participant are difficult to isolate and assess. To date, there remains a dearth of research investigating the intricate interplay between diet, exercise, and the gut microbiota. Additionally, the responses of the gut microbiota to diet may vary among individuals, indicating that the formulation of diet regimens should shift from standardized diet guidelines to flexible recommendations tailored to individual preference and local customs, and the regular reassessment of these dietary regimens is essential. Moreover, the significance of nutrients or compounds in diets that have traditionally been regarded as non-nutritive cannot be disregarded, necessitating an exploration into whether these nutrients exert their effects independently or synergistically. Future research should focus on personalized nutrition strategies for different populations and the combined effects of different nutrients. The aforementioned findings will contribute to a comprehensive understanding of the intricate interplay among exercise, diet, and human health, which has implications not only for athletes' well-being but also for that of the general population.

Key Points

- The interactions between exercise and the gut microbiota play a role in the sports performance of athletes.
- The ketogenic diet, plant-based diet, high-protein diet, and Mediterranean diet may improve sports performance from different aspects.
- The gut microbiota and its metabolites play an important role in the effects of dietary patterns on sports performance.

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References

1. Burke, L.M.; Read, R.S. Sports Nutrition. Approaching the Nineties. *Sports Med.* **1989**, *8*, 80–100. [CrossRef] [PubMed]
2. Burke, L.M.; Kiens, B.; Ivy, J.L. Carbohydrates and Fat for Training and Recovery. *J. Sports Sci.* **2004**, *22*, 15–30. [CrossRef] [PubMed]
3. Kaufman, M.; Nguyen, C.; Shetty, M.; Oppezzo, M.; Barrack, M.; Fredericson, M. Popular Dietary Trends' Impact on Athletic Performance: A Critical Analysis Review. *Nutrients* **2023**, *15*, 3511. [CrossRef] [PubMed]
4. Griffiths, A.; Matu, J.; Whyte, E.; Akin-Nibosun, P.; Clifford, T.; Stevenson, E.; Shannon, O.M. The Mediterranean Dietary Pattern for Optimising Health and Performance in Competitive Athletes: A Narrative Review. *Br. J. Nutr.* **2022**, *128*, 1285–1298. [CrossRef] [PubMed]
5. Nieman, D.C. Vegetarian Dietary Practices and Endurance Performance. *Am. J. Clin. Nutr.* **1988**, *48*, 754–761. [CrossRef] [PubMed]

6. Marttinen, M.; Ala-Jaakkola, R.; Laitila, A.; Lehtinen, M.J. Gut Microbiota, Probiotics and Physical Performance in Athletes and Physically Active Individuals. *Nutrients* **2020**, *12*, 2936. [CrossRef] [PubMed]
7. Bedu-Ferrari, C.; Biscarrat, P.; Langella, P.; Cherbuy, C. Prebiotics and the Human Gut Microbiota: From Breakdown Mechanisms to the Impact on Metabolic Health. *Nutrients* **2022**, *14*, 2096. [CrossRef] [PubMed]
8. Whitman, J.A.; Doherty, L.A.; Pantoja-Feliciano de Goodfellow, I.G.; Racicot, K.; Anderson, D.J.; Kensil, K.; Karl, J.P.; Gibson, G.R.; Soares, J.W. In Vitro Fermentation Shows Polyphenol and Fiber Blends Have an Additive Beneficial Effect on Gut Microbiota States. *Nutrients* **2024**, *16*, 1159. [CrossRef] [PubMed]
9. Mancin, L.; Amatori, S.; Caprio, M.; Sattin, E.; Bertoldi, L.; Cenci, L.; Sisti, D.; Bianco, A.; Paoli, A. Effect of 30 Days of Ketogenic Mediterranean Diet with Phytoextracts on Athletes' Gut Microbiome Composition. *Front. Nutr.* **2022**, *9*, 979651. [CrossRef]
10. Willems, M.E.T.; Blacker, S.D. Anthocyanin-Rich Supplementation: Emerging Evidence of Strong Potential for Sport and Exercise Nutrition. *Front. Nutr.* **2022**, *9*, 864323. [CrossRef]
11. Sender, R.; Fuchs, S.; Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.* **2016**, *14*, e1002533. [CrossRef] [PubMed]
12. Matijašić, M.; Meštrović, T.; Čipčić Paljetak, H.; Perić, M.; Barešić, A.; Verbanac, D. Gut Microbiota beyond Bacteria—Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD. *Int. J. Mol. Sci.* **2020**, *21*, 2668. [CrossRef] [PubMed]
13. Sender, R.; Fuchs, S.; Milo, R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell* **2016**, *164*, 337–340. [CrossRef]
14. Santacroce, L.; Man, A.; Charitos, I.A.; Haxhirexha, K.; Topi, S. Current Knowledge about the Connection between Health Status and Gut Microbiota from Birth to Elderly. A Narrative Review. *Front. Biosci. Landmark Ed.* **2021**, *26*, 135–148.
15. Thursby, E.; Juge, N. Introduction to the Human Gut Microbiota. *Biochem. J.* **2017**, *474*, 1823–1836. [CrossRef] [PubMed]
16. Frank, D.N.; Pace, N.R. Gastrointestinal Microbiology Enters the Metagenomics Era. *Curr. Opin. Gastroenterol.* **2008**, *24*, 4. [CrossRef]
17. Sung, J.; Kim, N.; Kim, J.; Jo, H.J.; Park, J.H.; Nam, R.H.; Seok, Y.-J.; Kim, Y.-R.; Lee, D.H.; Jung, H.C. Comparison of Gastric Microbiota Between Gastric Juice and Mucosa by Next Generation Sequencing Method. *J. Cancer Prev.* **2016**, *21*, 60–65. [CrossRef]
18. Nardone, G.; Compare, D. The Human Gastric Microbiota: Is It Time to Rethink the Pathogenesis of Stomach Diseases? *United Eur. Gastroenterol. J.* **2015**, *3*, 255–260. [CrossRef] [PubMed]
19. Wegierska, A.E.; Charitos, I.A.; Topi, S.; Potenza, M.A.; Montagnani, M.; Santacroce, L. The Connection Between Physical Exercise and Gut Microbiota: Implications for Competitive Sports Athletes. *Sports Med.* **2022**, *52*, 2355–2369. [CrossRef]
20. Fouhy, F.; Ross, R.P.; Fitzgerald, G.F.; Stanton, C.; Cotter, P.D. Composition of the Early Intestinal Microbiota: Knowledge, Knowledge Gaps and the Use of High-Throughput Sequencing to Address These Gaps. *Gut Microbes* **2012**, *3*, 203–220. [CrossRef]
21. Rinninella, E.; Raoul, P.; Cintoni, M.; Franceschi, F.; Miggiano, G.A.D.; Gasbarrini, A.; Mele, M.C. What Is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* **2019**, *7*, 14. [CrossRef] [PubMed]
22. Dieterich, W.; Schink, M.; Zopf, Y. Microbiota in the Gastrointestinal Tract. *Med. Sci.* **2018**, *6*, 116. [CrossRef] [PubMed]
23. Sommer, F.; Bäckhed, F. The Gut Microbiota—Masters of Host Development and Physiology. *Nat. Rev. Microbiol.* **2013**, *11*, 227–238. [CrossRef] [PubMed]
24. Cho, I.; Blaser, M.J. The Human Microbiome: At the Interface of Health and Disease. *Nat. Rev. Genet.* **2012**, *13*, 260–270. [CrossRef]
25. O'Hara, A.M.; Shanahan, F. The Gut Flora as a Forgotten Organ. *EMBO Rep.* **2006**, *7*, 688–693. [CrossRef] [PubMed]
26. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut Microbiota Functions: Metabolism of Nutrients and Other Food Components. *Eur. J. Nutr.* **2018**, *57*, 1–24. [CrossRef] [PubMed]
27. Nicholson, J.K.; Holmes, E.; Kinross, J.; Burcelin, R.; Gibson, G.; Jia, W.; Pettersson, S. Host-Gut Microbiota Metabolic Interactions. *Science* **2012**, *336*, 1262–1267. [CrossRef]
28. Tang, W.H.W.; Kitai, T.; Hazen, S.L. Gut Microbiota in Cardiovascular Health and Disease. *Circ. Res.* **2017**, *120*, 1183–1196. [CrossRef] [PubMed]
29. Bischoff, S.C. "Gut Health": A New Objective in Medicine? *BMC Med.* **2011**, *9*, 24. [CrossRef]
30. Santacroce, L.; Charitos, I.A.; Ballini, A.; Inchingolo, F.; Luperto, P.; De Nitto, E.; Topi, S. The Human Respiratory System and Its Microbiome at a Glimpse. *Biology* **2020**, *9*, 318. [CrossRef]
31. Alam, A.; Neish, A. Role of Gut Microbiota in Intestinal Wound Healing and Barrier Function. *Tissue Barriers* **2018**, *6*, 1539595. [CrossRef] [PubMed]
32. Wegienka, G.; Havstad, S.; Zoratti, E.M.; Woodcroft, K.J.; Bobbitt, K.R.; Ownby, D.R.; Johnson, C.C. Regulatory T Cells in Prenatal Blood Samples: Variability with Pet Exposure and Sensitization. *J. Reprod. Immunol.* **2009**, *81*, 74–81. [CrossRef]
33. Santacroce, L.; Sardaro, N.; Topi, S.; Pettini, F.; Bottalico, L.; Cantore, S.; Cascella, G.; Del Prete, R.; Dipalma, G.; Inchingolo, F. The Pivotal Role of Oral Microbiota in Health and Disease. *J. Biol. Regul. Homeost. Agents* **2020**, *34*, 733–737. [PubMed]
34. Kho, Z.Y.; Lal, S.K. The Human Gut Microbiome—A Potential Controller of Wellness and Disease. *Front. Microbiol.* **2018**, *91*, 1835. [CrossRef]
35. Jandhyala, S.M.; Talukdar, R.; Subramanyam, C.; Vuyyuru, H.; Sasikala, M.; Reddy, D.N. Role of the Normal Gut Microbiota. *World J. Gastroenterol.* **2015**, *21*, 8787–8803. [CrossRef]
36. Hawrelak, J.A.; Myers, S.P. The Causes of Intestinal Dysbiosis: A Review. *Altern. Med. Rev.* **2004**, *9*, 180–197.
37. Bull, M.J.; Plummer, N.T. Part 1: The Human Gut Microbiome in Health and Disease. *Integr. Med.* **2014**, *13*, 17–22.

38. Almeida, A.; Mitchell, A.L.; Boland, M.; Forster, S.C.; Gloor, G.B.; Tarkowska, A.; Lawley, T.D.; Finn, R.D. A New Genomic Blueprint of the Human Gut Microbiota. *Nature* **2019**, *568*, 499–504. [CrossRef]
39. Tamboli, C.P.; Neut, C.; Desreumaux, P.; Colombel, J.F. Dysbiosis in Inflammatory Bowel Disease. *Gut* **2004**, *53*, 1–4. [CrossRef]
40. Perler, B.K.; Friedman, E.S.; Wu, G.D. The Role of the Gut Microbiota in the Relationship Between Diet and Human Health. *Annu. Rev. Physiol.* **2023**, *85*, 449–468. [CrossRef]
41. Purdel, C.; Margină, D.; Adam-Dima, I.; Ungurianu, A. The Beneficial Effects of Dietary Interventions on Gut Microbiota-An Up-to-Date Critical Review and Future Perspectives. *Nutrients* **2023**, *15*, 5005. [CrossRef] [PubMed]
42. Hughes, R.L.; Holscher, H.D. Fueling Gut Microbes: A Review of the Interaction between Diet, Exercise, and the Gut Microbiota in Athletes. *Adv. Nutr.* **2021**, *12*, 2190–2215. [CrossRef] [PubMed]
43. Ping, Z. Influence of Foods and Nutrition on the Gut Microbiome and Implications for Intestinal Health. *Int. J. Mol. Sci.* **2022**, *231*, 9588.
44. Clarke, S.F.; Murphy, E.F.; O'Sullivan, O.; Lucey, A.J.; Humphreys, M.; Hogan, A.; Hayes, P.; O'Reilly, M.; Jeffery, I.B.; Wood-Martin, R.; et al. Exercise and Associated Dietary Extremes Impact on Gut Microbial Diversity. *Gut* **2014**, *63*, 1913–1920. [CrossRef] [PubMed]
45. Petersen, L.M.; Bautista, E.J.; Nguyen, H.; Hanson, B.M.; Chen, L.; Lek, S.H.; Sodergren, E.; Weinstock, G.M. Community Characteristics of the Gut Microbiomes of Competitive Cyclists. *Microbiome* **2017**, *5*, 98. [CrossRef]
46. Scheiman, J.; Luber, J.M.; Chavkin, T.A.; MacDonald, T.; Tung, A.; Pham, L.-D.; Wibowo, M.C.; Wurth, R.C.; Punthambaker, S.; Tierney, B.T.; et al. Meta-Omics Analysis of Elite Athletes Identifies a Performance-Enhancing Microbe That Functions via Lactate Metabolism. *Nat. Med.* **2019**, *25*, 1104–1109. [CrossRef] [PubMed]
47. Barton, W.; Penney, N.C.; Cronin, O.; Garcia-Perez, I.; Molloy, M.G.; Holmes, E.; Shanahan, F.; Cotter, P.D.; O'Sullivan, O. The Microbiome of Professional Athletes Differs from That of More Sedentary Subjects in Composition and Particularly at the Functional Metabolic Level. *Gut* **2018**, *67*, 625–633. [CrossRef] [PubMed]
48. Moreno-Pérez, D.; Bressa, C.; Bailén, M.; Hamed-Bousdar, S.; Naclerio, F.; Carmona, M.; Pérez, M.; González-Soltero, R.; Montalvo-Lominchar, M.G.; Carabaña, C.; et al. Effect of a Protein Supplement on the Gut Microbiota of Endurance Athletes: A Randomized, Controlled, Double-Blind Pilot Study. *Nutrients* **2018**, *10*, 337. [CrossRef] [PubMed]
49. Jang, L.-G.; Choi, G.; Kim, S.-W.; Kim, B.-Y.; Lee, S.; Park, H. The Combination of Sport and Sport-Specific Diet Is Associated with Characteristics of Gut Microbiota: An Observational Study. *J. Int. Soc. Sports Nutr.* **2019**, *16*, 21. [CrossRef]
50. Munukka, E.; Ahtiainen, J.P.; Puigbó, P.; Jalkanen, S.; Pahkala, K.; Keskitalo, A.; Kujala, U.M.; Pietilä, S.; Hollmén, M.; Elo, L.; et al. Six-Week Endurance Exercise Alters Gut Metagenome That Is Not Reflected in Systemic Metabolism in Over-Weight Women. *Front. Microbiol.* **2018**, *9*, 2323. [CrossRef]
51. Fernández, J.; Fernández-Sanjurjo, M.; Iglesias-Gutiérrez, E.; Martínez-Camblor, P.; Villar, C.J.; Tomás-Zapico, C.; Fernández-García, B.; Lombó, F. Resistance and Endurance Exercise Training Induce Differential Changes in Gut Microbiota Composition in Murine Models. *Front. Physiol.* **2021**, *12*, 748854. [CrossRef] [PubMed]
52. Rohwer, N.; El Hage, R.; Smyl, C.; Ocvirk, S.; Goris, T.; Grune, T.; Swidsinski, A.; Weylandt, K.-H. Ketogenic Diet Has Moderate Effects on the Fecal Microbiota of Wild-Type Mice. *Nutrients* **2023**, *15*, 4629. [CrossRef] [PubMed]
53. Xu, Y.; Zhong, F.; Zheng, X.; Lai, H.Y.; Wu, C.; Huang, C. Disparity of Gut Microbiota Composition Among Elite Athletes and Young Adults with Different Physical Activity Independent of Dietary Status: A Matching Study. *Front. Nutr.* **2022**, *9*, 843076. [CrossRef]
54. Hintikka, J.E.; Munukka, E.; Valtonen, M.; Luoto, R.; Ihalainen, J.K.; Kallonen, T.; Waris, M.; Heinonen, O.J.; Ruuskanen, O.; Pekkala, S. Gut Microbiota and Serum Metabolome in Elite Cross-Country Skiers: A Controlled Study. *Metabolites* **2022**, *12*, 335. [CrossRef]
55. Humińska-Lisowska, K.; Zielińska, K.; Mieszkowski, J.; Michałowska-Sawczyn, M.; Cięszczyk, P.; Łabaj, P.P.; Wasąg, B.; Frączek, B.; Grzywacz, A.; Kochanowicz, A.; et al. Microbiome Features Associated with Performance Measures in Athletic and Non-Athletic Individuals: A Case-Control Study. *PLoS ONE* **2024**, *19*, e0297858. [CrossRef]
56. Dao, M.C.; Everard, A.; Aron-Wisnewsky, J.; Sokolovska, N.; Prifti, E.; Verger, E.O.; Kayser, B.D.; Levenez, F.; Chilloux, J.; Hoyles, L.; et al. Akkermansia Muciniphila and Improved Metabolic Health during a Dietary Intervention in Obesity: Relationship with Gut Microbiome Richness and Ecology. *Gut* **2016**, *65*, 426–436. [CrossRef] [PubMed]
57. Smith, K.S.; Morris, M.M.; Morrow, C.D.; Novak, J.R.; Roberts, M.D.; Frugé, A.D. Associations between Changes in Fat-Free Mass, Fecal Microbe Diversity, and Mood Disturbance in Young Adults after 10-Weeks of Resistance Training. *Microorganisms* **2022**, *10*, 2344. [CrossRef]
58. Dupuit, M.; Rance, M.; Morel, C.; Bouillon, P.; Boscaro, A.; Martin, V.; Vazeille, E.; Barnich, N.; Chassaing, B.; Boisseau, N. Effect of Concurrent Training on Body Composition and Gut Microbiota in Postmenopausal Women with Overweight or Obesity. *Med. Sci. Sports Exerc.* **2022**, *54*, 517–529. [CrossRef]
59. Allen, J.M.; Mailing, L.J.; Niemiro, G.M.; Moore, R.; Cook, M.D.; White, B.A.; Holscher, H.D.; Woods, J.A. Exercise Alters Gut Microbiota Composition and Function in Lean and Obese Humans. *Med. Sci. Sports Exerc.* **2018**, *50*, 747. [CrossRef]
60. Hsu, Y.J.; Chiu, C.C.; Li, Y.P.; Huang, W.C.; Huang, Y.T.; Huang, C.C.; Chuang, H.L. Effect of Intestinal Microbiota on Exercise Performance in Mice. *J. Strength Cond. Res.* **2015**, *29*, 552. [CrossRef]
61. Coffey, V.G.; Hawley, J.A. Concurrent Exercise Training: Do Opposites Distract? *J. Physiol.* **2017**, *595*, 2883–2896. [CrossRef] [PubMed]

62. West, N.P.; Horn, P.L.; Pyne, D.B.; Gebski, V.J.; Lahtinen, S.J.; Fricker, P.A.; Cripps, A.W. Probiotic Supplementation for Respiratory and Gastrointestinal Illness Symptoms in Healthy Physically Active Individuals. *Clin. Nutr.* **2014**, *33*, 581–587. [CrossRef]
63. Huang, W.-C.; Lee, M.-C.; Lee, C.-C.; Ng, K.-S.; Hsu, Y.-J.; Tsai, T.-Y.; Young, S.-L.; Lin, J.-S.; Huang, C.-C. Effect of Lactobacillus Plantarum TWK10 on Exercise Physiological Adaptation, Performance, and Body Composition in Healthy Humans. *Nutrients* **2019**, *11*, 2836. [CrossRef] [PubMed]
64. Huang, W.-C.; Wei, C.-C.; Huang, C.-C.; Chen, W.-L.; Huang, H.-Y. The Beneficial Effects of Lactobacillus Plantarum PS128 on High-Intensity, Exercise-Induced Oxidative Stress, Inflammation, and Performance in Triathletes. *Nutrients* **2019**, *11*, 353. [CrossRef] [PubMed]
65. Sawada, D.; Kuwano, Y.; Tanaka, H.; Hara, S.; Uchiyama, Y.; Sugawara, T.; Fujiwara, S.; Rokutan, K.; Nishida, K. Daily Intake of Lactobacillus Gasseri CP2305 Relieves Fatigue and Stress-Related Symptoms in Male University Ekiden Runners: A Double-Blind, Randomized, and Placebo-Controlled Clinical Trial. *J. Funct. Foods* **2019**, *57*, 465–476. [CrossRef]
66. Singh, R.K.; Chang, H.-W.; Yan, D.; Lee, K.M.; Ucmak, D.; Wong, K.; Abrouk, M.; Farahnik, B.; Nakamura, M.; Zhu, T.H.; et al. Influence of Diet on the Gut Microbiome and Implications for Human Health. *J. Transl. Med.* **2017**, *15*, 73. [CrossRef]
67. Makki, K.; Deehan, E.C.; Walter, J.; Bäckhed, F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell Host Microbe* **2018**, *23*, 705–715. [CrossRef] [PubMed]
68. David, L.A.; Maurice, C.F.; Carmody, R.N.; Gootenberg, D.B.; Button, J.E.; Wolfe, B.E.; Ling, A.V.; Devlin, A.S.; Varma, Y.; Fischbach, M.A.; et al. Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. *Nature* **2014**, *505*, 559–563. [CrossRef] [PubMed]
69. Medawar, E.; Huhn, S.; Villringer, A.; Veronica Witte, A. The Effects of Plant-Based Diets on the Body and the Brain: A Systematic Review. *Transl. Psychiatry* **2019**, *9*, 226. [CrossRef] [PubMed]
70. Przewłócka, K.; Folwarski, M.; Kaźmierczak-Siedlecka, K.; Skonieczna-Żydecka, K.; Kaczor, J.J. Gut-Muscle AxisExists and May Affect Skeletal Muscle Adaptation to Training. *Nutrients* **2020**, *12*, 1451. [CrossRef]
71. Ticinesi, A.; Nouvenne, A.; Cerundolo, N.; Catania, P.; Prati, B.; Tana, C.; Meschi, T. Gut Microbiota, Muscle Mass and Function in Aging: A Focus on Physical Frailty and Sarcopenia. *Nutrients* **2019**, *11*, 1633. [CrossRef] [PubMed]
72. Peng, J.; Narasimhan, S.; Marchesi, J.R.; Benson, A.; Wong, F.S.; Wen, L. Long Term Effect of Gut Microbiota Transfer on Diabetes Development. *J. Autoimmun.* **2014**, *53*, 85–94. [CrossRef]
73. Rivière, A.; Selak, M.; Lantin, D.; Leroy, F.; De Vuyst, L. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. *Front. Microbiol.* **2016**, *7*, 979. [CrossRef]
74. Cullen, A.E.; Centner, A.M.; Deitado, R.; Fernandez, J.; Salazar, G. The Impact of Dietary Supplementation of Whole Foods and Polyphenols on Atherosclerosis. *Nutrients* **2020**, *12*, 2069. [CrossRef] [PubMed]
75. Burton-Freeman, B.M.; Sesso, H.D. Whole Food versus Supplement: Comparing the Clinical Evidence of Tomato Intake and Lycopene Supplementation on Cardiovascular Risk Factors. *Adv. Nutr.* **2014**, *5*, 457–485. [CrossRef] [PubMed]
76. Seel, W.; Reiners, S.; Kipp, K.; Simon, M.-C.; Dawczynski, C. Role of Dietary Fiber and Energy Intake on Gut Microbiome in Vegans, Vegetarians, and Flexitarians in Comparison to Omnivores—Insights from the Nutritional Evaluation (NuEva) Study. *Nutrients* **2023**, *15*, 1914. [CrossRef]
77. Szurkowska, J.; Wiącek, J.; Laparidis, K.; Karolkiewicz, J. A Comparative Study of Selected Gut Bacteria Abundance and Fecal pH in Bodybuilders Eating High-Protein Diet and More Sedentary Controls. *Nutrients* **2021**, *13*, 4093. [CrossRef] [PubMed]
78. Merra, G.; Noce, A.; Marrone, G.; Cintoni, M.; Tarsitano, M.G.; Capacci, A.; De Lorenzo, A. Influence of Mediterranean Diet on Human Gut Microbiota. *Nutrients* **2020**, *13*, 7. [CrossRef]
79. Jin, Q.; Black, A.; Kales, S.N.; Vattem, D.; Ruiz-Canela, M.; Sotos-Prieto, M. Metabolomics and Microbiomes as Potential Tools to Evaluate the Effects of the Mediterranean Diet. *Nutrients* **2019**, *11*, 207. [CrossRef]
80. Haro, C.; Garcia-Carpintero, S.; Alcala-Diaz, J.F.; Gomez-Delgado, F.; Delgado-Lista, J.; Perez-Martinez, P.; Rangel Zuñiga, O.A.; Quintana-Navarro, G.M.; Landa, B.B.; Clemente, J.C.; et al. The Gut Microbial Community in Metabolic Syndrome Patients Is Modified by Diet. *J. Nutr. Biochem.* **2016**, *27*, 27–31. [CrossRef]
81. Odell, O.J.; Wallis, G.A. The Application of Lactose in Sports Nutrition. *Int. Dairy J.* **2021**, *116*, 104970. [CrossRef]
82. Whitney, R.; Nair, R.R. Expanding Dietary Therapy Beyond the Classic Ketogenic Diet: On the Use of the Modified Atkins Diet and Low Glycemic Index Treatment in Pediatric Epilepsy. *Indian Pediatr.* **2021**, *58*, 811–812. [CrossRef] [PubMed]
83. Pérez-Guisado, J.; Muñoz-Serrano, A.; Alonso-Moraga, Á. Spanish Ketogenic Mediterranean Diet: A Healthy Cardiovascular Diet for Weight Loss. *Nutr. J.* **2008**, *7*, 30. [CrossRef] [PubMed]
84. Murphy, P.; Likhodii, S.S.; Hatamian, M.; Burnham, W.M. Effect of the Ketogenic Diet on the Activity Level of Wistar Rats. *Pediatr. Res.* **2005**, *57*, 353–357. [CrossRef] [PubMed]
85. Paoli, A.; Tinsley, G.M.; Mattson, M.P.; De Vivo, I.; Dhawan, R.; Moro, T. Common and Divergent Molecular Mechanisms of Fasting and Ketogenic Diets. *Trends Endocrinol. Metab.* **2024**, *35*, 125–141. [CrossRef] [PubMed]
86. Antonio Paoli, A.; Mancin, L.; Caprio, M.; Monti, E.; Narici, M.V.; Cenci, L.; Piccini, F.; Pincella, M.; Grigoletto, D.; Marcolin, G. Effects of 30 Days of Ketogenic Diet on Body Composition, Muscle Strength, Muscle Area, Metabolism, and Performance in Semi-Professional Soccer Players. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 62. [CrossRef] [PubMed]
87. Ashtary-Larky, D.; Bagheri, R.; Asbaghi, O.; Tinsley, G.M.; Kooti, W.; Abbasnezhad, A.; Afrisham, R.; Wong, A. Effects of Resistance Training Combined with a Ketogenic Diet on Body Composition: A Systematic Review and Meta-Analysis. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 5717–5732. [CrossRef] [PubMed]

88. Burke, L.M. Ketogenic low-CHO, High-fat Diet: The Future of Elite Endurance Sport? *J. Physiol.* **2021**, *599*, 819–843. [CrossRef] [PubMed]
89. Ang, Q.Y.; Alexander, M.; Newman, J.C.; Tian, Y.; Cai, J.; Upadhyay, V.; Turnbaugh, J.A.; Verdin, E.; Hall, K.D.; Leibel, R.L.; et al. Ketogenic Diets Alter the Gut Microbiome Resulting in Decreased Intestinal Th17 Cells. *Cell* **2020**, *181*, 1263–1275.e16. [CrossRef]
90. Cheng, L.; Jin, H.; Qiang, Y.; Wu, S.; Yan, C.; Han, M.; Xiao, T.; Yan, N.; An, H.; Zhou, X.; et al. High Fat Diet Exacerbates Dextran Sulfate Sodium Induced Colitis through Disturbing Mucosal Dendritic Cell Homeostasis. *Int. Immunopharmacol.* **2016**, *40*, 1–10. [CrossRef]
91. Devkota, S.; Wang, Y.; Musch, M.W.; Leone, V.; Fehlner-Peach, H.; Nadimpalli, A.; Antonopoulos, D.A.; Jabri, B.; Chang, E.B. Dietary-Fat-Induced Taurocholic Acid Promotes Pathobiont Expansion and Colitis in Il10-/- Mice. *Nature* **2012**, *487*, 104–108. [CrossRef] [PubMed]
92. Wolters, M.; Ahrens, J.; Romaní-Pérez, M.; Watkins, C.; Sanz, Y.; Benítez-Páez, A.; Stanton, C.; Günther, K. Dietary Fat, the Gut Microbiota, and Metabolic Health—A Systematic Review Conducted within the MyNewGut Project. *Clin. Nutr.* **2019**, *38*, 2504–2520. [CrossRef] [PubMed]
93. Rl, H. A Review of the Role of the Gut Microbiome in Personalized Sports Nutrition. *Front. Nutr.* **2020**, *6*, 504337.
94. Bueno, N.B.; de Melo, I.S.V.; de Oliveira, S.L.; Ataide, T.d.R. Very-Low-Carbohydrate Ketogenic Diet v. Low-Fat Diet for Long-Term Weight Loss: A Meta-Analysis of Randomised Controlled Trials. *Br. J. Nutr.* **2013**, *110*, 1178–1187. [CrossRef] [PubMed]
95. Paoli, A.; Grimaldi, K.; Bianco, A.; Lodi, A.; Cenci, L.; Parmagnani, A. Medium Term Effects of a Ketogenic Diet and a Mediterranean Diet on Resting Energy Expenditure and Respiratory Ratio. *BMC Proc.* **2012**, *6*, P37. [CrossRef]
96. Zambrano, A.K.; Cadena-Ullauri, S.; Guevara-Ramírez, P.; Frias-Toral, E.; Ruiz-Pozo, V.A.; Paz-Cruz, E.; Tamayo-Trujillo, R.; Chapela, S.; Montalván, M.; Sarno, G.; et al. The Impact of a Very-Low-Calorie Ketogenic Diet in the Gut Microbiota Composition in Obesity. *Nutrients* **2023**, *15*, 2728. [CrossRef] [PubMed]
97. Muscogiuri, G.; Barrea, L.; Laudisio, D.; Pugliese, G.; Salzano, C.; Savastano, S.; Colao, A. The Management of Very Low-Calorie Ketogenic Diet in Obesity Outpatient Clinic: A Practical Guide. *J. Transl. Med.* **2019**, *17*, 356. [CrossRef] [PubMed]
98. Sundgot-Borgen, J.; Garthe, I. Elite Athletes in Aesthetic and Olympic Weight-Class Sports and the Challenge of Body Weight and Body Compositions. *J. Sports Sci.* **2011**, *29* (Suppl. S1), S101–S114. [CrossRef] [PubMed]
99. Donnelly, J.E.; Jakicic, J.; Gunderson, S. Diet and Body Composition. Effect of Very Low Calorie Diets and Exercise. *Sports Med.* **1991**, *12*, 237–249. [CrossRef]
100. Rogerson, D. Vegan Diets: Practical Advice for Athletes and Exercisers. *J. Int. Soc. Sports Nutr.* **2017**, *14*, 36. [CrossRef]
101. Thomas, M.S.; Calle, M.; Fernandez, M.L. Healthy Plant-Based Diets Improve Dyslipidemias, Insulin Resistance, and Inflammation in Metabolic Syndrome. A Narrative Review. *Adv. Nutr.* **2023**, *14*, 44–54. [CrossRef] [PubMed]
102. Aidoo, R.; Abe-Inge, V.; Kwofie, E.M.; Baum, J.I.; Kubow, S. Sustainable Healthy Diet Modeling for a Plant-Based Dietary Transitioning in the United States. *NPJ Sci. Food* **2023**, *7*, 61. [CrossRef]
103. Casas, R.; Castro-Barquero, S.; Estruch, R.; Sacanella, E. Nutrition and Cardiovascular Health. *Int. J. Mol. Sci.* **2018**, *19*, 3988. [CrossRef] [PubMed]
104. Dawczynski, C.; Weidauer, T.; Richert, C.; Schlattmann, P.; Dawczynski, K.; Kiehntopf, M. Corrigendum: Nutrient Intake and Nutrition Status in Vegetarians and Vegans in Comparison to Omnivores-the Nutritional Evaluation (NuEva) Study. *Front. Nutr.* **2022**, *9*, 975159. [CrossRef] [PubMed]
105. Key, T.J.; Appleby, P.N.; Rosell, M.S. Health Effects of Vegetarian and Vegan Diets. *Proc. Nutr. Soc.* **2006**, *65*, 35–41. [CrossRef] [PubMed]
106. Craig, W.J. Nutrition Concerns and Health Effects of Vegetarian Diets. *Nutr. Clin. Pract.* **2010**, *25*, 613–620. [CrossRef] [PubMed]
107. Klein, L.; Dawczynski, C.; Schwarz, M.; Maares, M.; Kipp, K.; Haase, H.; Kipp, A.P. Selenium, Zinc, and Copper Status of Vegetarians and Vegans in Comparison to Omnivores in the Nutritional Evaluation (NuEva) Study. *Nutrients* **2023**, *15*, 3538. [CrossRef] [PubMed]
108. Meehan, C.J.; Beiko, R.G. A Phylogenomic View of Ecological Specialization in the Lachnospiraceae, a Family of Digestive Tract-Associated Bacteria. *Genome Biol. Evol.* **2014**, *6*, 703–713. [CrossRef] [PubMed]
109. Ai, D.; Pan, H.; Li, X.; Gao, Y.; Liu, G.; Xia, L.C. Identifying Gut Microbiota Associated with Colorectal Cancer Using a Zero-Inflated Lognormal Model. *Front. Microbiol.* **2019**, *10*, 826. [CrossRef]
110. Barnard, N.D.; Goldman, D.M.; Loomis, J.F.; Kahleova, H.; Levin, S.M.; Neabore, S.; Batts, T.C. Plant-Based Diets for Cardiovascular Safety and Performance in Endurance Sports. *Nutrients* **2019**, *11*, 130. [CrossRef]
111. Tipton, K.D.; Wolfe, R.R. Protein and Amino Acids for Athletes. *J. Sports Sci.* **2004**, *22*, 65–79. [CrossRef] [PubMed]
112. Oliphant, K.; Allen-Vercoe, E. Macronutrient Metabolism by the Human Gut Microbiome: Major Fermentation by-Products and Their Impact on Host Health. *Microbiome* **2019**, *7*, 91. [CrossRef] [PubMed]
113. Blachier, F.; Beaumont, M.; Portune, K.J.; Steuer, N.; Lan, A.; Audebert, M.; Khodorova, N.; Andriamihaja, M.; Airinei, G.; Benamouzig, R.; et al. High-Protein Diets for Weight Management: Interactions with the Intestinal Microbiota and Consequences for Gut Health. A Position Paper by the My New Gut Study Group. *Clin. Nutr.* **2019**, *38*, 1012–1022. [CrossRef] [PubMed]
114. Lowery, L.; Forsythe, C.E. Protein and Overtraining: Potential Applications for Free-Living Athletes. *J. Int. Soc. Sports Nutr.* **2006**, *3*, 42–50. [CrossRef] [PubMed]

115. Davis, C.; Bryan, J.; Hodgson, J.; Murphy, K. Definition of the Mediterranean Diet; A Literature Review. *Nutrients* **2015**, *7*, 9139–9153. [CrossRef] [PubMed]
116. Barber, T.M.; Kabisch, S.; Pfeiffer, A.F.H.; Weickert, M.O. The Effects of the Mediterranean Diet on Health and Gut Microbiota. *Nutrients* **2023**, *15*, 2150. [CrossRef] [PubMed]
117. Bellastella, G.; Scappaticcio, L.; Caiazzo, F.; Tomasuolo, M.; Carotenuto, R.; Caputo, M.; Arena, S.; Caruso, P.; Maiorino, M.I.; Esposito, K. Mediterranean Diet and Thyroid: An Interesting Alliance. *Nutrients* **2022**, *14*, 4130. [CrossRef] [PubMed]
118. Scoditti, E.; Tumolo, M.R.; Garbarino, S. Mediterranean Diet on Sleep: A Health Alliance. *Nutrients* **2022**, *14*, 2998. [CrossRef] [PubMed]
119. Lotfi, K.; Saneei, P.; Hajhashemy, Z.; Esmaillzadeh, A. Adherence to the Mediterranean Diet, Five-Year Weight Change, and Risk of Overweight and Obesity: A Systematic Review and Dose-Response Meta-Analysis of Prospective Cohort Studies. *Adv. Nutr.* **2022**, *13*, 152–166. [CrossRef]
120. Jimenez-Torres, J.; Alcalá-Diaz, J.F.; Torres-Peña, J.D.; Gutierrez-Mariscal, F.M.; Leon-Acuña, A.; Gómez-Luna, P.; Fernández-Gandara, C.; Quintana-Navarro, G.M.; Fernandez-Garcia, J.C.; Perez-Martinez, P.; et al. Mediterranean Diet Reduces Atherosclerosis Progression in Coronary Heart Disease: An Analysis of the CORDIOPREV Randomized Controlled Trial. *Stroke* **2021**, *52*, 3440–3449. [CrossRef]
121. Zhang, C.; Zhang, M.; Wang, S.; Han, R.; Cao, Y.; Hua, W.; Mao, Y.; Zhang, X.; Pang, X.; Wei, C.; et al. Interactions between Gut Microbiota, Host Genetics and Diet Relevant to Development of Metabolic Syndromes in Mice. *ISME J.* **2010**, *4*, 232–241. [CrossRef] [PubMed]
122. Djuric, Z.; Bassis, C.M.; Plegue, M.A.; Ren, J.; Chan, R.; Sidahmed, E.; Turgeon, D.K.; Ruffin, M.T.; Kato, I.; Sen, A. Colonic Mucosal Bacteria Are Associated with Inter-Individual Variability in Serum Carotenoid Concentrations. *J. Acad. Nutr. Diet.* **2018**, *118*, 606–616.e3. [CrossRef] [PubMed]
123. Hargreaves, M. Skeletal Muscle Metabolism during Exercise in Humans. *Clin. Exp. Pharmacol. Physiol.* **2000**, *27*, 225–228. [CrossRef] [PubMed]
124. Clark, A.; Mach, N. Exercise-Induced Stress Behavior, Gut-Microbiota-Brain Axis and Diet: A Systematic Review for Athletes. *J. Int. Soc. Sports Nutr.* **2016**, *13*, 43. [CrossRef] [PubMed]
125. Jeukendrup, A. A Step Towards Personalized Sports Nutrition: Carbohydrate Intake During Exercise. *Sports Med.* **2014**, *44*, 25–33. [CrossRef] [PubMed]
126. Jeukendrup, A.E. Carbohydrate Intake during Exercise and Performance. *Nutrition* **2004**, *20*, 669–677. [CrossRef] [PubMed]
127. Spriet, L.L. Diet and Nutraceuticals for Mental and Physical Performance in Athletes. *Sports Med.* **2022**, *52*, 1–3. [CrossRef] [PubMed]
128. Rollo, I.; Williams, C. Carbohydrate Nutrition and Skill Performance in Soccer. *Sports Med.* **2023**, *53*, 7–14. [CrossRef] [PubMed]
129. Faits, T.; Walker, M.E.; Rodriguez-Morato, J.; Meng, H.; Gervis, J.E.; Galluccio, J.M.; Lichtenstein, A.H.; Johnson, W.E.; Matthan, N.R. Exploring Changes in the Human Gut Microbiota and Microbial-Derived Metabolites in Response to Diets Enriched in Simple, Refined, or Unrefined Carbohydrate-Containing Foods: A Post Hoc Analysis of a Randomized Clinical Trial. *Am. J. Clin. Nutr.* **2020**, *112*, 1631–1641. [CrossRef]
130. Holscher, H.D.; Chumpitazi, B.P.; Dahl, W.J.; Fahey, G.C.; Liska, D.J.; Slavin, J.L.; Verbeke, K. Perspective: Assessing Tolerance to Nondigestible Carbohydrate Consumption. *Adv. Nutr.* **2022**, *13*, 2084–2097. [CrossRef]
131. Sonnenburg, E.D.; Smits, S.A.; Tikhonov, M.; Higginbottom, S.K.; Wingreen, N.S.; Sonnenburg, J.L. Diet-Induced Extinctions in the Gut Microbiota Compound over Generations. *Nature* **2016**, *529*, 212–215. [CrossRef] [PubMed]
132. Yang, L.; Chen, X.; Chen, D.; Yu, B.; He, J.; Luo, Y.; Zheng, P.; Chen, H.; Yan, H.; Huang, Z. Effects of Protocatechuic Acid on Antioxidant Capacity, Mitochondrial Biogenesis and Skeletal Muscle Fiber Transformation. *J. Nutr. Biochem.* **2023**, *116*, 109327. [CrossRef] [PubMed]
133. Caesar, R.; Tremaroli, V.; Kovatcheva-Datchary, P.; Cani, P.D.; Bäckhed, F. Crosstalk between Gut Microbiota and Dietary Lipids Aggravates WAT Inflammation through TLR Signaling. *Cell Metab.* **2015**, *22*, 658–668. [CrossRef] [PubMed]
134. Gutiérrez-Repiso, C.; Hernández-García, C.; García-Almeida, J.M.; Bellido, D.; Martín-Núñez, G.M.; Sánchez-Alcoholado, L.; Alcaide-Torres, J.; Sajoux, I.; Tinahones, F.J.; Moreno-Indias, I. Effect of Synbiotic Supplementation in a Very-Low-Calorie Ketogenic Diet on Weight Loss Achievement and Gut Microbiota: A Randomized Controlled Pilot Study. *Mol. Nutr. Food Res.* **2019**, *63*, e1900167.
135. Kimmel, M.; Keller, D.; Farmer, S.; Warrino, D.E. A Controlled Clinical Trial to Evaluate the Effect of GanedenBC(30) on Immunological Markers. *Methods Find Exp. Clin. Pharmacol.* **2010**, *32*, 129–132. [CrossRef] [PubMed]
136. Minevich, J.; Olson, M.A.; Mannion, J.P.; Boublik, J.H.; McPherson, J.O.; Lowery, R.P.; Shields, K.; Sharp, M.; De Souza, E.O.; Wilson, J.M.; et al. Digestive Enzymes Reduce Quality Differences between Plant and Animal Proteins: A Double-Blind Crossover Study. *J. Int. Soc. Sports Nutr.* **2015**, *12*, P26. [CrossRef]
137. Jäger, R.; Purpura, M.; Farmer, S.; Cash, H.A.; Keller, D. Probiotic Bacillus Coagulans GBI-30, 6086 Improves Protein Absorption and Utilization. *Probiotics Antimicrob. Proteins* **2018**, *10*, 611–615. [CrossRef] [PubMed]
138. Albracht-Schulte, K.; Islam, T.; Johnson, P.; Moustaid-Moussa, N. Systematic Review of Beef Protein Effects on Gut Microbiota: Implications for Health. *Adv. Nutr.* **2020**, *12*, 102–114. [CrossRef] [PubMed]
139. Zhu, Y.; Shi, X.; Lin, X.; Ye, K.; Xu, X.; Li, C.; Zhou, G. Beef, Chicken, and Soy Proteins in Diets Induce Different Gut Microbiota and Metabolites in Rats. *Front. Microbiol.* **2017**, *8*, 1395. [CrossRef] [PubMed]

140. Zhu, Y.; Lin, X.; Zhao, F.; Shi, X.; Li, H.; Li, Y.; Zhu, W.; Xu, X.; Li, C.; Zhou, G. Meat, Dairy and Plant Proteins Alter Bacterial Composition of Rat Gut Bacteria. *Sci. Rep.* **2015**, *5*, 15220. [CrossRef]
141. Zhu, Y.; Lin, X.; Li, H.; Li, Y.; Shi, X.; Zhao, F.; Xu, X.; Li, C.; Zhou, G. Intake of Meat Proteins Substantially Increased the Relative Abundance of Genus Lactobacillus in Rat Feces. *PLoS ONE* **2016**, *11*, e0152678. [CrossRef] [PubMed]
142. Lang, J.M.; Pan, C.; Cantor, R.M.; Tang, W.H.W.; Garcia-Garcia, J.C.; Kurtz, I.; Hazen, S.L.; Bergeron, N.; Krauss, R.M.; Lusis, A.J. Impact of Individual Traits, Saturated Fat, and Protein Source on the Gut Microbiome. *mBio* **2018**, *9*, e01604-18. [CrossRef] [PubMed]
143. Losasso, C.; Eckert, E.M.; Mastrorilli, E.; Villiger, J.; Mancin, M.; Patuzzi, I.; Di Cesare, A.; Cibin, V.; Barrucci, F.; Pernthaler, J.; et al. Assessing the Influence of Vegan, Vegetarian and Omnivore Oriented Westernized Dietary Styles on Human Gut Microbiota: A Cross Sectional Study. *Front. Microbiol.* **2018**, *9*, 317. [CrossRef] [PubMed]
144. Ticinesi, A.; Lauretani, F.; Tana, C.; Nouvenne, A.; Ridolo, E.; Meschi, T. Exercise and Immune System as Modulators of Intestinal Microbiome: Implications for the Gut-Muscle Axis Hypothesis. *Exerc. Immunol. Rev.* **2019**, *25*, 84–95. [PubMed]
145. Casati, M.; Ferri, E.; Azzolino, D.; Cesari, M.; Arosio, B. Gut Microbiota and Physical Frailty through the Mediation of Sarcopenia. *Exp. Gerontol.* **2019**, *124*, 110639. [CrossRef] [PubMed]
146. Mao, Y.-H.; Wang, M.; Yuan, Y.; Yan, J.-K.; Peng, Y.; Xu, G.; Weng, X. Konjac Glucomannan Counteracted the Side Effects of Excessive Exercise on Gut Microbiome, Endurance, and Strength in an Overtraining Mice Model. *Nutrients* **2023**, *15*, 4206. [CrossRef] [PubMed]
147. Xu, B.; Fu, J.; Qiao, Y.; Cao, J.; Deehan, E.C.; Li, Z.; Jin, M.; Wang, X.; Wang, Y. Higher Intake of Microbiota-Accessible Carbohydrates and Improved Cardiometabolic Risk Factors: A Meta-Analysis and Umbrella Review of Dietary Management in Patients with Type 2 Diabetes. *Am. J. Clin. Nutr.* **2021**, *113*, 1515–1530. [CrossRef] [PubMed]
148. den Besten, G.; van Eunen, K.; Groen, A.K.; Venema, K.; Reijngoud, D.-J.; Bakker, B.M. The Role of Short-Chain Fatty Acids in the Interplay between Diet, Gut Microbiota, and Host Energy Metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340. [CrossRef] [PubMed]
149. Gao, Z.; Yin, J.; Zhang, J.; Ward, R.E.; Martin, R.J.; Lefevre, M.; Cefalu, W.T.; Ye, J. Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice. *Diabetes* **2009**, *58*, 1509–1517. [CrossRef]
150. Yamashita, H.; Fujisawa, K.; Ito, E.; Idei, S.; Kawaguchi, N.; Kimoto, M.; Hiemori, M.; Tsuji, H. Improvement of Obesity and Glucose Tolerance by Acetate in Type 2 Diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) Rats. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 1236–1243. [CrossRef]
151. Jäger, S.; Handschin, C.; St-Pierre, J.; Spiegelman, B.M. AMP-Activated Protein Kinase (AMPK) Action in Skeletal Muscle via Direct Phosphorylation of PGC-1alpha. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12017–12022. [CrossRef] [PubMed]
152. Lin, J.; Handschin, C.; Spiegelman, B.M. Metabolic Control through the PGC-1 Family of Transcription Coactivators. *Cell Metab.* **2005**, *1*, 361–370. [CrossRef] [PubMed]
153. Holscher, H.D. Dietary Fiber and Prebiotics and the Gastrointestinal Microbiota. *Gut Microbes* **2017**, *8*, 172–184. [CrossRef] [PubMed]
154. Di Rienzi, S.C.; Britton, R.A. Adaptation of the Gut Microbiota to Modern Dietary Sugars and Sweeteners. *Adv. Nutr.* **2020**, *11*, 616–629. [CrossRef] [PubMed]
155. Payne, A.N.; Chassard, C.; Lacroix, C. Gut Microbial Adaptation to Dietary Consumption of Fructose, Artificial Sweeteners and Sugar Alcohols: Implications for Host-Microbe Interactions Contributing to Obesity. *Obes. Rev.* **2012**, *13*, 799–809. [CrossRef] [PubMed]
156. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food Sources and Bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747. [CrossRef]
157. Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Identification of the 100 Richest Dietary Sources of Polyphenols: An Application of the Phenol-Explorer Database. *Eur. J. Clin. Nutr.* **2010**, *64*, S112–S120. [CrossRef]
158. Bowtell, J.; Kelly, V. Fruit-Derived Polyphenol Supplementation for Athlete Recovery and Performance. *Sports Med.* **2019**, *49*, 3–23. [CrossRef] [PubMed]
159. Pereira Panza, V.S.; Diefenthaeler, F.; da Silva, E.L. Benefits of Dietary Phytochemical Supplementation on Eccentric Exercise-Induced Muscle Damage: Is Including Antioxidants Enough? *Nutrition* **2015**, *31*, 1072–1082. [CrossRef]
160. Carey, C.C.; Lucey, A.; Doyle, L. Flavonoid Containing Polyphenol Consumption and Recovery from Exercise-Induced Muscle Damage: A Systematic Review and Meta-Analysis. *Sports Med.* **2021**, *51*, 1293–1316. [CrossRef]
161. Somerville, V.; Bringans, C.; Braakhuis, A. Polyphenols and Performance: A Systematic Review and Meta-Analysis. *Sports Med.* **2017**, *47*, 1589–1599. [CrossRef] [PubMed]
162. Mattioli, R.; Francioso, A.; Mosca, L.; Silva, P. Anthocyanins: A Comprehensive Review of Their Chemical Properties and Health Effects on Cardiovascular and Neurodegenerative Diseases. *Molecules* **2020**, *25*, 3809. [CrossRef] [PubMed]
163. Liang, A.; Leonard, W.; Beasley, J.T.; Fang, Z.; Zhang, P.; Ranadheera, C.S. Anthocyanins-Gut Microbiota-Health Axis: A Review. *Crit. Rev. Food Sci. Nutr.* **2023**, *15*, 2367. [CrossRef] [PubMed]
164. Eker, M.E.; Aaby, K.; Budic-Leto, I.; Brnčić, S.R.; El, S.N.; Karakaya, S.; Simsek, S.; Manach, C.; Wiczkowski, W.; de Pascual-Teresa, S. A Review of Factors Affecting Anthocyanin Bioavailability: Possible Implications for the Inter-Individual Variability. *Foods* **2019**, *9*, 2. [CrossRef] [PubMed]

165. Varillas-Delgado, D.; Morencos, E.; Gutiérrez-Hellín, J.; Aguilar-Navarro, M.; Muñoz, A.; Mendoza Láiz, N.; Perucho, T.; Maestro, A.; Tellería-Orriols, J.J. Genetic Profiles to Identify Talents in Elite Endurance Athletes and Professional Football Players. *PLoS ONE* **2022**, *17*, e0274880. [CrossRef]
166. Mayta-Apaza, A.C.; Pottgen, E.; De Bodt, J.; Papp, N.; Marasini, D.; Howard, L.; Abranko, L.; Van de Wiele, T.; Lee, S.-O.; Carbonero, F. Impact of Tart Cherries Polyphenols on the Human Gut Microbiota and Phenolic Metabolites in Vitro and in Vivo. *J. Nutr. Biochem.* **2018**, *59*, 160–172. [CrossRef] [PubMed]
167. Bresciani, L.; Angelino, D.; Vivas, E.I.; Kerby, R.L.; García-Viguera, C.; Del Rio, D.; Rey, F.E.; Mena, P. Differential Catabolism of an Anthocyanin-Rich Elderberry Extract by Three Gut Microbiota Bacterial Species. *J. Agric. Food Chem.* **2020**, *68*, 1837–1843. [CrossRef] [PubMed]
168. Koerich, A.C.C.; Borszcz, F.K.; Thives Mello, A.; de Lucas, R.D.; Hansen, F. Effects of the Ketogenic Diet on Performance and Body Composition in Athletes and Trained Adults: A Systematic Review and Bayesian Multivariate Multilevel Meta-Analysis and Meta-Regression. *Crit. Rev. Food Sci. Nutr.* **2023**, *63*, 11399–11424. [CrossRef] [PubMed]
169. West, S.; Monteyne, A.J.; van der Heijden, I.; Stephens, F.B.; Wall, B.T. Nutritional Considerations for the Vegan Athlete. *Adv. Nutr.* **2023**, *14*, 774–795. [CrossRef]
170. Phillips, S.M.; Van Loon, L.J.C. Dietary Protein for Athletes: From Requirements to Optimum Adaptation. *J. Sports Sci.* **2011**, *29* (Suppl. S1), S29–S38. [CrossRef]

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Review

Targeting the Gut Microbiota for Prevention and Management of Type 2 Diabetes

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Abstract: Type 2 diabetes (T2D) is a chronic metabolic disorder with a heterogeneous etiology encompassing societal and behavioral risk factors in addition to genetic and environmental susceptibility. The cardiovascular consequences of diabetes account for more than two-thirds of mortality among people with T2D. Not only does T2D shorten life expectancy, but it also lowers quality of life and is associated with extremely high health expenditures since diabetic complications raise both direct and indirect healthcare costs. An increasing body of research indicates a connection between T2D and gut microbial traits, as numerous alterations in the intestinal microorganisms have been noted in pre-diabetic and diabetic individuals. These include pro-inflammatory bacterial patterns, increased intestinal permeability, endotoxemia, and hyperglycemia-favoring conditions, such as the alteration of glucagon-like peptide-1 (GLP-1) secretion. Restoring microbial homeostasis can be very beneficial for preventing and co-treating T2D and improving antidiabetic therapy outcomes. This review summarizes the characteristics of a "diabetic" microbiota and the metabolites produced by microbial species that can worsen or ameliorate T2D risk and progression, suggesting gut microbiota-targeted strategies to restore eubiosis and regulate blood glucose. Nutritional supplementation, diet, and physical exercise are known to play important roles in T2D, and here their effects on the gut microbiota are discussed, suggesting non-pharmacological approaches that can greatly help in diabetes management and highlighting the importance of tailoring treatments to individual needs.

Keywords: gut microbiota; type 2 diabetes; glucagon-like peptide-1; diet; supplements; lifestyle intervention; physical exercise

1. Introduction

The gut microbiota is a complex ecosystem within the gastrointestinal tract environment, comprising bacteria, viruses, fungi, archaea, and protozoa [1]. It was previously believed that there were almost ten times as many bacterial cells as human cells [2]; however, current research indicates that there are actually about the same number of them [3]. Gut bacteria are primarily composed of five phyla: 90% of them are represented by *Firmicutes* and *Bacteroidetes*, the remaining 10% mainly by *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* [4]. Great interindividual differences in gut microbial composition exist,

and this makes each person's bacterial community distinct and unique [5]. This complex and dynamic ecosystem is modulated by not modifiable factors, such as host genetics and sex, and modifiable ones such as environmental factors, medications, and lifestyle choices, which affect diversity and richness [6,7]. There is mounting evidence that the gut microbiota is essential to human health and that an "imbalance" in the gut microbial community, namely dysbiosis, is linked to a number of clinical conditions, including autoimmune disease [8], inflammatory intestinal diseases [9], mental disorders [10], and metabolic diseases [11]. Obesity, non-alcoholic fatty liver, insulin resistance (IR), and chronic inflammation, which are influenced by the gut microbiota, are associated with the development of diabetes [12,13].

Diabetes mellitus (DM) refers to a range of metabolic dysfunctions marked by chronic hyperglycemia due to impaired insulin efficacy, insufficient insulin secretion, or both of them [14]. Diabetes can be classified into general categories, which are gestational diabetes, type 1 (T1D) and type 2 diabetes (T2D), and specific types of diabetes [15]. Gestational diabetes is a glucose use disorder first diagnosed during pregnancy [14,15]. In T1D, antibodies are generated against several components of pancreatic β-cells, which results in the islets that make insulin deteriorating and ultimately being totally destroyed, leading to a shortage of insulin [16]. This type also includes checkpoint inhibitor-induced diabetes and latent autoimmune diabetes in adults (LADA) [14,15]. T2D is characterized by non-autoimmune insulin deficiency associated with IR and frequently metabolic syndrome, while specific types of diabetes are due to several causes, such as genetic defects, genetic syndromes, disease of the exocrine pancreas, endocrinopathies, infections, and drug use [15]. The International Diabetes Federation (IDF) reported that, worldwide, 536.6 million adults (20–79 years), which represented 9.2% of the total, were diabetic in 2021, and T1D affected an additional 1.2 million children and adolescents aged 0 to 19 [17]. Furthermore, by 2045, the number of diabetics is expected to reach 700 million [18]. More than 90% of cases of DM are T2D, a disorder characterized by non-autoimmune insulin deficiency associated with IR and often metabolic syndrome, often undiagnosed for many years but with considerable associated risks [15].

For a large population, this is a serious issue because T2D can cause severe comorbidities, including microvascular and macrovascular complications, accounting for the majority of patient morbidity and death [19,20]. It is important to note that prediabetes, a condition characterized by slightly elevated blood glucose level, is also associated with cardiovascular events and death [21], confirming that these complications can arise before T2D develops. T2D complications cause patients' physical and emotional suffering in addition to a significant financial burden on medical care. This chronic disease's development is heavily influenced by both hereditary and environmental factors interacting with each other and determining T2D prevalence. Environmental factors that contribute to its ongoing rise in prevalence include diet, ambient air pollution, and physical inactivity [22,23]. In terms of the pathophysiology of T2D, IR causes the liver to produce more glucose and absorb less from the muscles. Additionally, IR-induced β-cell dysfunction lowers insulin secretion, creating a feedback loop that leads to hyperglycemia and further exacerbates the condition. As obesity is linked to metabolic abnormalities that cause IR, it is a significant risk factor for T2D. Engaging in physical exercise helps to prevent obesity and acts on insulin receptors to increase muscle glucose uptake from plasma [24].

Some of the suggested causes for impaired insulin production and sensitivity in T2D include oxidative stress, endoplasmic reticulum stress, ectopic lipid deposition, amyloid deposition in the pancreas, lipotoxicity, and glucotoxicity. Oxidative stress, defined as an imbalance between the body's antioxidant defense systems and the generation of reactive oxygen species (ROS), plays a key role in the etiology, development, and consequences

of T2D. Hyperglycemia and mitochondrial dysfunction associated with T2D promote ROS production, and oxidative stress is known to enhance IR and decrease β-cell activity, thereby altering glucose homeostasis. Oxidative stress can harm any organ or tissue in the body, leading to diabetic retinopathy, neuropathy, and nephropathy and resulting in vascular consequences and cardiovascular illnesses [25]. ROS are produced more readily when oxidative stress triggers the release of inflammatory mediators. Numerous studies have demonstrated that IR and the traits of metabolic syndrome, such as hyperglycemia, are connected to sub-clinical inflammation and that low-grade inflammation increases the risk of T2D, which can be considered an inflammatory disease [26]. In order to understand the complex pathophysiology of this multifactorial disease, the alteration of metabolic interorgan crosstalk (the signaling between tissue and secreted factors) should also be considered. Hormones, insulin, glucagon, glucagon-like peptide-1 (GLP-1), organokines, and other metabolic molecules, including amino acids, lipids, and free fatty acids (FFAs), are among the regulators known to influence metabolism that are involved in T2D development and progression [27].

The large number of individuals who are at risk for T2D, or suffer from it, suggests the need to look for further reasons for the pathophysiology of the illness. This can result in the development of strategies aimed at reducing its risk and novel treatments targeted at stopping or reversing the disease's progression. The aim of this review is to explore the complex relationship between the gut microbiota and T2D, highlighting how an integrated and individualized approach, based on diet, exercise, and supplementation, could help in disease prevention and management.

2. Interaction Between the Gut Microbiota and T2D

The gut microbiota has been demonstrated to be altered in diabetic patients, indicating its significant role in the pathophysiology of T2D [28–30]. A fecal metagenomic analysis in European women revealed that T2D patients had four *Lactobacillus* species that were enriched and five additional *Clostridium* species that were decreased in abundance compared to healthy individuals [31]. It should be mentioned that the *Lactobacillus* species, which could be associated with augmented glucose presence in the intestine, correlated positively with hemoglobin A1c (HbA1c) and fasting glucose, whereas the *Clostridium* species linked negatively with insulin, plasma triglycerides, fasting glucose, and HbA1c and positively with adiponectin and high-density lipoprotein (HDL). Furthermore, prototypical butyrate producers *Faecalibacterium prausnitzii* and *Roseburia intestinalis* were very discriminant for T2D [31]. The authors also reported that metagenomic markers for T2D differed between people of different ages and population, suggesting that they greatly depend on the context and should be better considered as a whole [31]. It has also been discovered that metabolic parameters are linked to the changed gut microbiota in T2D. For example, a strong and positive correlation exists between the ratio of *Bacteroidetes* to *Firmicutes,* the ratio of *Bacteroides-Prevotella* group to *C. coccoides-E. rectale* group, and plasma glucose levels [32]. *Fusobacterium*, *Ruminococcus*, and *Blautia* were positively correlated with T2D, whereas *Bacteroidetes*, *Bifidobacterium*, *Akkermansia*, *Faecalibacterium*, and *Roseburia* were negatively correlated [33]. It should be underlined that *Akkermansia* are a very beneficial bacteria for host metabolism, known for protecting the gut barrier. Interestingly, prediabetics also have not-common gut microbiota [34–36]; furthermore, the microbial composition varies across phases of T2D.

Although the role of individual bacterial species in promoting or counteracting T2D has not yet been clarified, except in a few cases, the reported studies provide a general picture of the gut microbiota in diabetics and healthy people. It should always be kept in mind that the microbiota is an ecosystem that affects its host and can be greatly influenced

by lifestyle. It has been shown that, in obesity and related metabolic illnesses, dietary modifications quickly cause alterations in both *Firmicutes* and *Bacteroidetes* levels. Hwang et al. demonstrated that depletion of *Firmicutes* and *Bacteroidetes* caused by antibiotics in diet-induced obesity in mice alleviated systemic glucose intolerance, hyperinsulinemia, and IR via GLP-1 production [37]. In response to a meal, enteroendocrine L cells release GLP-1, an insulinotropic hormone that is essential for blood sugar levels, controlling secretions of insulin and glucagon, emptying of the stomach, blood flow, and food consumption. GLP-1 is quickly degraded and deactivated by the enzyme dipeptidyl peptidase 4 (DPP4) [38] and acts in a paracrine manner by binding locally to its receptor in the vagus nerve and enteric nervous system, stimulating the gut–brain axis, and in an endocrine manner by triggering β-cells [39]. Currently, GLP-1 receptor agonists like liraglutide, lixisenatide, exenatide, dulaglutide, albiglutide, and semaglutide are used for the therapy of T2D [40]. Through metabolites that control enteroendocrine cells (EECs) and, subsequently, hormonal activity, the gut microbiota influences host GLP-1 synthesis [41]. Furthermore, GLP-1 release was more prompt in the morning than in the afternoon in humans, indicating that GLP-1 secretion possesses temporal variability [42]. The gut microbiota appears to be crucial in regulating the rhythm of GLP-1 secretion since insulin production did not follow a diurnal pattern in germ-free animals lacking a gut microbiome, but it does after fecal transplantation from mice consuming a regular diet [43]. GLP-1 is stimulated by metabolites from the gut microbiota, but the gut microbiota is also regulated by GLP-1 through appetite, satiety, nutrient regulation, and mechanisms involving inflammatory responses [41]. Below, several mechanisms that elucidate the close relationship between T2D and gut bacteria are described, and the strategies that can help in obtaining a healthier microbial profile are reported.

2.1. Gut Barrier Permeability and Inflammation

The intestinal epithelium barrier interacts strongly with immune system cells and the gut microbiota rather than acting as a static physical barrier. The gut microbiome's composition influences immune system development and modifies immunological mediators, both of which have an impact [44] on the intestinal epithelium. Dysbiosis, which is an imbalance of gut microbial species, may also promote intestinal barrier breakdown and be linked to a higher risk of contracting specific illnesses [45]. The leaky gut syndrome (LGS) hypothesis states that intestinal hyperpermeability could permit pathogens and toxins to enter through intestinal epithelium junctions into the bloodstream, impacting immune, respiratory, reproductive, hormonal, or neurological systems [45]. The intestinal protective layer serves as a barrier between the body and gut contents; when it malfunctions, bacteria or bacterial products can seep into the body, increasing the risk of T2D [46]. The lipopolysaccharide (LPS) produced by gut bacteria causes metabolic endotoxemia and IR in addition to acting as a trigger for chronic low-grade inflammation through the LPS-CD14 pathway [47]. Furthermore, studies conducted in vitro and in vivo confirmed that hyperglycemia alters the integrity of tight and adherent junctions, increasing intestinal barrier permeability and allowing microbial compounds to enter into the bloodstream [48]. A key role for GLP-1 in suppressing inflammation and restoring mucosal integrity has been suggested by Lebrun et al.'s demonstration that, after gut barrier injury, enteroendocrine L cells recognize LPS, enhancing the release of GLP-1 via a Toll-like receptor 4 (TLR4)-dependent mechanism, which precedes the onset of significant modifications in inflammatory status and LPS levels [44]. Enhancing gut barrier function and ameliorating inflammation can greatly help in counteracting T2D and its complications. Several studies confirmed improved gut permeability and reduced LPS infiltration using *Akkermansia muciniphila*, a bacterial strain usually with reduced abundance in diabetes. *A. muciniphila* has been reported to enhance

Mucin 2 gene expression, strengthening mucus barrier function, and to preserve gut cell layer health, promoting intestinal epithelium renewing and tight junction protein expression. Furthermore, its abundance positively correlates with anti-inflammatory cytokines and negatively with pro-inflammatory factors [49]. Given its protective effect against endotoxemia, combined with its ability to improve metabolism and maintain microbiota homeostasis, *A. muciniphila* has recently been proposed as a "next-generation probiotic" for alleviating metabolic disorders [49].

2.2. Short-Chain Fatty Acids (SCFAs)

The intestinal microbiota breaks down and ferments dietary fibers that are ingested with food, which aids in the metabolism of carbohydrates, producing monosaccharides and short-chain fatty acids (SCFAs), which become important energy sources for the host and can act as substrates for lipogenesis and gluconeogenesis. The principal SCFAs are acetic acid, propionic acid, and butyric acid. According to earlier research, diabetic individuals have less bacteria that produce SCFAs, and consequently less SCFAs produced [50]. Dietary fibers are mostly broken down by *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Alistipes* species belonging to the phylum of *Bacteroidetes.* In addition to having a local effect on the colon and acting as energy source, SCFAs have the ability to enter the bloodstream and alter the metabolism of other organs. Their mode of action involves the stimulation of G protein-coupled receptors (GPCRs), such as GPR43 and GPR41, which are expressed in a number of different tissues, including adipose tissue, distal ileum, colon, lymph nodes, and the inhibition of histone deacetylases (HDAs), causing gene transcription and metabolic modifications with a pleiotropic effect [51,52]. Specifically, SCFAs can act in T2D prevention and management in several manners, for example, preserving the integrity of the gut epithelium by stimulating the synthesis of mucin [53] and enhancing the host immune system by affecting the activities of macrophages [54], preventing the entry of pathogens. Furthermore, acetate can promote immunometabolism, acting as an energy source for immune cells [55]. Interestingly, SCFAs, through GPR43 and HDAs, promote Peptide-YY (PYY) and GLP-1 expression and/or secretion, regulating food intake and preventing obesity [56,57]. Additionally, it was demonstrated that human adipose-derived mesenchymal stem cells expressed GPR43 and that upon propionate binding, these cells' reduction in lipid accumulation indicated the prevention of adipogenesis [58]. This obesity prevention effect represents another antidiabetic action of SCFAs.

Diabetics exhibit lower numbers of SCFA-producing bacteria and circulating SCFAs compared to healthy individuals, with fewer genes involved in SCFA synthesis due to microbiota disturbance. Specifically, reduced levels of butyrate and butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, are associated with T2D due to increased inflammation. This bacterium contributes to butyrate production and has shown potential in reducing IR by inducing GLP-1 secretion through the fatty acid receptor FFAR2 [59]. The butyrate biosynthetic pathway of *F. prausnutzi* is shared by *Roseburia* spp., hypothesized to be a marker of health due to the decrease observed in several diseases, including T2D [60]. Butyrate has been shown to slow down the onset of T2D via a variety of processes, including preserving the integrity of the intestinal epithelial barrier [61], enhancing insulin sensitivity, and reducing inflammation and appetite. Butyrate is also important for transcriptional regulation and post-translational modifications, as it significantly inhibits lysine and HDA activity [62]. This inhibition results in histone hyperacetylation, increasing the accessibility of transcription factors to gene promoter regions. Additionally, butyrate acts as a ligand for two transcription factors: peroxisome proliferator-activated receptor γ (PPARγ) and the aryl hydrocarbon receptor [63,64]. For these reasons, several strategies have been

described to increase butyrate levels in T2D patients who are often butyrate-producing bacteria deficient [65].

2.3. Branched-Chain Amino Acids (BCAAs)

In addition to SCFAs, functional products of the gut microbiota, such as amino acids, are linked to the pathophysiology of obesity, IR, and T2D. The gut microbiota can affect the amount of amino acids that are bioavailable from either endogenous or alimentary proteins and can thus provide the host with amino acids [66]. Crucially, new research indicates that increased systemic concentrations of branched-chain amino acids (BCAAs), namely leucine, isoleucine, and valine, in adult humans are associated with obesity, IR, and T2D [67] and might be thought as markers of IR and predictors of the onset of T2D. Zou et al. offered proof-of-concept data for the therapeutic feasibility of modifying BCAA metabolism to treat diabetes and showed that a BCAA catabolic deficiency and increased abundance of BCAAs and BCKAs play a crucial causative role in obesity-associated IR [64]. Moreover, Liu et al. revealed that branched-chain α-keto acids (BCKAs), which are endogenous metabolites of BCAAs, might exacerbate inflammation and organ damage in T2D by inducing mitochondrial oxidative stress and cytokine release in macrophages [68]. A positive correlation was found between the amounts of BCAAs, SCFAs, *Prevotella*, *Alistipes*, and *Barnesiella*; however, a negative correlation was observed with *Bacteroides* and *Enterococcus*. It is interesting to note that *Prevotellaceae* have been discovered to be considerably enriched in obesity [69]. Yoshida et al. demonstrated a favorable correlation between the gut microbiota and BCAA catabolism in brown adipose tissue, which is a modulator of metabolic and cardiovascular disorders [70]. Through an anti-inflammatory action, they demonstrated that treatment with *Bacteroides* spp. like *B. dorei* and *B. vulgatus* reduced obesity-induced BCAA catabolic deficiencies in this tissue and, therefore, obesity, underscoring the significance of specific bacterial species in BCAA metabolism and metabolic health [70].

2.4. Bile Acids (BAs)

The liver produces primary bile acids (BAs) when cholesterol is broken down, which are involved in the uptake and transportation of fats and fat-soluble vitamins. Cell death, apoptosis, and inflammation can all be brought on by toxic bile acids. Conversely, bile acid-stimulated nuclear and GPCR signaling protect against hepatic, intestinal, and macrophage inflammatory responses. Depending on where receptor activation occurs—in the intestine or the liver—it may either prevent or contribute to steatosis and obesity [71]. Abnormal concentrations of BAs and fecal metabolites have been reported in people suffering of metabolic disorders and in T2D patients [72]. In this context, the gut microbiota plays a central role since it converts primary BAs into secondary BAs when they reach the colon [73], preventing accumulation. An impairment in the conversion from primary to secondary BAs may contribute to intestinal inflammation since secondary BAs can exert anti-inflammatory effects [74]. In addition, certain secondary BAs preserve the intestinal tract's barrier function by impeding the development and migration of gut bacteria into host cells [75]. Deoxycholic acid and lithocholic acid can also be harmful to the host, causing oxidative stress, membrane damage, and colonic carcinogenesis [76], emphasizing the various and different functions of microbial-generated secondary BAs. Finally, secondary BAs can either activate the intestinal L cell's Takeda G protein-coupled receptor 5 to increase secretion of GPL-1 [77] or inhibit it by the farnesoid X receptor (FXR) [78], meaning they have dual regulatory effects on GPL-1 secretion. On the other hand, BAs are strong antibacterial substances that are differently tolerated by bacteria and have a significant impact on the gut's microbial ecology [79]. For this reason, the complex metabolism of BAs

and the dual role of secondary BAs in gut health should be considered in evaluating the gut microbiota's effects on T2D.

2.5. Tryptophan and Its Metabolites

Tryptophan is an essential aromatic amino acid that is obtained from foods, including milk, cheese, chicken, fish, and oats. Tryptophan plays a key function in the production of proteins and is a precursor to several important metabolites. Even if most of the tryptophan in food is processed locally in the gut by host enzymes, gut microorganisms digest around 5% of it. The three main metabolic routes for tryptophan are kynurenine (KYN) and its derivatives, indole, and serotonin [80].

IR and T2D are linked to tryptophan dysmetabolism. KYN and KYN metabolite production is correlated with the inflammatory status in metabolic syndrome through the activation of indoleamine 2,3-dioxygenase 1. Concurrently, there is a deficiency in the gut microbiota's ability to produce indole-3-propionic acid (IPA) and other ligands for the aryl hydrocarbon receptor (AhR). Inadequate activation of the AhR pathway modifies gut permeability and facilitates LPS translocation by reducing GLP-1 and interleukin (IL)-22. Furthermore, gut bacteria constitute a crucial modulator of the biosynthesis of serotonin, which affects food behavior and satiety, and in consequence, body mass index (BMI), and is disrupted in metabolic syndrome [81]. Chimerel et al. demonstrated that indole is important for gut microbiota–mouse colonic L cells communication. Indole, on the one hand, it caused an acute stimulation of GLP-1 secretion by inhibiting voltage-gated K^+ channels and improving Ca^{2+} entry during short exposure; on the other hand, indole decreased the rate at which ATP was created by inhibiting NADH dehydrogenase, which led to a long-lasting reduction in GLP-1 secretion over longer period [82]. In this context, diet plays an important role. For instance, increased intake of fiber and milk (which have higher levels of gut *Bifidobacterium*) is linked to a better-circulating tryptophan metabolite profile with enhanced IPA synthesis [83]. These data underline the importance of a targeted diet in achieving an equilibrium microbial state.

2.6. Trimethylamine N-Oxide (TMAO)

L-carnitine and phosphatidylcholine-rich foods are broken down by gut bacteria into trimethylamine, which the liver then converts to trimethylamine N-oxide (TMAO). Greater TMAO concentrations were related to T2D and increased mortality risk independent of glycemic control in T2D patients [84]. Gao et al. demonstrated that, in mice given a high-fat diet, TMAO worsened poor glucose tolerance, blocked the hepatic insulin signaling pathway, and induced inflammation in adipose tissue [85]. It is worth noting that individuals with dysbiosis produce more TMAO than those with eubiosis while ingesting the same food [86]. Increasing data suggest that TMAO is linked to increased risks of cardiovascular disease and renal failure. In mice, dietary treatment with TMAO, carnitine, or choline modified the cecal microbial composition, raising the risk of atherosclerosis [87]. Strategies to reestablish eubiosis and certain dietary adjustments might help not only in counteracting the onset and progression of T2D but also the comorbidities, such as cardiovascular diseases, related to it.

2.7. Antidiabetic Drugs

T2D and obesity are known to induce gut dysbiosis, while antidiabetic drugs can help to restore a healthy microbiota [88]. Alpha-glucosidase inhibitors slow carbohydrate uptake in the distal intestine, favoring bacterial growth. The most commonly used of these inhibitors, Acarbose, increases the number of taxa that produce SCFAs, such as *Lactobacillus*, *Faecalibacterium*, *Prevotella* [89], and *Bifidobacterium* [90]. Sitagliptin, which is a dipeptidyl peptidase IV inhibitor, as well as metformin, commonly used to lower glucose

and enhance insulin sensitivity, have been demonstrated to increase the relative abundance of the genus *Lactobacillus* [90]. In T2D complicated with nonalcoholic fatty liver disease, the intestinal bacterial community exhibited significant increases in diversity and richness upon receiving treatment with liraglutide, a GLP-1 receptor agonist. Additionally, the relative abundances of *Bacteroidetes*, *Proteobacteria*, and *Bacilli* were significantly elevated, while those of *Fusobacteria* and *Actinobacteria* were significantly enhanced upon metformin treatment [91]. Thus, different antiglycemic drugs exert different effects on the composition of the gut microbiota, selectively increasing beneficial bacteria [90]. It is known that the efficacy of drugs greatly depends on gut microbiota features, and gut microbiota modulation could influence therapy outcomes [92]; for example, liraglutide responsiveness is likely to depend on gut microbiota dysbiosis [93]. Restoring eubiosis in the altered gut microbiota in T2D patients can greatly help in potentiating the effect and reducing the doses of traditional drugs.

3. Diet, T2D, and the Gut Microbiota

An unbalanced and unhealthy diet is one of the primary risk factors for chronic illness since it is widely acknowledged that eating habits have a substantial influence on our overall health and wellbeing. Diet is one of the most important lifestyle interventions in T2D prevention and management. A number of dietary strategies, such as a low-carbohydrate diet (LCD), very-low-calorie diet (VLCD), fasting-mimicking diet (FMD), and Mediterranean diet (MedDiet), have been suggested to reduce the occurrence of chronic diseases [94].

3.1. Low-Carb, Very-Low-Calorie, and Fasting-Mimicking Diets

Given their positive effects on weight reduction and glycemic management, research shows that low-carb (<130 g/day of carbs) and very low-carb diets (usually <50 g/day of carbs) can be useful strategies for treating T2D. In clinical trials and standard care, LCDs have shown weight and HbA1c-lowering effects [95]. A primary care practice in England using an LCD approach saw 46% of people with T2D achieve remission, with improvements in lipid profile, blood pressure, and weight loss. International researchers defined T2D remission as HbA1c <6.5% at 3 months post antidiabetic medication cessation, without bariatric surgery [96]. Significant weight loss through LCDs and VLCDs led to a 25–77% remission rate in overweight and obese individuals, with reduced HbA1c and decreased medical support [97,98]. Higher remission rates were directly associated with greater weight reduction, according to recent results from the Diabetes Remission Clinical Trial (DiRECT). Individuals who lost more than 10 kg were far more likely to achieve remission than those who lost less. People with T2D experienced a remission rate of 46–60% after one year [99]. Significant weight reduction was accomplished using an LCD. Consequently, 20% of all practicing T2D patients achieved remission. It seems that a time frame of less than one year for T2D treatment constitutes a significant window of opportunity for reaching drug-free diabetic remission [100,101].

Studies show that most patients experienced remission because of dietary strategies, with significant changes in blood lipids, HbA1c, blood glucose, and quality of life. Remission was more likely in those who lost more weight and had a shorter T2D diagnosis duration [102]. VLCD caused weight loss by 20–30%, sometimes in just 12–16 weeks when daily energy intake was restricted to 400–800 kcal/day [103]. Steven et al. reported that for the 40% of patients who reacted to a VLCD by reaching a fasting plasma glucose of <7 mmol/L, a comprehensive and long-lasting weight reduction program resulted in a continuous remission of T2D for at least 6 months [104]. Currently, available data indi-

cates that only people who have been diagnosed with T2D relatively recently may benefit from VLCD or other calorie restriction techniques to induce T2D remission.

While there is evidence that extensive behavioral support can increase the durability of weight reduction with VLCD, people who do not receive the best possible assistance for sustained behavior change will still find it difficult to successfully adhere to these dietary methods over the long term [103]. In T2D patients using diet alone or metformin for glycemic management, Van der Butg et al. found that a 5-day monthly fasting-mimicking diet over a 12-month period increased HbA1c levels while requiring fewer pharmaceuticals, looked safe in standard clinical practice, and decreased the need for glucose-lowering drugs [105].

3.2. Mediterranean Diet

Among diets, MedDiet, one of the world's most popular and researched regimens, has been linked to several health advantages [106]. The Mediterranean diet provides several benefits on metabolic illness and T2D risk. Molecules within the Mediterranean diet (MedDiet) can counteract mechanisms involved in T2D progression, including inflammation and oxidation. In particular, MedDiet, adding another piece to the puzzle of metabolic pathology prevention, can modulate the gut microbiota. A diet that emphasizes eating more fruits, vegetables, whole grains, and seafood while consuming fewer starches, sugary beverages, and red and processed meats can postpone the onset of T2D, according to epidemiological research [107]. Moreover, a high intake of dietary fiber (particularly cereal fiber), antioxidants, and monounsaturated fatty acids (MUFAs), along with foods containing these nutrients, has been linked to improved insulin sensitivity, the ability of pancreatic β-cells to secrete insulin, and a lower risk of developing T2D (Figure 1), according to consistent epidemiological and clinical evidence [108,109]. MedDiet is praised for its antidiabetic properties, making it a suitable alternative to low-fat, high-carb diets for managing blood glucose in T2D patients [110].

By increasing antioxidant capacity and decreasing inflammation, Filippatos et al. discovered that moderate to high adherence to MedDiet could prevent T2D [111]. More adherence to MedDiet might reduce the incidence of T2D by 19%, according to a meta-analysis of trials including 122,810 individuals [112]. High-MUFA diets have been demonstrated in recent years to have a beneficial impact on glycemic management and total triglyceride (TG) levels in T2D patients [109]. Additionally, two prospective studies have connected the risks or outcomes of gestational T2D to MedDiet adherence. Participants in the highest quartile of MedDiet adherence had a 40% reduced chance of developing T2D compared to those in the lowest quartile, according to a nurses' health survey that found 491 occurrences of incident T2D among 4413 females followed for 14 years and aged 22–44 with a history of gestational diabetes [113]. Adherence to the Mediterranean diet (MedDiet) was associated with a lower prevalence of gestational diabetes and better glucose tolerance, even in women without the disease, according to research including 1076 pregnant women from ten different countries [113]. Thus far, the vast majority of research has evidenced the value of MedDiet in T2D prevention. What is even more exciting is the fact that studies on pregnant women have increased the group for whom MedDiet adoption is appropriate for T2D prevention. MedDiet education, depending on the population size, may be a safe public health strategy to stop or postpone the onset of T2D. To elucidate the mechanisms of T2D risk reduction that are not reliant on weight loss, more study is needed [106], but it is likely that most of the diet-induced benefits in managing T2D pass through gut microbiota modulation. Mediterranean diet components have an important impact on the gut microbiota's development. The major intake of fiber, vitamins, antioxidants, and mono-

and poly-unsaturated fatty acids and minor consumption of processed foods, saturated fatty acids, and amino acids induce an increase in microbiome diversity.

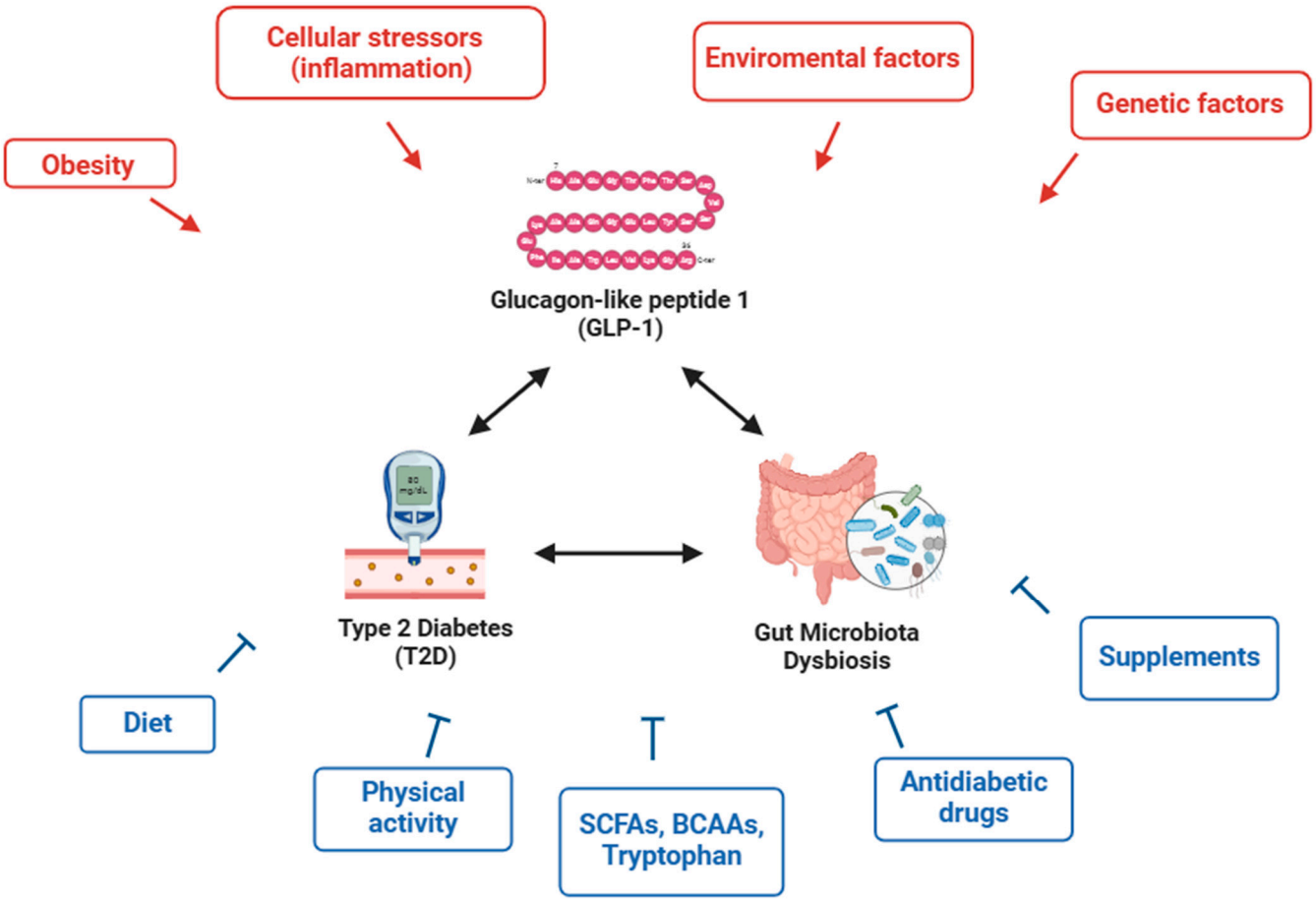

Figure 1. An integrated approach to maintaining a healthy gut microbiota for T2D prevention and management.

3.3. Effects of Diet on the Gut Microbiota

According to human studies, MedDiet can change the composition of gut microbes. Donati Zeppa et al. reported a significant decrease in the abundance of *Proteobacteria* in 20 BC survivors of the MoviS clinical trial following a 12-week home-based lifestyle intervention based on MedDiet and exercise [114]. The adherence of eating habits to MedDiet led to a reduction of the abundances of *Proteobacteria* and *Firmicutes* and an increase in the abundance of advantageous bacterial communities such as *Bacteroidetes*, *Lactobacilli*, *Bifidobacteria*, and *Faecalibacterium*. These bacterial modifications associated with enhanced production of microbiota-derived metabolites have been observed in human studies. SCFAs and MedDiet itself lead to oxidative stress, inflammation, obesity, and TD2 reductions [115].

Wang et al. [116] reported the potential connections between the gut microbiome, MedDiet, and T2D. Participants in this study comprised 394 normoglycemic, 805 prediabetic, and 543 diabetic individuals from a cohort study of Hispanic/Latino men and women in the United States. Increased adherence to MedDiet was linked to increased abundance of major dietary fiber metabolizers, such as *Faecalibacterium prausnitzii*, *Coprococcus*, and *Lachnospira*. The majority of adherents to MedDiet were found to have more or less abundant microbial activities linked to the metabolism of amino acids and carbohydrates, with the exception of those involved in the breakdown of galactose and lactose. A depletion of bacteria related to dietary sulfur reduction to H_2S has also been associated with the higher adherence to

MedDiet. In particular, MedDiet showed a stronger protective effect against T2D in participants with lower levels of *Prevotella*. The microbial adaptations induced by MedDiet led to increased microbiota-mediated metabolites, intestinal homeostasis, decreased dysbiosis, and decreased intestinal permeability, contributing to the prevention of T2D onset [117].

Previous analysis has indicated a correlation between eating vegetarian and improvements in T2D glycemic management [118]. In this context, Panhigrai et al. [119] proposed a lifestyle intervention that promoted adherence to a plant-predominant diet in T2D patients. This diet was rich in phytochemicals and bioactive nutritional molecules, such as vitamins and fiber, and low in fat. A dietary fiber intake of 50 g per day (25 g soluble and 25 g insoluble fiber) induced an improvement in glucose homeostasis and insulin secretion in T2D patients, probably due to the increase in SCFA products by the anaerobic microbial fermentation of fiber. Total SCFA, acetate, and butyrate levels in human feces were found to be negatively associated with the risk of developing T2D in cross-sectional research from the Henan rural cohort [120]. SCFAs stimulate GLP-1 and PYY in the stomach, which improves glucose homeostasis [121]. The authors also identified SCFA interval levels associated with decrease in T2D prevalence, although several studies will be needed to identify a healthy individual SCFA concentration to prevent T2D. Whole grains and legumes are known to lower postprandial blood glucose levels, both during the meal they are consumed and at subsequent meals. This has significant consequences for blood glucose regulation throughout the day and the avoidance of T2D [122].

In conclusion, more accurate and successful dietary interventions for the prevention of T2D should arise from the personalization of diets based on the gut microbial compositions of each individual.

4. Supplements, T2D, and the Gut Microbiota

Research on animals suggests that the evolution of IR to T2D may be influenced by the nature of intestinal microbes. By lowering intestinal endotoxin concentrations, altering the structure of the gut microbial population, and lowering energy harvest, probiotics and/or prebiotics may be a viable strategy for improving insulin sensitivity [123]. Probiotics have antidiabetic properties via decreasing oxidative stress, intestinal permeability, and pro-inflammatory cytokines through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. To create guidelines for the use of pro- and prebiotics in the prevention of T2D, better-planned human clinical trials are needed (Table 1). Furthermore, other nutraceuticals, such as antioxidants and traditional Chinese medicine, have been reported to exert antidiabetic activity, although most of them need to be metabolized and activated by gut microorganisms.

4.1. Probiotics

Probiotics are live microorganisms that, when administered in the appropriate amounts and for sufficient duration, can benefit the host's health [124]. They work by reducing inflammation, strengthening the intestinal barrier, and ultimately restoring gut health. Probiotics are currently undergoing extensive research as potential biotherapeutics thanks to their health-promoting properties and their capacity to combat certain diseases [125]. Given the gut microbiota characteristics in patients with T2D and the resulting consequences, probiotics are recognized as beneficial and complementary. Probiotics usually improve T2D symptoms, enhancing intestinal integrity and restoring the host's intestinal barrier function through their surface molecules and metabolites, reducing systemic LPS levels, lowering endoplasmic reticulum stress, and improving peripheral insulin sensitivity [126]. Probiotics, specifically *Lactobacilli* and *Bifidobacteria*, have recently gained attention as promising biotherapeutics with proven efficacy demonstrated in different experimental

models. Some studies have reported reductions in the abundances of *Bifidobacterium* spp. and *Lactobacillus* spp. with increased plasma LPS, which caused metabolic endotoxemia via NF-kB activation at the molecular onset of IR. One hypothesis is that T2D can be improved by decreasing the concentration of LPS in the blood [125,127]. Ma et al. investigated the activation of NF-kB signaling in the progression of inflammation using a HeLa cell line preincubated with live *Lactobacillus reuteri* cells for 1–2 h. This treatment prevented translocation of NF-kB to the nucleus and inhibited the expression of various pro-inflammatory cytokines regulated by NF-kB [128].

In a double-blind, randomized, placebo-controlled trial, 50 volunteers consumed 120 g/d of fermented milk daily for 6 wks. The subjects were divided into two groups: the probiotic group, who consumed fermented milk containing 109 colony-forming units/d of *Bifidobacterium animalis* subsp lactis BB-12 and *Lactobacillus acidophilus* La-5, and the control group, who had regular fermented milk. After 6 weeks, the intervention arm showed an improvement in their glycemic profile. There was a significant decrease in fructosamine levels, and HbA1c levels also appeared to be reduced. By contrast, the control group did not show significantly affected glycemic control [129]. In addition, *A. muciniphila* has a beneficial role in glycemic control and IR. Furthermore, its important roles in promoting intestinal barrier function, lowering inflammation, boosting metabolism, and preserving microbial homeostasis have received recent research interest [49]. Everard et al. proved that treatment with *A. muciniphila* for 4 weeks reversed metabolic disorders induced by a high-fat diet. This included fat mass gain, metabolic endotoxemia, adipose tissue inflammation, and IR in male C57BL/6 *ob*/*ob*, HF-fed obese, and T2D mice. *A. muciniphila* administration increased the levels of endocannabinoids in the intestine, which control inflammation, gut barrier function, and gut peptide secretion [130].

Several studies demonstrated the effect of probiotics in GLP-1 normalization. A commercial probiotic mixture called VSL#3, which contained four strains of *Lactobacillus*, three strains of *Bifidobacterium*, and one strain of *Streptococcus*, boosted butyrate levels, which in turn promoted the production of GLP-1 in a mouse model. GLP-1 level normalization was associated with decreased meal consumption and enhanced glucose tolerance [131]. Also, Pegah et al. demonstrated that probiotics, as well as resveratrol, decreased glucose and IR in diabetic rats by increasing GLP-1 levels and reducing oxidative stress [132]. Han and colleagues reported that the process by which fecal microbiota transplantation from a normal glucose tolerance donor improved glycolipid abnormality in db/db mice involved modifications to the bacterial composition that generates SCFAs and triggers the GPR43/GLP-1 pathway [133]. For an exhaustive review on the mechanisms, applications, and challenges of probiotics useful for counteracting T2D, see Shen et al. [134].

4.2. Antioxidants

IR and hyperglycemia—which is brought on by oxidative stress, disrupting glucose homeostasis—can both contribute to T2D [135]. Antioxidants, both dietary and endogenous, can alter gut microbiota composition and function, improving metabolic health. Dietary polyphenols, which are naturally occurring substances found in fruits, vegetables, cereals, tea, coffee, and wine, play an important role in the interaction between exogenous antioxidants and the gut microbiota, regulating the incidence and progression of T2D. Because of this, the small intestine absorbs only a tiny portion (5–10%) of the total amount of polyphenols consumed. Approximately 90–95% of the total amount of polyphenols consumed are deposited in the large intestine, where they are broken down into smaller phenolic metabolites by the gut microbial population. Consuming meals high in polyphenols may have health advantages since these metabolites are absorbable [136]. Research has shown that higher consumption of polyphenol-rich foods is linked to improved glycemic

control and reduced inflammation in patients with type 2 diabetes (T2D) [135]. Furthermore, the intake of polyphenols and the primary foods that contain them may help to lower IR and reduce risk factors for T2D, including oxidative stress and inflammation, as evidenced by numerous human studies.

The pertinent research relating dietary polyphenols to prediabetes and T2D is reviewed by Guasch-Ferrè et al. [137], who concentrated on a number of clinical trials examining the impact of polyphenols on cardiometabolic parameters in T2D patients. For instance, in a meta-analysis including data from 22 randomized clinical trials, catechins from green tea were found to have a positive effect on decreasing fasting glucose; however, its effects on fasting insulin, HbA1c, and the Homeostatic Model Assessment for IR (HOMA-IR) were not significant.

Green tea has been shown to correct microbial dysbiosis associated with various conditions, including obesity, which negatively impacts the onset of T2D. In particular, the abundance of the phylum *Bacteroidetes* significantly decreased in obese mice, whereas that of the phylum *Firmicutes* significantly increased. Observational studies have indicated that regular consumption of green tea is associated with improved glycemic control and a lowered risk of T2D [138]. Furthermore, the administration of green tea was able to influence the composition of the gut microbial community by elevating the relative abundances of some advantageous (and adversely correlated with obesity) bacteria, such *Akkermansia*, *Lachnospiraceae*, and *Alistipes* [139].

Numerous extracts rich in polyphenol content have shown antidiabetic effects. Rutin is the primary flavonoid glycoside found in *djulis husk* crude extract, which was the source of one such polyphenol. In order to evaluate the preventive impact of rutin and *djulis husk* crude extract on glucose tolerance, mice were given a high-fat diet (HFD) for 16 weeks in order to cause hyperglycemia. Tung et al. found that the crude extract significantly reduced HFD-induced diabetogenic effects. Furthermore, the crude extract markedly elevated the phosphorylation of insulin receptor substrate 1 (pIRS1) and glucose transporter type 4 (GLUT4) protein expression in epididymal white adipose tissue and the liver. Moreover, the crude extract restored the HFD-induced decrease in catalase (CAT) and glutathione peroxidase (GPX) activities [140]. Furthermore, in HFD-fed animals, the expression of Zonula occludens-1 (ZO-1) and occludin proteins in the colon was significantly downregulated, reducing intestinal permeability and LPS translocation. However, these effects were reverted after the application of the crude extract. Furthermore, the crude extract intervention significantly influenced the gut microbiota community and its alpha diversity. Therefore, in cases of hyperglycemia brought on by a high-fat diet, the crude extract of *djulis* shell may raise blood glucose levels and improve insulin receptor sensitivity. Its capacity to control the gut microbiota, preserve intestinal barrier integrity to lower body inflammation, boost antioxidant activity, and alter insulin signaling may be the reason for this action [140].

Glycyrrhiza uralensis polysaccharide extract (GUP) is another extract that shows promise for improving the gut microbiota in connection with T2D. It has exceptional antioxidant properties, inhibits alpha-glucosidase activity, and enhances glycemic management in T2D. The genus *Glycyrrhiza* has many health benefits and contains bioactive substances that are important for T2D treatment. A study showed that *G. uralensis* polysaccharide extract (GUP) reduced liver lipid levels, oxidative stress, IR, and high blood sugar in T2 diabetic mice. GUP also improved gut microbiota diversity, reducing harmful species such as *Bacteroides*, *Escherichia-Shigella*, and *Clostridium sensu stricto 1*, and increasing beneficial ones like *Akkermansia*, *Lactobacillus*, *Romboutsia*, and *Faecalibaculum*. This research provides valuable information on dietary approaches for managing T2D and promoting overall well-being [141].

Melatonin interacts with gut bacteria to reduce T2D as well. Huang et al.'s case–control research examined the relationship between T2D risk and serum melatonin in a community of southern Chinese people, emphasizing the importance of the gut flora. Higher blood melatonin levels were linked to a decreased risk of T2D and lower levels of fasting glucose, according to research including 2034 people with T2D (cases) and healthy people (controls). A reduced risk of T2D was associated with greater blood melatonin levels. The gut microbiota and melatonin signaling mediated the connection; tryptophan metabolites may play a particular role in this process. These results highlight the significance of melatonin and associated microorganisms and metabolites as possible targets for T2D treatment. The study also discovered that the gut microbiota of people with T2D was altered, showing decreased levels of serum melatonin, a less diverse gut microbiota, a greater abundance of *Bifidobacterium*, and a lower abundance of *Coprococcus*. Seven genera were also discovered to be linked. Furthermore, a correlation between melatonin and features associated with T2D was discovered to exist for seven genera. Among these, serum LPS and interleukin-10 (IL-10) showed positive correlations with *Bifidobacterium*, whereas serum interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17), α tumor necrosis factor TNF-α, and LPS showed negative correlations with *Coprococcus* [142]. It is worth mentioning a study that investigated the impact of a dietary intervention involving high-fiber, polyphenol-rich, and vegetable protein functional foods on the fecal microbiota and various biochemical parameters in patients with T2D. These parameters included LPS, BCAAs, TMAO, HbA1c, and FFAs.

Patients with T2D showed imbalances in their intestinal bacteria, with an increase in the abundance of *Prevotella copri*. Dietary intervention with functional foods significantly enhanced the fecal microbiota compared with the placebo, leading to increased alpha diversity and modifying the abundances of specific bacteria. This change occurred regardless of any T2D medication they were taking. The patients had fewer *P. copri* bacteria and more of the bacteria *F. prausnitzii* and *A. muciniphila*, which are known to have anti-inflammatory effects. The group who changed their diet also saw significant reductions in glucose, total and low-density lipoprotein (LDL) cholesterol, FFAs, HbA1c, triglycerides, and CRP, as well as an improvement in antioxidant activity, compared to the group who did not change their diet. Long-term adherence to a high-fiber, polyphenol-enriched, and vegetable protein-based diet provided benefits for gut microbiota composition and may offer potential therapies for to improve glycemic control, dyslipidemia, and inflammation [143].

4.3. Chinese Medicine

Traditional Chinese medicine (TCM) has emerged as a promising approach to the management of T2D [144,145]. Because TCM is mostly taken orally, it has a direct impact on the gut microbiome. Nevertheless, a number of active components found in herbal remedies, including tannins, flavonoids, and triterpene glycosides, have characteristics such as high molecular flexibility, low lipophilicity, and hydrogen bonding capability, which restrict TCM bioavailability. Its pharmacology is significantly influenced by absorption, which is facilitated by a change in the gut microbiota. Furthermore, because different TCM components function as nutrients for the growth of particular bacteria, TCM modulates the population of host intestinal microbes. T2D can be treated with a particular Chinese medicine formula called Huang-Qi-Ling-Hua-San (HQLHS). *Astragalus Membranaceus*, *Ganoderma lucidum*, *Inonotus obliquus*, and *Momordica charantia* make up its composition. Using a T2D mouse model induced by streptozotocin and a high-fat diet, researchers were able to better understand how HQLHS reduced high blood sugar and high cholesterol levels. HQLHS enhanced the abundances of *Bifidobacterium*, *Turicibacter*, *Alistipes*, *Romboutsia*, and *Christensenella*. Next, fecal microbiota transplantation (FMT) was employed to determine

whether these bacteria had any therapeutic value, with a focus on two particular strains of *Christensenella*. The results demonstrated that feeding these strains to diabetic rats improved their ability to metabolize fat and blood sugar, raised GLP-1 production, strengthened their antioxidant defenses, and induced other positive benefits. Overall, the evidence pointed to *Christensenella* being a major target for HQLHS treatment with a beneficial effect on T2D [146].

The fruit of *Lycium barbarum* is another plant used in traditional Chinese medicine. It is abundant in flavonoids, which have strong exploration potential and are linked to anti-inflammatory and antioxidant properties that may lower the incidence of T2D. In T2D mice induced with a high-fat diet (HFD) and streptozotocin (STZ), its therapeutic impact was examined. Significant antidiabetic activity was demonstrated by the study, which included decreased water intake, liver index, fasting blood glucose, HOMA-IR, HOMA-IS, HbA1c levels, and Oral Glucose Tolerance Test (OGTT) levels, along with improved lipid and glucose metabolism. Additionally, the study demonstrated the restoration of liver tissue structure, as evidenced by a decrease in fat vesicles, and a remission of hepatocyte swelling, as well as a reduction in the expression of proinflammatory cytokines and related mRNAs. The study also looked at alterations in the gut microbiome of mice with T2D induced by HFD/STZ. Bacteria including *Bacteroidales*_S24-7_group, *Allobaculum*, *Turicibacter*, *Coriobacteriaceae*, *Ruminococcaceae*, *Clostridiales*_vadinBB60_group, and *Enterococcus* were shown to be common in T2D patients. These findings showed improvements in the metabolic profile, gut microbiota health, and glucose levels [147].

In a separate study, Wang et al. administered oral treatments for constipation to HFD-fed mice and db/db mice using the Chinese formula Shouhuitongbian (SHTB; 200 and 100 mg/kg/d) and the standard medication metformin (100 mg/kg/d). The results showed that SHTB successfully reduced inflammation, improved dysfunctional lipid metabolism, and enhanced IR and glucose tolerance. Furthermore, SHTB demonstrated effectiveness in treating T2D by altering the composition of the gut microbiota, particularly by increasing the abundances of *Akkermansia* and *Parabacteroides*, and by promoting the production of SCFAs and the breakdown of BCAAs [148].

Table 1. Supplements demonstrating efficacy T2D management.

Supplements	Model	Administration (Dose/Day, Duration) of Supplemetation	General Effects and Possible Mechanism of Action	Effect on Microbiome Composition	Refs.
			Probiotics		
	HeLa cell line	1–2 h treatment	↓ translocation of NF-kB to the nucleus and expression of inflammatory cytokines	↑ *Lactobacillus* and blocked metabolic endotoxemia via NF-kB activation	[128]
Fermented milk with *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp *lactis* BB-12	T2D patients	120 g/d for 6 weeks	Improved glycemic profile: ↓ fructosamine and HbA1c levels		[129]
Akkermansia muciniphila	Male C57BL/6 *ob/ob*, HF-fed obese, and type 2 diabetic mice	4 weeks	Improved metabolic profile: ↓ fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and IR. ↑ levels of endocannabinoids in the intestine.		[130,131]
VSL#3	*Animal model*: C57J/B6 male mice (n = 7 in each group) 4–6-week-old male mice fed with a low-fat diet or a high-fat diet, with and without VSL#3. In vitro *model:* NCI-H716 cells line	5 mg/kg body weight by oral gavage for 8 weeks	Using a cell culture system, it has been demontrated that butyrate stimulated the release of GLP-1 from intestinal L-cells. Potential therapeutic utility to counter obesity and T2D.	↑ levels of a short-chain fatty acid (SCFA), butyrate.	[131]
			Antioxidants—Polyphenols		
Green Tea	Obese mice	Regular consumption of green tea	Improved glycemic control	↓ *Bacteroidetes* and ↑ *Firmicutes*; ↑ *Akkermansia*, *Lachnospiraceae*, and *Alistipes*	[138,139]
Djulis hull crude extract—rutin	HFD mice with hyperglycemia	16 weeks	↑ pIRS1 and GLUT4 protein expression in eWAT and liver; ↓ AUC, OGTT, HOMA-IR, and AGE levels; ↓ CAT and GPX activities; upregulated expression of ZO-1 and occluding proteins in the colon	↑ microbial community and alpha diversity; preserved intestinal barrier integrity	[140]
Glycyrrhiza uralensis polysaccharide extract	HFD/STZ-induced T2D mice	400 mg/kg for 4 weeks	↓ liver lipid levels, oxidative stress, IR, and hyperglycemia	↑ alpha diversity; enhanced intestinal barrier integrity; ↓ *Bacteroidetes*, *Escherichia-Shigella*, and *Clostridium*, and ↑ *Akkermansia*, *Lactobacillus*, *Romboutsia*, and *Faecalibaculum*	[141]
Dietary intervention with functional food	81 T2D patients	3 months	↓ glucose, total and LDL cholesterol, free fatty acids, HbA1c, triglycerides, and CRP; ↑ antioxidant activity, glycemic control, dyslipidemia, and inflammation	↑ alpha diversity; ↓ *Prevotella copri* (increased in T2D patients), and ↑ *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*	[143]

Table 1. *Cont.*

Supplements	Model	Administration (Dose/Day, Duration) of Supplemetation	General Effects and Possible Mechanism of Action	Effect on Microbiome Composition	Refs.
Chinese medicine					
Huang-Qi-Ling-Hua-San (HQLHS)	T2D mouse	300 ul once a day	Improved fat and blood sugar metabolism, ↑GLP-1 production, strengthened their antioxidant defenses	↑ *Bifidobacterium*, *Turicibacter*, *Alistipes*, *Romboutsia*, and *Christensenella*	[146]
Flavonoids from *Lycium barbarum* (LBFs)	HFD/STZ-induced T2D mice		Antidiabetic activity: ↓ water intake, liver index, fasting blood glucose, HOMA-IR, HOMA-IS, HbA1c levels, and OGTT levels; ↓ proinflammatory cytokine expression		[147]
Shouhuitongbian (SHTB)	HFD-fed mice and db/db mice	200 and 100 mg/kg/d	↓ inflammation, repaired dysfunctional lipid metabolism, and improved IR and glucose intolerance; IRS-1/PI3K/AKT signaling pathway overexpression	↑ *Akkermansia* and *Parabacteroides*; ↓ BCAAs and ↑ BSCFAs and SCFAs	[148]

Abbreviations. AGE: advanced glycation end product; AUC: area under the curve; BCAAs: branched-chain amino acids; BSCFAs: branched short-chain fatty acids; CAT: catalase; CRP: C-reactive protein; eWAT: epididymal white adipose tissue; GLP-1: glucagon-like peptide 1; GLUT4: glucose transporter type 4; GPX: glutathione peroxidase; HbA1c: glycosylated hemoglobin A1C; HFD/STZ: streptozotocin high-fat diet; HFD: high-fat diet; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance; HOMA-IS: Homeostatic Model Assessment for Insulin Sensivity; HQLHS: Huang-Qi-Ling-Hua-San; IR: insulin resistance; LBFs: flavonoids from *Lycium barbarum*; LDL: low-density lipoprotein; NCI-H716: cell line derived from ascites fluid of a colorectal adenocarcinoma from a 33-year-old Caucasian male; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; *ob/ob* mice: obese mice; OGTT: Oral Glucose Tolerance Test; PI3K: phosphatidylinositol 3-kinase; pIRS1: phosphorylation of insulin receptor substrate 1; SCFA: short-chain fatty acid; SHTB: Shouhuitongbian; T2D: type 2 diabetes; VSL#3: Probiotic "Very Safe Lactobacilli#3"; ZO-1: zonula occludens-1; ↓: Decrease; ↑: Increase.

5. Physical Exercise, T2D, and the Gut Microbiota

Physical activity may have a protective effect on gut health and T2D. Not only is exercise often recommended for weight loss and maintenance, but some studies suggest that both acute and chronic exercise may reduce the risk of fatty liver and gastrointestinal disorders, as well as inflammatory mediators and apoptotic markers in gut lymphocytes, as observed in older animals [149]. This review looked at research exploring how exercise might protect against or reduce mechanisms associated with the microbiota that are believed to be responsible for triggering the processes underlying T2D.

5.1. The Role of Physical Activity in Intestinal Barrier Function and Metabolic Health

Yu et al. [150] recently investigated the relationship between intestinal permeability and physical activity. Specifically, they showed that aerobic exercise, through its ability to induce a redistribution of hypoxia-inducible factor-1 α (HIF-1α), modulated the transcription of many barrier-protective genes and anti-inflammatory and tissue-protective pathways [151]. In this context, chronic aerobic exercise stimulated AMP-activated protein kinase (AMPK), an important downstream signaling pathway of sextrin2 (SESN2)-mediated activity [152–157]. The latter is a stress-inducible protein that promotes epithelial cell survival and recovery and acts as a positive regulator of exercise-induced improvements in glycolipid metabolism [151]. SESN2 is critical in controlling different types of stressors, allowing epithelial cells to recover from inflammatory damage [153–155]. According to research by Yu et al. [150], chronic aerobic exercise significantly reduced weight gain induced by a high-fat diet, improved body composition in mice, and increased fasting glucose levels. Notably, the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the control and SENS2 ablation groups were significantly reduced with chronic aerobic exercise [158]. Interestingly, chronic aerobic exercise restored the levels of alpha diversity by increasing the abundance of *Bacteroidetes* and reducing the abundances of *Firmicutes* and *Actinobacteria* that the high-fat diet (HFD) had significantly increased in the mice [150]. Moreover, regular exercise tends to increase several genera of bacteria, contributing to improved gut health and metabolic benefits. *Bifidobacterium*, *Lactobacillus*, and *Akkermansia* are associated with enhanced metabolic health. The abundance of *Bacteroides*, which play a role in breaking down complex molecules in the gut, also increases with exercise. Additionally, *Roseburia*, known for producing short-chain fatty acids (SCFAs) that support gut health, tends to proliferate with physical activity [159,160]. In this way, exercise significantly protected the intestinal epithelium from bacterial invasion. In addition, the colon villus musculature thickened significantly after chronic aerobic exercise, showing a large increase in TJ protein expression, as well as improved motility, as evidenced by more frequent stools. Although these are early findings, the results on the role of chronic aerobic exercise in protecting the gut barrier are promising and exciting.

5.2. The Importance of Chronic Endurance Exercises in SCFA Production and Consumption for the Prevention of T2D

Numerous studies have examined the different functions of SCFAs in IR and T2D. These functions include controlling immunomodulatory processes, maintaining intestinal epithelial integrity, and controlling insulin secretion and pancreatic cell proliferation [161]. Owing to the therapeutic functions of SCFAs, studies on how exercise affects the production and consumption of these fats were conducted [162,163]. Specifically, Allen J.M. [164] studied whether a 6-week aerobic exercise program may alter gut microbial populations and fecal SCFAs in previously inactive, lean, and obese adults. The authors found that fecal concentrations of SCFAs (acetate, propionate, and butyrate) increased mostly in the lean subjects after three sessions per week (30 to 60 min) of moderate-to-vigorous aerobic

exercise over a period of six weeks, and this effect appeared to be dependent on BMI status. After six weeks of returning to sedentary habits, the concentrations of SCFAs in the obese group remained unchanged. Nevertheless, the washout period was associated with a decline in the gut microbiome's capacity to produce SCFAs in the obese group, as evidenced by a decrease in the relative abundances of butyrate- and propionate-regulating genes [164]. These results, although interesting, need further study with exercise protocols of greater duration, intensity, and complexity. Finally, with the current state of the art, we can conclude that, although the effect on obese people was not as strong as that on thin people, it appears that constant physical activity plays a significant role in the regulation of SCFAs and that the latter has a direct relationship with T2D.

The abundances of several genera of bacteria that produce SCFAs tend to increase after exercise. These include *Roseburia*, known for producing butyrate, a type of SCFA that supports gut health; *Faecalibacterium*, another butyrate producer, which is often associated with anti-inflammatory effects; *Akkermansia*, primarily known for its role in maintaining the gut lining, but also contributes to SCFA production; and *Bifidobacterium*, which produces acetate, another important SCFA [159,160].

5.3. Exercise Modulation in the Reduction of LPS

As previously described (Section 2.1), LPS causes metabolic endotoxemia and IR and represents a trigger for chronic low-grade inflammation through the LPS–CD14 pathway. Following the consumption of high-fat, high-carbohydrate meals, LPS plasma concentrations greatly increase [165], indicating that the source of this fat-soluble LPS is the gastrointestinal tract. Furthermore, dietary fat has been demonstrated to raise intestinal permeability to LPS [166]. In a study [167], researchers examined the hypothesis that a regimen of swimming for 60 minutes per session, 5 days per week for 8 weeks, ameliorates insulin resistance in high-fat diet (HFD)-induced obese (DIO) rats through the regulation of TAK1-dependent signaling and its hepatic regulators. HFD feeding led to increased body weight, visceral fat mass, serum free fatty acids (FFAs), and hepatic lipid deposition, while reducing hepatic glycogen content and insulin sensitivity. Both chronic and acute exercise training improved insulin resistance. Exercise training resulted in decreased phosphorylation of TAK1, c-Jun N-terminal kinase 1 (JNK1), and insulin receptor substrate 1 (IRS1), while enhancing Akt phosphorylation in the liver. Furthermore, exercise elevated the protein levels of USP4 and DUSP14 and reduced the protein levels of TRIM8 in the liver of obese rats. These findings demonstrate that exercise induces significant modulation of TAK1-dependent signaling and its regulators in the liver, leading to marked improvements in insulin sensitivity. This study provides novel insights into the mechanisms by which physical exercise mitigates insulin resistance. Exercise robustly reversed the activation of this pathway and enhanced insulin signaling, presenting a novel mechanism by which exercise improves insulin action in obesity and T2D. Thus, exercise, both acute and chronic, promoted a reduction in serum LPS in rats that had been induced to eat HFD. This pathway has been validated in various models examining the impact of exercise on LPS-induced lung inflammation [168] and LPS-induced inflammatory responses in rat cardiac tissue [169]. In conclusion, the available in vivo and in vitro evidence supports the need for clinical studies to investigate the effects of moderate- and low-intensity exercise on different inflammatory LPS-induced conditions.

5.4. The Effect of Exercise in Counteracting the Production of TMAO Associated with T2D

Recent results suggest that food has little bearing on the positive effects of regular exercise on gut microbial populations [170–172]. Consequently, physical activity may encourage the production of less dangerous bioactive metabolites, such as TMAO, by improving the

profile of the gut microbiome, especially in older or obese people who are more prone to have a dysfunctional gut microbiota [173]. Argyridou et al. [174] analyzed baseline and 12-month follow-up data from the Walking Away from T2D study, which recruited adults at high risk for T2D in primary care in 2009–2010. During this period, 316 men and 167 women were analyzed. Moderate to vigorous physical activity (about 30 min per day) was associated with TMAO in all models. The results showed that each 30 min difference in moderate to vigorous physical activity was associated with less TMAO, while sedentariness and light physical activity were not associated with TMAO in any model. Thus, engagement in physical activity was associated with lower TMAO levels, suggesting a possible new mechanism underlying the inverse relationship between physical activity and cardiometabolic health [174]. In addition, Battillo and Malin [175] demonstrated that a low-calorie diet plus high-intensity interval exercise intervention reduced TMAO more than a low-calorie diet-only program. The study involved 23 sedentary women with obesity (age: 48.4 $\pm$ 2.4 years; BMI: 37.9 $\pm$ 1.4 kg/m^2), who were randomized to complete 12 supervised high-intensity interval exercise sessions (3 min 50% peak heart rate alternating with 3 min at 90% for 60 min) over 13 days. The exercise duration was progressively increased so that participants completed 30 and 45 min of interval training on the first and second days, respectively, and subsequently 60 min of exercise per session, with 1 rest day over the 13 days. Overall, only in women with higher levels of circulating TMAO at baseline did both treatments reduce plasma TMAO [176]. Finally, according to the above findings, Erickson et al. demonstrated that the change in TMAO after diet and exercise intervention was inversely related to visceral adipose tissue at baseline ($r = -0.63$, $p = 0.009$) and glucose disposal rates ($r = 0.58$, $p = 0.002$) [176]. In conclusion, we can state that a low-calorie diet and exercise approach appears to be effective in reducing TMAO.

5.5. The Effect of Exercise on GLP-1 and the Gut Microbiota in T2D

Exercise influences GLP-1 through several mechanisms. Physical activity stimulates the secretion of GLP-1 from intestinal L cells, partly due to the increased production of SCFAs by beneficial gut bacteria, which are more abundant in physically active individuals [159,160,177]. Exercise also enhances insulin sensitivity, amplifying the effects of GLP-1, which helps to regulate blood glucose levels by promoting insulin secretion and inhibiting glucagon release [178]. Additionally, regular physical activity increases the population of bacteria that produce SCFAs, which not only stimulates GLP-1 secretion but also improves gut barrier function and reduce inflammation [177]. Moreover, exercise aids in weight loss and maintenance, enhancing the effectiveness of GLP-1 by reducing IR and inflammation [179]. These mechanisms collectively contribute to better glycemic control and overall metabolic health in individuals with T2D. Different types of exercise influence GLP-1 levels in various ways, enhancing its beneficial effects on glucose metabolism and overall health. Aerobic exercises, such as running and cycling, significantly increase GLP-1 secretion by improving gut microbiota composition and increasing the production of SCFAs [180]. Resistance training, including weight-lifting and body weight exercises, boosts GLP-1 levels by enhancing muscle insulin sensitivity and promoting lean muscle mass, which is crucial for glucose uptake [180]. High-intensity interval training (HIIT) is particularly effective in rapidly increasing GLP-1 levels due to its intense bursts of activity followed by short rest periods, which improve cardiovascular fitness and insulin sensitivity [181]. These exercises collectively contribute to better glycemic control, weight management, and overall metabolic health in individuals with T2D [182].

6. Conclusions

Although the important role of hereditary components in determining the onset of T2D is acknowledged, the disease primarily results from the common sedentary lifestyle and the sharp rise in obesity worldwide. T2D comorbidities can cause early death, hasten the course of the disease, and induce mental and physical illnesses. These comorbidities have an adverse effect on general health, well-being, exacerbating diabetes-related outcomes, and related expenditures in patients with T2D, leading to a substantial treatment burden, higher healthcare utilization, costs, and lost economic output.

T2D is influenced by the gut microbiota, which also affects IR, glucose homeostasis, and inflammation, major contributors to the development of T2D. It also affects how the intestinal tract and extraintestinal tissues respond to antidiabetic medications. In this review, we focused on the role that lifestyle plays in maintaining a healthy microbiota and the implications for the development and treatment of T2D. Nonetheless, further clinical research is required to elucidate the possible therapeutic impact of gut microbes and their byproducts on T2D. Given the complexity of the variables that may affect clinical outcomes, including basal conditions, nutrition, lifestyle, and medications, translating the understanding of the microbiota for clinical benefit in diabetics is both a challenging and exciting task for scientists. Figure 1 summarizes the factors that predispose or aggravate T2D and those that improve the situation, also acting through the microbiota, highlighting the central role of GLP-1. Emphasizing the need for a tailored approach is crucial, particularly when addressing a diverse and specific group of individuals, which includes pre-diabetics, confirmed patients, and those at risk of T2D. In order to minimize side effects and increase benefits, the same level of attention to individual features—made possible by technical progress—should be given to both identifying pharmaceutical interventions and non-pharmacological ones.

A diet that is tailored to individual needs, whether it includes supplements or not, a well thought out and meticulously implemented exercise chronic regimen, and customized drug therapies, paying particular attention to gut microbiota status and changes, represents an integrated strategy against T2D. In addition, it is important to keep an eye on how things are developing to guarantee that the appropriate course of action is taken. This review, far from being exhaustive, aims to provide useful insights for the implementation of an integrated and individualized strategy for T2D prevention and management.

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Abbreviations

AGE	advanced glycation end product
AMPK	AMP-activated protein kinase
AUC	area under the curve
Bas	bile acids
BCAAs	branched-chain amino acids
BCKAs	branched-chain α-keto acids
BMI	body mass index
BSCFAs	branched short-chain fatty acids
CAT	catalase
CRP	C-reactive protein

db/db	diabetic mice
DiRECT	Diabetes Remission Clinical Trial
DM	diabetes mellitus
DPP4	dipeptidyl peptidase 4
EECs	control enteroendocrine cells
eWAT	epididymal white adipose tissue
FFAs	fatty acids
FMD	fasting-mimicking diet
FMT	fecal microbiota transplantation
FXR	farnesoid X receptor
GLP-1	glucagon-like peptide 1
GLUT4	glucose transporter type 4
GPCRs	G protein-coupled receptors
GPX	glutathione peroxidase
GUP	*Glycyrrhiza uralensis* polysaccharide
HbA1c	glycosylated hemoglobin A1C
HDAs	histone deacetylases
HDL	high-density lipoprotein
HFD/STZ	Streptozotocin high-fat diet
HFD	high-fat diet
HIF-1α	hypoxia-inducible factor-1 α
HIIT	high-intensity interval training
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HOMA-IS	Homeostatic Model Assessment for Insulin Sensitivity
HQLHS	Huang-Qi-Ling-Hua-San
IDF	International Diabetes Federation
IL	interleukin
IL-10	interleukin-10
IL-17	interleukin-17
IL-1β	interleukin-1β
IL-6	interleukin-6
IPA	indole-3-propionic acid
IR	insulin resistance
KYN	kynurenine
LBFs	flavonoids from *Lycium barbarum*
LCD	low-carbohydrate diet
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MUFA	monounsaturated fatty acids
NCI-H716	cell line derived from ascites fluid of a colorectal adenocarcinoma from a 33-year-old Caucasian male
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
ob/*ob* mice	obese mice
OGTT	Oral Glucose Tolerance Test
PI3K	phosphatidylinositol 3-kinase
pIRS1	phosphorylation of insulin receptor substrate 1
PYY	peptide-YY
ROS	reactive oxygen species
SCFA	Short-chain fatty acid
SESN2	sextrin2
SHTB	Shouhuitongbian
STZ	streptozotocin
T1D	type 1 T1D diabetes
T2D	type 2 diabetes

TCM	traditional Chinese medicine
TLR4	Toll-like receptor 4
TMAO	trimethylamine N-oxide
TNF-α	tumor necrosis factor α
VLCD	very-low-calorie Diet
VSL#3	probiotic "Very Safe Lactobacilli#3"
ZO-1	zonula occludens-1

References

1. Portincasa, P.; Bonfrate, L.; Vacca, M.; De Angelis, M.; Farella, I.; Lanza, E.; Khalil, M.; Wang, D.Q.-H.; Sperandio, M.; Di Ciaula, A. Gut Microbiota and Short Chain Fatty Acids: Implications in Glucose Homeostasis. *Int. J. Mol. Sci.* **2022**, *23*, 1105. [CrossRef] [PubMed]
2. Savage, D.C. Microbial ecology of the gastrointestinal tract. *Annu. Rev. Microbiol.* **1977**, *31*, 107–133. [CrossRef] [PubMed]
3. Sender, R.; Fuchs, S.; Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.* **2016**, *14*, e1002533. [CrossRef]
4. Qin, J.; Li, R.; Raes, J.; Arumugam, M.; Burgdorf, K.S.; Manichanh, C.; Nielsen, T.; Pons, N.; Levenez, F.; Yamada, T.; et al. A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing. *Nature* **2010**, *464*, 59–65. [CrossRef] [PubMed]
5. Fassarella, M.; Blaak, E.E.; Penders, J.; Nauta, A.; Smidt, H.; Zoetendal, E.G. Gut Microbiome Stability and Resilience: Elucidating the Response to Perturbations in Order to Modulate Gut Health. *Gut* **2021**, *70*, 595–605. [CrossRef]
6. Coman, V.; Vodnar, D.C. Gut Microbiota and Old Age: Modulating Factors and Interventions for Healthy Longevity. *Exp. Gerontol.* **2020**, *141*, 111095. [CrossRef]
7. Donati Zeppa, S.; Agostini, D.; Ferrini, F.; Gervasi, M.; Barbieri, E.; Bartolacci, A.; Piccoli, G.; Saltarelli, R.; Sestili, P.; Stocchi, V. Interventions on Gut Microbiota for Healthy Aging. *Cells* **2022**, *12*, 34. [CrossRef]
8. Xu, Q.; Ni, J.-J.; Han, B.-X.; Yan, S.-S.; Wei, X.-T.; Feng, G.-J.; Zhang, H.; Zhang, L.; Li, B.; Pei, Y.-F. Causal Relationship Between Gut Microbiota and Autoimmune Diseases: A Two-Sample Mendelian Randomization Study. *Front. Immunol.* **2022**, *12*, 746998. [CrossRef]
9. Qiu, P.; Ishimoto, T.; Fu, L.; Zhang, J.; Zhang, Z.; Liu, Y. The Gut Microbiota in Inflammatory Bowel Disease. *Front. Cell Infect. Microbiol.* **2022**, *12*, 733992. [CrossRef]
10. Donati Zeppa, S.; Ferrini, F.; Agostini, D.; Amatori, S.; Barbieri, E.; Piccoli, G.; Sestili, P.; Stocchi, V. Nutraceuticals and Physical Activity as Antidepressants: The Central Role of the Gut Microbiota. *Antioxidants* **2022**, *11*, 236. [CrossRef]
11. Fan, Y.; Pedersen, O. Gut Microbiota in Human Metabolic Health and Disease. *Nat. Rev. Microbiol.* **2021**, *19*, 55–71. [CrossRef] [PubMed]
12. Huang, F.; Lyu, B.; Xie, F.; Li, F.; Xing, Y.; Han, Z.; Lai, J.; Ma, J.; Zou, Y.; Zeng, H.; et al. From Gut to Liver: Unveiling the Differences of Intestinal Microbiota in NAFL and NASH Patients. *Front. Microbiol.* **2024**, *15*, 1366744. [CrossRef] [PubMed]
13. Scheithauer, T.P.M.; Rampanelli, E.; Nieuwdorp, M.; Vallance, B.A.; Verchere, C.B.; van Raalte, D.H.; Herrema, H. Gut Microbiota as a Trigger for Metabolic Inflammation in Obesity and Type 2 Diabetes. *Front. Immunol.* **2020**, *11*, 571731. [CrossRef]
14. Pleus, S.; Tytko, A.; Landgraf, R.; Heinemann, L.; Werner, C.; Müller-Wieland, D.; Ziegler, A.-G.; Müller, U.A.; Freckmann, G.; Kleinwechter, H.; et al. Definition, Classification, Diagnosis and Differential Diagnosis of Diabetes Mellitus: Update 2023. *Exp. Clin. Endocrinol. Diabetes* **2024**, *132*, 112–124. [CrossRef] [PubMed]
15. ElSayed, N.A.; Aleppo, G.; Aroda, V.R.; Bannuru, R.R.; Brown, F.M.; Bruemmer, D.; Collins, B.S.; Gaglia, J.L.; Hilliard, M.E.; Isaacs, D.; et al. 2. Classification and Diagnosis of Diabetes: Standards of Care in Diabetes—2023. *Diabetes Care* **2023**, *46*, S19–S40. [CrossRef]
16. Bluestone, J.A.; Herold, K.; Eisenbarth, G. Genetics, Pathogenesis and Clinical Interventions in Type 1 Diabetes. *Nature* **2010**, *464*, 1293–1300. [CrossRef]
17. International Diabetes Federation. *IDF Diabetes Atlas*, 10th ed.; International Diabetes Federation: Brussels, Belgium, 2021; Available online: https://www.diabetesatlas.org (accessed on 24 October 2024).
18. Saeedi, P.; Petersohn, I.; Salpea, P.; Malanda, B.; Karuranga, S.; Unwin, N.; Colagiuri, S.; Guariguata, L.; Motala, A.A.; Ogurtsova, K.; et al. Global and Regional Diabetes Prevalence Estimates for 2019 and Projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th Edition. *Diabetes Res. Clin. Pract.* **2019**, *157*, 107843. [CrossRef]
19. Carracher, A.M.; Marathe, P.H.; Close, K.L. International Diabetes Federation 2017. *J. Diabetes* **2018**, *10*, 353–356. [CrossRef]
20. Khan, R.; Chua, Z.; Tan, J.; Yang, Y.; Liao, Z.; Zhao, Y. From Pre-Diabetes to Diabetes: Diagnosis, Treatments and Translational Research. *Medicina* **2019**, *55*, 546. [CrossRef]
21. Echouffo-Tcheugui, J.B.; Perreault, L.; Ji, L.; Dagogo-Jack, S. Diagnosis and Management of Prediabetes. *JAMA* **2023**, *329*, 1206. [CrossRef]

22. Kahn, S.E.; Cooper, M.E.; Del Prato, S. Pathophysiology and Treatment of Type 2 Diabetes: Perspectives on the Past, Present, and Future. *Lancet* **2014**, *383*, 1068–1083. [CrossRef] [PubMed]
23. Kang, N.; Chen, G.; Tu, R.; Liao, W.; Liu, X.; Dong, X.; Li, R.; Pan, M.; Yin, S.; Hu, K.; et al. Adverse Associations of Different Obesity Measures and the Interactions with Long-Term Exposure to Air Pollutants with Prevalent Type 2 Diabetes Mellitus: The Henan Rural Cohort Study. *Environ. Res.* **2022**, *207*, 112640. [CrossRef] [PubMed]
24. Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K.B.; Ostolaza, H.; Martín, C. Pathophysiology of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* **2020**, *21*, 6275. [CrossRef]
25. Caturano, A.; D'Angelo, M.; Mormone, A.; Russo, V.; Mollica, M.P.; Salvatore, T.; Galiero, R.; Rinaldi, L.; Vetrano, E.; Marfella, R.; et al. Oxidative Stress in Type 2 Diabetes: Impacts from Pathogenesis to Lifestyle Modifications. *Curr. Issues Mol. Biol.* **2023**, *45*, 6651–6666. [CrossRef]
26. Oguntibeju, O.O. Type 2 Diabetes Mellitus, Oxidative Stress and Inflammation: Examining the Links. *Int. J. Physiol. Pathophysiol. Pharmacol.* **2019**, *11*, 45.
27. Sanches, J.M.; Zhao, L.N.; Salehi, A.; Wollheim, C.B.; Kaldis, P. Pathophysiology of Type 2 Diabetes and the Impact of Altered Metabolic Interorgan Crosstalk. *FEBS J.* **2023**, *290*, 620–648. [CrossRef]
28. Horie, M.; Miura, T.; Hirakata, S.; Hosoyama, A.; Sugino, S.; Umeno, A.; Murotomi, K.; Yoshida, Y.; Koike, T. Comparative Analysis of the Intestinal Flora in Type 2 Diabetes and Nondiabetic Mice. *Exp. Anim.* **2017**, *66*, 405–416. [CrossRef]
29. Bielka, W.; Przezak, A.; Pawlik, A. The Role of the Gut Microbiota in the Pathogenesis of Diabetes. *Int. J. Mol. Sci.* **2022**, *23*, 480. [CrossRef] [PubMed]
30. Zhou, Z.; Sun, B.; Yu, D.; Zhu, C. Gut Microbiota: An Important Player in Type 2 Diabetes Mellitus. *Front. Cell Infect. Microbiol.* **2022**, *12*, 834485. [CrossRef]
31. Karlsson, F.H.; Tremaroli, V.; Nookaew, I.; Bergström, G.; Behre, C.J.; Fagerberg, B.; Nielsen, J.; Bäckhed, F. Gut Metagenome in European Women with Normal, Impaired and Diabetic Glucose Control. *Nature* **2013**, *498*, 99–103. [CrossRef]
32. Larsen, N.; Vogensen, F.K.; van den Berg, F.W.J.; Nielsen, D.S.; Andreasen, A.S.; Pedersen, B.K.; Al-Soud, W.A.; Sørensen, S.J.; Hansen, L.H.; Jakobsen, M. Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults. *PLoS ONE* **2010**, *5*, e9085. [CrossRef] [PubMed]
33. He, Y.; Wu, W.; Wu, S.; Zheng, H.-M.; Li, P.; Sheng, H.-F.; Chen, M.-X.; Chen, Z.-H.; Ji, G.-Y.; Zheng, Z.-D.-X.; et al. Linking Gut Microbiota, Metabolic Syndrome and Economic Status Based on a Population-Level Analysis. *Microbiome* **2018**, *6*, 172. [CrossRef] [PubMed]
34. Zhou, W.; Sailani, M.R.; Contrepois, K.; Zhou, Y.; Ahadi, S.; Leopold, S.R.; Zhang, M.J.; Rao, V.; Avina, M.; Mishra, T.; et al. Longitudinal Multi-Omics of Host–Microbe Dynamics in Prediabetes. *Nature* **2019**, *569*, 663–671. [CrossRef] [PubMed]
35. Chávez-Carbajal, A.; Pizano-Zárate, M.L.; Hernández-Quiroz, F.; Ortiz-Luna, G.F.; Morales-Hernández, R.M.; De Sales-Millán, A.; Hernández-Trejo, M.; García-Vite, A.; Beltrán-Lagunes, L.; Hoyo-Vadillo, C.; et al. Characterization of the Gut Microbiota of Individuals at Different T2D Stages Reveals a Complex Relationship with the Host. *Microorganisms* **2020**, *8*, 94. [CrossRef] [PubMed]
36. Wang, L.; Yu, X.; Xu, X.; Ming, J.; Wang, Z.; Gao, B.; Xing, Y.; Zhou, J.; Fu, J.; Liu, T.; et al. The Fecal Microbiota Is Already Altered in Normoglycemic Individuals Who Go on to Have Type 2 Diabetes. *Front. Cell Infect. Microbiol.* **2021**, *11*, 598672. [CrossRef]
37. Hwang, I.; Park, Y.J.; Kim, Y.; Kim, Y.N.; Ka, S.; Lee, H.Y.; Seong, J.K.; Seok, Y.; Kim, J.B. Alteration of Gut Microbiota by Vancomycin and Bacitracin Improves Insulin Resistance via Glucagon-like Peptide 1 in Diet-induced Obesity. *FASEB J.* **2015**, *29*, 2397–2411. [CrossRef]
38. Holst, J.J. The Physiology of Glucagon-like Peptide 1. *Physiol. Rev.* **2007**, *87*, 1409–1439. [CrossRef]
39. Richards, P.; Parker, H.E.; Adriaenssens, A.E.; Hodgson, J.M.; Cork, S.C.; Trapp, S.; Gribble, F.M.; Reimann, F. Identification and Characterization of GLP-1 Receptor–Expressing Cells Using a New Transgenic Mouse Model. *Diabetes* **2014**, *63*, 1224–1233. [CrossRef]
40. Nauck, M.A.; Quast, D.R.; Wefers, J.; Meier, J.J. GLP-1 Receptor Agonists in the Treatment of Type 2 Diabetes—State-of-the-Art. *Mol. Metab.* **2021**, *46*, 101102. [CrossRef]
41. Zeng, Y.; Wu, Y.; Zhang, Q.; Xiao, X. Crosstalk between Glucagon-like Peptide 1 and Gut Microbiota in Metabolic Diseases. *mBio* **2024**, *15*, e0203223. [CrossRef]
42. Lindgren, O.; Mari, A.; Deacon, C.F.; Carr, R.D.; Winzell, M.S.; Vikman, J.; Ahrén, B. Differential Islet and Incretin Hormone Responses in Morning Versus Afternoon after Standardized Meal in Healthy Men. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 2887–2892. [CrossRef] [PubMed]
43. Martchenko, S.E.; Martchenko, A.; Cox, B.J.; Naismith, K.; Waller, A.; Gurges, P.; Sweeney, M.E.; Philpott, D.J.; Brubaker, P.L. Circadian GLP-1 Secretion in Mice Is Dependent on the Intestinal Microbiome for Maintenance of Diurnal Metabolic Homeostasis. *Diabetes* **2020**, *69*, 2589–2602. [CrossRef] [PubMed]

44. Lebrun, L.J.; Lenaerts, K.; Kiers, D.; Pais de Barros, J.-P.; Le Guern, N.; Plesnik, J.; Thomas, C.; Bourgeois, T.; Dejong, C.H.C.; Kox, M.; et al. Enteroendocrine L Cells Sense LPS after Gut Barrier Injury to Enhance GLP-1 Secretion. *Cell Rep.* **2017**, *21*, 1160–1168. [CrossRef] [PubMed]
45. Takiishi, T.; Fenero, C.I.M.; Câmara, N.O.S. Intestinal Barrier and Gut Microbiota: Shaping Our Immune Responses throughout Life. *Tissue Barriers* **2017**, *5*, e1373208. [CrossRef] [PubMed]
46. Tilg, H.; Moschen, A.R. Microbiota and Diabetes: An Evolving Relationship. *Gut* **2014**, *63*, 1513–1521. [CrossRef]
47. Cani, P.D.; Amar, J.; Iglesias, M.A.; Poggi, M.; Knauf, C.; Bastelica, D.; Neyrinck, A.M.; Fava, F.; Tuohy, K.M.; Chabo, C.; et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes* **2007**, *56*, 1761–1772. [CrossRef]
48. Arnone, D.; Chabot, C.; Heba, A.-C.; Kökten, T.; Caron, B.; Hansmannel, F.; Dreumont, N.; Ananthakrishnan, A.N.; Quilliot, D.; Peyrin-Biroulet, L. Sugars and Gastrointestinal Health. *Clin. Gastroenterol. Hepatol.* **2022**, *20*, 1912–1924.e7. [CrossRef]
49. Li, J.; Yang, G.; Zhang, Q.; Liu, Z.; Jiang, X.; Xin, Y. Function of Akkermansia Muciniphila in Type 2 Diabetes and Related Diseases. *Front. Microbiol.* **2023**, *14*, 1172400. [CrossRef]
50. Caesar, R. Pharmacologic and Nonpharmacologic Therapies for the Gut Microbiota in Type 2 Diabetes. *Can. J. Diabetes* **2019**, *43*, 224–231. [CrossRef]
51. Tan, J.K.; Macia, L.; Mackay, C.R. Dietary Fiber and SCFAs in the Regulation of Mucosal Immunity. *J. Allergy Clin. Immunol.* **2023**, *151*, 361–370. [CrossRef]
52. Lymperopoulos, A.; Suster, M.S.; Borges, J.I. Short-Chain Fatty Acid Receptors and Cardiovascular Function. *Int. J. Mol. Sci.* **2022**, *23*, 3303. [CrossRef] [PubMed]
53. Burger-van Paassen, N.; Vincent, A.; Puiman, P.J.; van der Sluis, M.; Bouma, J.; Boehm, G.; van Goudoever, J.B.; van Seuningen, I.; Renes, I.B. The Regulation of Intestinal Mucin MUC2 Expression by Short-Chain Fatty Acids: Implications for Epithelial Protection. *Biochem. J.* **2009**, *420*, 211–219. [CrossRef] [PubMed]
54. Smolinska, S.; Groeger, D.; O'Mahony, L. Biology of the Microbiome 1. *Gastroenterol. Clin. N. Am.* **2017**, *46*, 19–35. [CrossRef] [PubMed]
55. Tan, J.; Ni, D.; Ribeiro, R.V.; Pinget, G.V.; Macia, L. How Changes in the Nutritional Landscape Shape Gut Immunometabolism. *Nutrients* **2021**, *13*, 823. [CrossRef]
56. Tolhurst, G.; Heffron, H.; Lam, Y.S.; Parker, H.E.; Habib, A.M.; Diakogiannaki, E.; Cameron, J.; Grosse, J.; Reimann, F.; Gribble, F.M. Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2. *Diabetes* **2012**, *61*, 364–371. [CrossRef]
57. Larraufie, P.; Martin-Gallausiaux, C.; Lapaque, N.; Dore, J.; Gribble, F.M.; Reimann, F.; Blottiere, H.M. SCFAs Strongly Stimulate PYY Production in Human Enteroendocrine Cells. *Sci. Rep.* **2018**, *8*, 74. [CrossRef]
58. Iván, J.; Major, E.; Sipos, A.; Kovács, K.; Horváth, D.; Tamás, I.; Bay, P.; Dombrádi, V.; Lontay, B. The Short-Chain Fatty Acid Propionate Inhibits Adipogenic Differentiation of Human Chorion-Derived Mesenchymal Stem Cells Through the Free Fatty Acid Receptor 2. *Stem Cells Dev.* **2017**, *26*, 1724–1733. [CrossRef]
59. Yang, Q.; Ouyang, J.; Sun, F.; Yang, J. Short-Chain Fatty Acids: A Soldier Fighting Against Inflammation and Protecting From Tumorigenesis in People With Diabetes. *Front. Immunol.* **2020**, *11*, 590685. [CrossRef]
60. Tamanai-Shacoori, Z.; Smida, I.; Bousarghin, L.; Loreal, O.; Meuric, V.; Fong, S.B.; Bonnaure-Mallet, M.; Jolivet-Gougeon, A. *Roseburia* Spp.: A Marker of Health? *Future Microbiol.* **2017**, *12*, 157–170. [CrossRef]
61. Liang, L.; Liu, L.; Zhou, W.; Yang, C.; Mai, G.; Li, H.; Chen, Y. Gut Microbiota-Derived Butyrate Regulates Gut Mucus Barrier Repair by Activating the Macrophage/WNT/ERK Signaling Pathway. *Clin. Sci.* **2022**, *136*, 291–307. [CrossRef]
62. Donohoe, D.R.; Collins, L.B.; Wali, A.; Bigler, R.; Sun, W.; Bultman, S.J. The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation. *Mol. Cell* **2012**, *48*, 612–626. [CrossRef] [PubMed]
63. Alex, S.; Lange, K.; Amolo, T.; Grinstead, J.S.; Haakonsson, A.K.; Szalowska, E.; Koppen, A.; Mudde, K.; Haenen, D.; Al-Lahham, S.; et al. Short-Chain Fatty Acids Stimulate Angiopoietin-Like 4 Synthesis in Human Colon Adenocarcinoma Cells by Activating Peroxisome Proliferator-Activated Receptor γ. *Mol. Cell Biol.* **2013**, *33*, 1303–1316. [CrossRef]
64. Marinelli, L.; Martin-Gallausiaux, C.; Bourhis, J.-M.; Béguet-Crespel, F.; Blottière, H.M.; Lapaque, N. Identification of the Novel Role of Butyrate as AhR Ligand in Human Intestinal Epithelial Cells. *Sci. Rep.* **2019**, *9*, 643. [CrossRef]
65. Arora, T.; Tremaroli, V. Therapeutic Potential of Butyrate for Treatment of Type 2 Diabetes. *Front. Endocrinol.* **2021**, *12*, 761834. [CrossRef] [PubMed]
66. Neis, E.; Dejong, C.; Rensen, S. The Role of Microbial Amino Acid Metabolism in Host Metabolism. *Nutrients* **2015**, *7*, 2930–2946. [CrossRef] [PubMed]
67. Wang, T.J.; Larson, M.G.; Vasan, R.S.; Cheng, S.; Rhee, E.P.; McCabe, E.; Lewis, G.D.; Fox, C.S.; Jacques, P.F.; Fernandez, C.; et al. Metabolite Profiles and the Risk of Developing Diabetes. *Nat. Med.* **2011**, *17*, 448–453. [CrossRef] [PubMed]
68. Liu, S.; Li, L.; Lou, P.; Zhao, M.; Wang, Y.; Tang, M.; Gong, M.; Liao, G.; Yuan, Y.; Li, L.; et al. Elevated Branched-Chain α-Keto Acids Exacerbate Macrophage Oxidative Stress and Chronic Inflammatory Damage in Type 2 Diabetes Mellitus. *Free Radic. Biol. Med.* **2021**, *175*, 141–154. [CrossRef]

69. Zhang, H.; DiBaise, J.K.; Zuccolo, A.; Kudrna, D.; Braidotti, M.; Yu, Y.; Parameswaran, P.; Crowell, M.D.; Wing, R.; Rittmann, B.E.; et al. Human Gut Microbiota in Obesity and after Gastric Bypass. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 2365–2370. [CrossRef]
70. Yoshida, N.; Yamashita, T.; Osone, T.; Hosooka, T.; Shinohara, M.; Kitahama, S.; Sasaki, K.; Sasaki, D.; Yoneshiro, T.; Suzuki, T.; et al. Bacteroides Spp. Promotes Branched-Chain Amino Acid Catabolism in Brown Fat and Inhibits Obesity. *iScience* **2021**, *24*, 103342. [CrossRef]
71. Chiang, J.Y.L. Bile Acid Metabolism and Signaling. In *Comprehensive Physiology*; Wiley: Hoboken, NJ, USA, 2013; pp. 1191–1212.
72. Yin, C.; Zhong, R.; Zhang, W.; Liu, L.; Chen, L.; Zhang, H. The Potential of Bile Acids as Biomarkers for Metabolic Disorders. *Int. J. Mol. Sci.* **2023**, *24*, 12123. [CrossRef]
73. Winston, J.A.; Theriot, C.M. Diversification of Host Bile Acids by Members of the Gut Microbiota. *Gut Microbes* **2020**, *11*, 158–171. [CrossRef] [PubMed]
74. Duboc, H.; Rajca, S.; Rainteau, D.; Benarous, D.; Maubert, M.-A.; Quervain, E.; Thomas, G.; Barbu, V.; Humbert, L.; Despras, G.; et al. Connecting Dysbiosis, Bile-Acid Dysmetabolism and Gut Inflammation in Inflammatory Bowel Diseases. *Gut* **2013**, *62*, 531–539. [CrossRef] [PubMed]
75. Sommer, F.; Anderson, J.M.; Bharti, R.; Raes, J.; Rosenstiel, P. The Resilience of the Intestinal Microbiota Influences Health and Disease. *Nat. Rev. Microbiol.* **2017**, *15*, 630–638. [CrossRef] [PubMed]
76. Barrasa, J.I.; Olmo, N.; Lizarbe, M.A.; Turnay, J. Bile Acids in the Colon, from Healthy to Cytotoxic Molecules. *Toxicol. Vitr.* **2013**, *27*, 964–977. [CrossRef]
77. Thomas, C.; Gioiello, A.; Noriega, L.; Strehle, A.; Oury, J.; Rizzo, G.; Macchiarulo, A.; Yamamoto, H.; Mataki, C.; Pruzanski, M.; et al. TGR5-Mediated Bile Acid Sensing Controls Glucose Homeostasis. *Cell Metab.* **2009**, *10*, 167–177. [CrossRef]
78. Trabelsi, M.-S.; Daoudi, M.; Prawitt, J.; Ducastel, S.; Touche, V.; Sayin, S.I.; Perino, A.; Brighton, C.A.; Sebti, Y.; Kluza, J.; et al. Farnesoid X Receptor Inhibits Glucagon-like Peptide-1 Production by Enteroendocrine L Cells. *Nat. Commun.* **2015**, *6*, 7629. [CrossRef]
79. An, C.; Chon, H.; Ku, W.; Eom, S.; Seok, M.; Kim, S.; Lee, J.; Kim, D.; Lee, S.; Koo, H.; et al. Bile Acids: Major Regulator of the Gut Microbiome. *Microorganisms* **2022**, *10*, 1792. [CrossRef]
80. Hyland, N.P.; Cavanaugh, C.R.; Hornby, P.J. Emerging Effects of Tryptophan Pathway Metabolites and Intestinal Microbiota on Metabolism and Intestinal Function. *Amino Acids* **2022**, *54*, 57–70. [CrossRef]
81. Agus, A.; Clément, K.; Sokol, H. Gut Microbiota-Derived Metabolites as Central Regulators in Metabolic Disorders. *Gut* **2021**, *70*, 1174–1182. [CrossRef]
82. Chimerel, C.; Emery, E.; Summers, D.K.; Keyser, U.; Gribble, F.M.; Reimann, F. Bacterial Metabolite Indole Modulates Incretin Secretion from Intestinal Enteroendocrine L Cells. *Cell Rep.* **2014**, *9*, 1202–1208. [CrossRef]
83. Sehgal, R.; de Mello, V.D.; Männistö, V.; Lindström, J.; Tuomilehto, J.; Pihlajamäki, J.; Uusitupa, M. Indolepropionic Acid, a Gut Bacteria-Produced Tryptophan Metabolite and the Risk of Type 2 Diabetes and Non-Alcoholic Fatty Liver Disease. *Nutrients* **2022**, *14*, 4695. [CrossRef] [PubMed]
84. Tang, W.H.W.; Wang, Z.; Li, X.S.; Fan, Y.; Li, D.S.; Wu, Y.; Hazen, S.L. Increased Trimethylamine N-Oxide Portends High Mortality Risk Independent of Glycemic Control in Patients with Type 2 Diabetes Mellitus. *Clin. Chem.* **2017**, *63*, 297–306. [CrossRef] [PubMed]
85. Gao, X.; Liu, X.; Xu, J.; Xue, C.; Xue, Y.; Wang, Y. Dietary Trimethylamine N-Oxide Exacerbates Impaired Glucose Tolerance in Mice Fed a High Fat Diet. *J. Biosci. Bioeng.* **2014**, *118*, 476–481. [CrossRef]
86. Cho, C.E.; Taesuwan, S.; Malysheva, O.V.; Bender, E.; Tulchinsky, N.F.; Yan, J.; Sutter, J.L.; Caudill, M.A. Trimethylamine-*N*.-oxide (TMAO) Response to Animal Source Foods Varies among Healthy Young Men and Is Influenced by Their Gut Microbiota Composition: A Randomized Controlled Trial. *Mol. Nutr. Food Res.* **2017**, *61*, 1600324. [CrossRef] [PubMed]
87. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. *Nat. Med.* **2013**, *19*, 576–585. [CrossRef]
88. Montandon, S.; Jornayvaz, F. Effects of Antidiabetic Drugs on Gut Microbiota Composition. *Genes.* **2017**, *8*, 250. [CrossRef] [PubMed]
89. Zhang, X.; Fang, Z.; Zhang, C.; Xia, H.; Jie, Z.; Han, X.; Chen, Y.; Ji, L. Effects of Acarbose on the Gut Microbiota of Prediabetic Patients: A Randomized, Double-Blind, Controlled Crossover Trial. *Diabetes Ther.* **2017**, *8*, 293–307. [CrossRef] [PubMed]
90. Zhang, M.; Feng, R.; Yang, M.; Qian, C.; Wang, Z.; Liu, W.; Ma, J. Effects of Metformin, Acarbose, and Sitagliptin Monotherapy on Gut Microbiota in Zucker Diabetic Fatty Rats. *BMJ Open Diabetes Res. Care* **2019**, *7*, e000717. [CrossRef]
91. Ying, X.; Rongjiong, Z.; Kahaer, M.; Chunhui, J.; Wulasihan, M. Therapeutic Efficacy of Liraglutide versus Metformin in Modulating the Gut Microbiota for Treating Type 2 Diabetes Mellitus Complicated with Nonalcoholic Fatty Liver Disease. *Front. Microbiol.* **2023**, *14*, 1088187. [CrossRef]
92. Liu, B.; Zhang, L.; Yang, H.; Zheng, H.; Liao, X. Microbiota: A Potential Orchestrator of Antidiabetic Therapy. *Front. Endocrinol.* **2023**, *14*, 973624. [CrossRef]

93. Tsai, C.-Y.; Lu, H.-C.; Chou, Y.-H.; Liu, P.-Y.; Chen, H.-Y.; Huang, M.-C.; Lin, C.-H.; Tsai, C.-N. Gut Microbial Signatures for Glycemic Responses of GLP-1 Receptor Agonists in Type 2 Diabetic Patients: A Pilot Study. *Front. Endocrinol.* **2022**, *12*, 814770. [CrossRef] [PubMed]
94. Gropper, S.S. The Role of Nutrition in Chronic Disease. *Nutrients* **2023**, *15*, 664. [CrossRef] [PubMed]
95. Merrill, J.D.; Soliman, D.; Kumar, N.; Lim, S.; Shariff, A.I.; Yancy, W.S. Low-Carbohydrate and Very-Low-Carbohydrate Diets in Patients with Diabetes. *Diabetes Spectr.* **2020**, *33*, 133–142. [CrossRef] [PubMed]
96. Riddle, M.C.; Cefalu, W.T.; Evans, P.H.; Gerstein, H.C.; Nauck, M.A.; Oh, W.K.; Rothberg, A.E.; le Roux, C.W.; Rubino, F.; Schauer, P.; et al. Consensus Report: Definition and Interpretation of Remission in Type 2 Diabetes. *J. Clin. Endocrinol. Metab.* **2022**, *107*, 1–9. [CrossRef] [PubMed]
97. Lean, M.E.; Leslie, W.S.; Barnes, A.C.; Brosnahan, N.; Thom, G.; McCombie, L.; Peters, C.; Zhyzhneuskaya, S.; Al-Mrabeh, A.; Hollingsworth, K.G.; et al. Primary Care-Led Weight Management for Remission of Type 2 Diabetes (DiRECT): An Open-Label, Cluster-Randomised Trial. *Lancet* **2018**, *391*, 541–551. [CrossRef]
98. Taheri, S.; Zaghloul, H.; Chagoury, O.; Elhadad, S.; Ahmed, S.H.; El Khatib, N.; Amona, R.A.; El Nahas, K.; Suleiman, N.; Alnaama, A.; et al. Effect of Intensive Lifestyle Intervention on Bodyweight and Glycaemia in Early Type 2 Diabetes (DIADEM-I): An Open-Label, Parallel-Group, Randomised Controlled Trial. *Lancet Diabetes Endocrinol.* **2020**, *8*, 477–489. [CrossRef]
99. Thom, G.; Messow, C.-M.; Leslie, W.S.; Barnes, A.C.; Brosnahan, N.; McCombie, L.; Al-Mrabeh, A.; Zhyzhneuskaya, S.; Welsh, P.; Sattar, N.; et al. Predictors of Type 2 Diabetes Remission in the Diabetes Remission Clinical Trial (DiRECT). *Diabet. Med.* **2021**, *38*, e14395. [CrossRef]
100. Unwin, D.; Delon, C.; Unwin, J.; Tobin, S.; Taylor, R. What Predicts Drug-Free Type 2 Diabetes Remission? Insights from an 8-Year General Practice Service Evaluation of a Lower Carbohydrate Diet with Weight Loss. *BMJ Nutr. Prev. Health* **2023**, *6*, 46–55. [CrossRef]
101. Hallberg, S.J.; McKenzie, A.L.; Williams, P.T.; Bhanpuri, N.H.; Peters, A.L.; Campbell, W.W.; Hazbun, T.L.; Volk, B.M.; McCarter, J.P.; Phinney, S.D.; et al. Effectiveness and Safety of a Novel Care Model for the Management of Type 2 Diabetes at 1 Year: An Open-Label, Non-Randomized, Controlled Study. *Diabetes Ther.* **2018**, *9*, 583–612. [CrossRef]
102. Aksoy, A.N.; Abayomi, J.; Relph, N.; Butler, T. Physiological and Psychological Determinants of Long-term Diet-induced Type 2 Diabetes (T2DM) Remission: A Narrative Review. *Obes. Rev.* **2024**, *25*, e13733. [CrossRef]
103. Juray, S.; Axen, K.V.; Trasino, S.E. Remission of Type 2 Diabetes with Very Low-Calorie Diets—A Narrative Review. *Nutrients* **2021**, *13*, 2086. [CrossRef] [PubMed]
104. Steven, S.; Hollingsworth, K.G.; Al-Mrabeh, A.; Avery, L.; Aribisala, B.; Caslake, M.; Taylor, R. Very Low-Calorie Diet and 6 Months of Weight Stability in Type 2 Diabetes: Pathophysiological Changes in Responders and Nonresponders. *Diabetes Care* **2016**, *39*, 808–815. [CrossRef]
105. van den Burg, E.L.; Schoonakker, M.P.; van Peet, P.G.; van den Akker-van Marle, E.M.; Lamb, H.J.; Longo, V.D.; Numans, M.E.; Pijl, H. Integration of a Fasting-Mimicking Diet Programme in Primary Care for Type 2 Diabetes Reduces the Need for Medication and Improves Glycaemic Control: A 12-Month Randomised Controlled Trial. *Diabetologia* **2024**, *67*, 1245–1259. [CrossRef] [PubMed]
106. Xiao, Y.; Xiao, X.; Zhang, X.; Yi, D.; Li, T.; Hao, Q.; Zhang, F.; Li, X.; Wang, N. Mediterranean Diet in the Targeted Prevention and Personalized Treatment of Chronic Diseases: Evidence, Potential Mechanisms, and Prospects. *EPMA J.* **2024**, *15*, 207–220. [CrossRef]
107. Cho, S.S.; Qi, L.; Fahey Jr, G.C.; Klurfeld, D.M. Consumption of Cereal Fiber, Mixtures of Whole Grains and Bran, and Whole Grains and Risk Reduction in Type 2 Diabetes, Obesity, and Cardiovascular Disease. *Am. J. Clin. Nutr.* **2013**, *98*, 594–619. [CrossRef] [PubMed]
108. Victor, V.M.; Rocha, M.; Herance, R.; Hernandez-Mijares, A. Oxidative Stress and Mitochondrial Dysfunction in Type 2 Diabetes. *Curr. Pharm. Des.* **2011**, *17*, 3947–3958. [CrossRef]
109. Shen, J.; Wilmot, K.A.; Ghasemzadeh, N.; Molloy, D.L.; Burkman, G.; Mekonnen, G.; Gongora, M.C.; Quyyumi, A.A.; Sperling, L.S. Mediterranean Dietary Patterns and Cardiovascular Health. *Annu. Rev. Nutr.* **2015**, *35*, 425–449. [CrossRef]
110. Georgoulis, M.; Kontogianni, M.; Yiannakouris, N. Mediterranean Diet and Diabetes: Prevention and Treatment. *Nutrients* **2014**, *6*, 1406–1423. [CrossRef]
111. Filippatos, T.D.; Panagiotakos, D.B.; Georgousopoulou, E.N.; Pitaraki, E.; Kouli, G.-M.; Chrysohoou, C.; Tousoulis, D.; Stefanadis, C.; Pitsavos, C. Mediterranean Diet and 10-Year (2002–2012) Incidence of Diabetes and Cardiovascular Disease in Participants with Prediabetes: The ATTICA Study. *Rev. Diabet. Stud.* **2016**, *13*, 226–235. [CrossRef]
112. Schwingshackl, L.; Missbach, B.; König, J.; Hoffmann, G. Adherence to a Mediterranean Diet and Risk of Diabetes: A Systematic Review and Meta-Analysis. *Public Health Nutr.* **2015**, *18*, 1292–1299. [CrossRef]
113. Tobias, D.K.; Hu, F.B.; Chavarro, J.; Rosner, B.; Mozaffarian, D.; Zhang, C. Healthful Dietary Patterns and Type 2 Diabetes Mellitus Risk Among Women with a History of Gestational Diabetes Mellitus. *Arch. Intern. Med.* **2012**, *172*, 1566. [CrossRef] [PubMed]

114. Donati Zeppa, S.; Natalucci, V.; Agostini, D.; Vallorani, L.; Amatori, S.; Sisti, D.; Rocchi, M.B.L.; Pazienza, V.; Perri, F.; Villani, A.; et al. Changes in Gut Microbiota Composition after 12 Weeks of a Home-Based Lifestyle Intervention in Breast Cancer Survivors during the COVID-19 Lockdown. *Front. Oncol.* **2023**, *13*, 1225645. [CrossRef] [PubMed]
115. Serpil Ozsoy, N.S.T.S. The Role of Mediterranean Diet and Gut Microbiota in Type-2 Diabetes Mellitus Associated with Obesity (Diabesity). *eCollection* **2022**, *63*, E87.
116. Wang, D.D.; Qi, Q.; Wang, Z.; Usyk, M.; Sotres-Alvarez, D.; Mattei, J.; Tamez, M.; Gellman, M.D.; Daviglus, M.; Hu, F.B.; et al. The Gut Microbiome Modifies the Association Between a Mediterranean Diet and Diabetes in USA Hispanic/ Latino Population. *J. Clin. Endocrinol. Metab.* **2022**, *107*, e924–e934. [CrossRef]
117. Nagpal, R.; Shively, C.A.; Register, T.C.; Craft, S.; Yadav, H. Gut Microbiome-Mediterranean Diet Interactions in Improving Host Health. *F1000Research* **2019**, *8*, 699. [CrossRef] [PubMed]
118. Yokoyama, Y.; Barnard, N.D.; Levin, S.M.; Watanabe, M. Vegetarian Diets and Glycemic Control in Diabetes: A Systematic Review and Meta-Analysis. *Cardiovasc. Diagn. Ther.* **2014**, *4*, 373.
119. Panigrahi, G.; Goodwin, S.M.; Staffier, K.L.; Karlsen, M. Remission of Type 2 Diabetes After Treatment with a High-Fiber, Low-Fat, Plant-Predominant Diet Intervention: A Case Series. *Am. J. Lifestyle Med.* **2023**, *17*, 839–846. [CrossRef]
120. Yang, T.; Wu, C.; Li, Y.; Wang, C.; Mao, Z.; Huo, W.; Li, J.; Li, Y.; Xing, W.; Li, L. Association of Short-Chain Fatty Acids and the Gut Microbiome with Type 2 Diabetes: Evidence from the Henan Rural Cohort. *Nutr. Metab. Cardiovasc. Dis.* **2024**, *34*, 1619–1630. [CrossRef]
121. Huda, M.N.; Kim, M.; Bennett, B.J. Modulating the Microbiota as a Therapeutic Intervention for Type 2 Diabetes. *Front. Endocrinol.* **2021**, *12*, 632335. [CrossRef]
122. Higgins, J.A. Whole Grains, Legumes, and the Subsequent Meal Effect: Implications for Blood Glucose Control and the Role of Fermentation. *J. Nutr. Metab.* **2012**, *2012*, 829238. [CrossRef]
123. Kim, Y.A.; Keogh, J.B.; Clifton, P.M. Probiotics, Prebiotics, Synbiotics and Insulin Sensitivity. *Nutr. Res. Rev.* **2018**, *31*, 35–51. [CrossRef] [PubMed]
124. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. The International Scientific Association for Probiotics and Prebiotics Consensus Statement on the Scope and Appropriate Use of the Term Probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef]
125. Panwar, H.; Rashmi, H.M.; Batish, V.K.; Grover, S. Probiotics as Potential Biotherapeutics in the Management of Type 2 Diabetes—Prospects and Perspectives. *Diabetes Metab. Res. Rev.* **2013**, *29*, 103–112. [CrossRef] [PubMed]
126. Liu, Q.; Yu, Z.; Tian, F.; Zhao, J.; Zhang, H.; Zhai, Q.; Chen, W. Surface Components and Metabolites of Probiotics for Regulation of Intestinal Epithelial Barrier. *Microb. Cell Fact.* **2020**, *19*, 23. [CrossRef] [PubMed]
127. Salgaço, M.K.; Oliveira, L.G.S.; Costa, G.N.; Bianchi, F.; Sivieri, K. Relationship between Gut Microbiota, Probiotics, and Type 2 Diabetes Mellitus. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 9229–9238. [CrossRef]
128. Ma, D.; Forsythe, P.; Bienenstock, J. Live *Lactobacillus reuteri* Is Essential for the Inhibitory Effect on Tumor Necrosis Factor Alpha-Induced Interleukin-8 Expression. *Infect. Immun.* **2004**, *72*, 5308–5314. [CrossRef]
129. Tonucci, L.B.; Olbrich dos Santos, K.M.; Licursi de Oliveira, L.; Rocha Ribeiro, S.M.; Duarte Martino, H.S. Clinical Application of Probiotics in Type 2 Diabetes Mellitus: A Randomized, Double-Blind, Placebo-Controlled Study. *Clin. Nutr.* **2017**, *36*, 85–92. [CrossRef]
130. Everard, A.; Belzer, C.; Geurts, L.; Ouwerkerk, J.P.; Druart, C.; Bindels, L.B.; Guiot, Y.; Derrien, M.; Muccioli, G.G.; Delzenne, N.M.; et al. Cross-Talk between *Akkermansia muciniphila* and Intestinal Epithelium Controls Diet-Induced Obesity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9066–9071. [CrossRef]
131. Yadav, H.; Lee, J.-H.; Lloyd, J.; Walter, P.; Rane, S.G. Beneficial Metabolic Effects of a Probiotic via Butyrate-Induced GLP-1 Hormone Secretion. *J. Biol. Chem.* **2013**, *288*, 25088–25097. [CrossRef]
132. Pegah, A.; Abbasi-Oshaghi, E.; Khodadadi, I.; Mirzaei, F.; Tayebinia, H. Probiotic and Resveratrol Normalize GLP-1 Levels and Oxidative Stress in the Intestine of Diabetic Rats. *Metabol. Open* **2021**, *10*, 100093. [CrossRef]
133. Han, X.; Wang, Y.; Zhang, P.; Zhu, M.; Li, L.; Mao, X.; Sha, X.; Li, L. Kazak Faecal Microbiota Transplantation Induces Short-Chain Fatty Acids That Promote Glucagon-like Peptide-1 Secretion by Regulating Gut Microbiota in *Db/Db* Mice. *Pharm. Biol.* **2021**, *59*, 1075–1085. [CrossRef] [PubMed]
134. Shen, X.; Ma, C.; Yang, Y.; Liu, X.; Wang, B.; Wang, Y.; Zhang, G.; Bian, X.; Zhang, N. The Role and Mechanism of Probiotics Supplementation in Blood Glucose Regulation: A Review. *Foods* **2024**, *13*, 2719. [CrossRef] [PubMed]
135. Zhang, X.; Zhang, B.; Li, L.; Li, X.; Zhang, J.; Chen, G. Fermented Noni (*Morinda citrifolia* L.) Fruit Juice Improved Oxidative Stress and Insulin Resistance under the Synergistic Effect of Nrf2/ARE Pathway and Gut Flora in Db/Db Mice and HepG2 Cells. *Food Funct.* **2022**, *13*, 8254–8273. [CrossRef] [PubMed]
136. Cardona, F.; Andrés-Lacueva, C.; Tulipani, S.; Tinahones, F.J.; Queipo-Ortuño, M.I. Benefits of Polyphenols on Gut Microbiota and Implications in Human Health. *J. Nutr. Biochem.* **2013**, *24*, 1415–1422. [CrossRef] [PubMed]

137. Guasch-Ferré, M.; Merino, J.; Sun, Q.; Fitó, M.; Salas-Salvadó, J. Dietary Polyphenols, Mediterranean Diet, Prediabetes, and Type 2 Diabetes: A Narrative Review of the Evidence. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 6723931. [CrossRef]
138. Pérez-Burillo, S.; Navajas-Porras, B.; López-Maldonado, A.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Green Tea and Its Relation to Human Gut Microbiome. *Molecules* **2021**, *26*, 3907. [CrossRef]
139. Zheng, X.-X.; Xu, Y.-L.; Li, S.-H.; Hui, R.; Wu, Y.-J.; Huang, X.-H. Effects of Green Tea Catechins with or without Caffeine on Glycemic Control in Adults: A Meta-Analysis of Randomized Controlled Trials. *Am. J. Clin. Nutr.* **2013**, *97*, 750–762. [CrossRef]
140. Tung, Y.-T.; Zeng, J.-L.; Ho, S.-T.; Xu, J.-W.; Lin, I.-H.; Wu, J.-H. Djulis Hull Improves Insulin Resistance and Modulates the Gut Microbiota in High-Fat Diet (HFD)-Induced Hyperglycaemia. *Antioxidants* **2021**, *11*, 45. [CrossRef]
141. Ye, J.; Ma, J.; Rozi, P.; Kong, L.; Zhou, J.; Luo, Y.; Yang, H. The Polysaccharides from Seeds of Glycyrrhiza Uralensis Ameliorate Metabolic Disorders and Restructure Gut Microbiota in Type 2 Diabetic Mice. *Int. J. Biol. Macromol.* **2024**, *264*, 130622. [CrossRef]
142. Huang, X.; Qiu, Y.; Gao, Y.; Zhou, R.; Hu, Q.; He, Z.; Lv, Y.; Wang, X.; Chen, W.; Deng, Y.; et al. Gut Microbiota Mediate Melatonin Signalling in Association with Type 2 Diabetes. *Diabetologia* **2022**, *65*, 1627–1641. [CrossRef]
143. Medina-Vera, I.; Sanchez-Tapia, M.; Noriega-López, L.; Granados-Portillo, O.; Guevara-Cruz, M.; Flores-López, A.; Avila-Nava, A.; Fernández, M.L.; Tovar, A.R.; Torres, N. A Dietary Intervention with Functional Foods Reduces Metabolic Endotoxaemia and Attenuates Biochemical Abnormalities by Modifying Faecal Microbiota in People with Type 2 Diabetes. *Diabetes Metab.* **2019**, *45*, 122–131. [CrossRef] [PubMed]
144. Wang, J.; Ma, Q.; Li, Y.; Li, P.; Wang, M.; Wang, T.; Wang, C.; Wang, T.; Zhao, B. Research Progress on Traditional Chinese Medicine Syndromes of Diabetes Mellitus. *Biomed. Pharmacother.* **2020**, *121*, 109565. [CrossRef] [PubMed]
145. He, L.; Yang, F.-Q.; Tang, P.; Gao, T.-H.; Yang, C.-X.; Tan, L.; Yue, P.; Hua, Y.-N.; Liu, S.-J.; Guo, J.-L. Regulation of the Intestinal Flora: A Potential Mechanism of Natural Medicines in the Treatment of Type 2 Diabetes Mellitus. *Biomed. Pharmacother.* **2022**, *151*, 113091. [CrossRef] [PubMed]
146. Pan, T.; Zheng, S.; Zheng, W.; Shi, C.; Ning, K.; Zhang, Q.; Xie, Y.; Xiang, H.; Xie, Q. Christensenella Regulated by Huang-Qi-Ling-Hua-San Is a Key Factor by Which to Improve Type 2 Diabetes. *Front. Microbiol.* **2022**, *13*, 1022403. [CrossRef]
147. Yang, T.; Zhou, W.; Xu, W.; Ran, L.; Yan, Y.; Lu, L.; Mi, J.; Zeng, X.; Cao, Y. Modulation of Gut Microbiota and Hypoglycemic/Hypolipidemic Activity of Flavonoids from the Fruits of *Lycium barbarum* on High-Fat Diet/Streptozotocin-Induced Type 2 Diabetic Mice. *Food Funct.* **2022**, *13*, 11169–11184. [CrossRef]
148. Wang, T.; Liao, H.; Lin, J.; Zhang, M.; Chen, B.; Yin, R.; Sun, J.; Dai, H.; Liu, H. Antidiabetic Action of the Chinese Formula Shouhuitongbian and the Underlying Mechanism Associated with Alteration of Gut Microbiota. *Phytomedicine* **2024**, *129*, 155575. [CrossRef]
149. Carbajo-Pescador, S.; Porras, D.; García-Mediavilla, M.V.; Martínez-Flórez, S.; Juarez-Fernández, M.; Cuevas, M.J.; Mauriz, J.L.; González-Gallego, J.; Nistal, E.; Sánchez-Campos, S. Beneficial Effects of Exercise on Gut Microbiota Functionality and Barrier Integrity, and Gut-Liver Crosstalk in an in Vivo Model of Early Obesity and Non-Alcoholic Fatty Liver Disease. *Dis. Model. Mech.* **2019**, *12*, dmm039206. [CrossRef]
150. Yu, C.; Liu, S.; Niu, Y.; Fu, L. Exercise Protects Intestinal Epithelial Barrier from High Fat Diet- Induced Permeabilization through SESN2/AMPKα1/HIF-1α Signaling. *J. Nutr. Biochem.* **2022**, *107*, 109059. [CrossRef]
151. Ro, S.-H.; Xue, X.; Ramakrishnan, S.K.; Cho, C.-S.; Namkoong, S.; Jang, I.; Semple, I.A.; Ho, A.; Park, H.-W.; Shah, Y.M.; et al. Tumor Suppressive Role of Sestrin2 during Colitis and Colon Carcinogenesis. *eLife* **2016**, *5*, e12204. [CrossRef]
152. Wu, D.; Cao, W.; Xiang, D.; Hu, Y.-P.; Luo, B.; Chen, P. Exercise Induces Tissue Hypoxia and HIF-1α Redistribution in the Small Intestine. *J. Sport Health Sci.* **2020**, *9*, 82–89. [CrossRef]
153. Shao, T.; Zhao, C.; Li, F.; Gu, Z.; Liu, L.; Zhang, L.; Wang, Y.; He, L.; Liu, Y.; Liu, Q.; et al. Intestinal HIF-1α Deletion Exacerbates Alcoholic Liver Disease by Inducing Intestinal Dysbiosis and Barrier Dysfunction. *J. Hepatol.* **2018**, *69*, 886–895. [CrossRef] [PubMed]
154. Kelly, C.J.; Glover, L.E.; Campbell, E.L.; Kominsky, D.J.; Ehrentraut, S.F.; Bowers, B.E.; Bayless, A.J.; Saeedi, B.J.; Colgan, S.P. Fundamental Role for HIF-1α in Constitutive Expression of Human β Defensin-1. *Mucosal Immunol.* **2013**, *6*, 1110–1118. [CrossRef] [PubMed]
155. Saeedi, B.J.; Kao, D.J.; Kitzenberg, D.A.; Dobrinskikh, E.; Schwisow, K.D.; Masterson, J.C.; Kendrick, A.A.; Kelly, C.J.; Bayless, A.J.; Kominsky, D.J.; et al. HIF-Dependent Regulation of Claudin-1 Is Central to Intestinal Epithelial Tight Junction Integrity. *Mol. Biol. Cell* **2015**, *26*, 2252–2262. [CrossRef]
156. Pan, Y.; Shao, D.; Zhao, Y.; Zhang, F.; Zheng, X.; Tan, Y.; He, K.; Li, J.; Chen, L. Berberine Reverses Hypoxia-Induced Chemoresistance in Breast Cancer through the Inhibition of AMPK-HIF-1α. *Int. J. Biol. Sci.* **2017**, *13*, 794–803. [CrossRef]
157. Sun, X.; Yang, Q.; Rogers, C.J.; Du, M.; Zhu, M.-J. AMPK Improves Gut Epithelial Differentiation and Barrier Function via Regulating Cdx2 Expression. *Cell Death Differ.* **2017**, *24*, 819–831. [CrossRef]
158. Lee, J.H.; Budanov, A.V.; Talukdar, S.; Park, E.J.; Park, H.L.; Park, H.-W.; Bandyopadhyay, G.; Li, N.; Aghajan, M.; Jang, I.; et al. Maintenance of Metabolic Homeostasis by Sestrin2 and Sestrin3. *Cell Metab.* **2012**, *16*, 311–321. [CrossRef] [PubMed]

159. Khaledi, M.; Darvishi, M.; Sameni, F.; Shahrjerdi, S.; Karami, E.; Barahui, N.; Hemmati, J.; Hasheminasab, M.S.; Sanae, M.-J.; Akhavan-Sigari, R.; et al. Association between Exercise and Changes in Gut Microbiota Profile: A Review. *Sport Sci. Health* **2024**, *20*, 273–286. [CrossRef]
160. Cullen, J.M.A.; Shahzad, S.; Dhillon, J. A Systematic Review on the Effects of Exercise on Gut Microbial Diversity, Taxonomic Composition, and Microbial Metabolites: Identifying Research Gaps and Future Directions. *Front. Physiol.* **2023**, *14*, 1292673. [CrossRef]
161. Ecklu-Mensah, G.; Choo-Kang, C.; Maseng, M.G.; Donato, S.; Bovet, P.; Viswanathan, B.; Bedu-Addo, K.; Plange-Rhule, J.; Oti Boateng, P.; Forrester, T.E.; et al. Gut Microbiota and Fecal Short Chain Fatty Acids Differ with Adiposity and Country of Origin: The METS-Microbiome Study. *Nat. Commun.* **2023**, *14*, 5160. [CrossRef]
162. Dziewiecka, H.; Buttar, H.S.; Kasperska, A.; Ostapiuk–Karolczuk, J.; Domagalska, M.; Cichoń, J.; Skarpańska-Stejnborn, A. Physical Activity Induced Alterations of Gut Microbiota in Humans: A Systematic Review. *BMC Sports Sci. Med. Rehabil.* **2022**, *14*, 122. [CrossRef]
163. Barton, W.; Penney, N.C.; Cronin, O.; Garcia-Perez, I.; Molloy, M.G.; Holmes, E.; Shanahan, F.; Cotter, P.D.; O'Sullivan, O. The Microbiome of Professional Athletes Differs from That of More Sedentary Subjects in Composition and Particularly at the Functional Metabolic Level. *Gut* **2017**, *67*, 625–633. [CrossRef] [PubMed]
164. Allen, J.M.; Mailing, L.J.; Niemiro, G.M.; Moore, R.; Cook, M.D.; White, B.A.; Holscher, H.D.; Woods, J.A. Exercise Alters Gut Microbiota Composition and Function in Lean and Obese Humans. *Med. Sci. Sports Exerc.* **2018**, *50*, 747–757. [CrossRef] [PubMed]
165. Ghanim, H.; Abuaysheh, S.; Sia, C.L.; Korzeniewski, K.; Chaudhuri, A.; Fernandez-Real, J.M.; Dandona, P. Increase in Plasma Endotoxin Concentrations and the Expression of Toll-Like Receptors and Suppressor of Cytokine Signaling-3 in Mononuclear Cells After a High-Fat, High-Carbohydrate Meal. *Diabetes Care* **2009**, *32*, 2281–2287. [CrossRef] [PubMed]
166. Cani, P.D.; Bibiloni, R.; Knauf, C.; Waget, A.; Neyrinck, A.M.; Delzenne, N.M.; Burcelin, R. Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. *Diabetes* **2008**, *57*, 1470–1481. [CrossRef] [PubMed]
167. Zhang, Y.; Wan, J.; Xu, Z.; Hua, T.; Sun, Q. Exercise ameliorates insulin resistance via regulating TGFβ-activated kinase 1 (TAK1)-mediated insulin signaling in liver of high-fat diet-induced obese rats. *J. Cell. Physiol.* **2019**, *234*, 7467–7474. [CrossRef]
168. Gholamnezhad, Z.; Safarian, B.; Esparham, A.; Mirzaei, M.; Esmaeilzadeh, M.; Boskabady, M.H. The Modulatory Effects of Exercise on Lipopolysaccharide-Induced Lung Inflammation and Injury: A Systemic Review. *Life Sci.* **2022**, *293*, 120306. [CrossRef]
169. Khoshkhouy, F.; Farshbaf, A.; Mahmoudabady, M.; Gholamnezhad, Z. Effects of Moderate Exercise on Lipopolysaccharide-Induced Inflammatory Responses in Rat's Cardiac Tissue. *Cytokine* **2021**, *138*, 155409. [CrossRef]
170. Evans, C.C.; LePard, K.J.; Kwak, J.W.; Stancukas, M.C.; Laskowski, S.; Dougherty, J.; Moulton, L.; Glawe, A.; Wang, Y.; Leone, V.; et al. Exercise Prevents Weight Gain and Alters the Gut Microbiota in a Mouse Model of High Fat Diet-Induced Obesity. *PLoS ONE* **2014**, *9*, e92193. [CrossRef]
171. Donati Zeppa, S.; Amatori, S.; Sisti, D.; Gervasi, M.; Agostini, D.; Piccoli, G.; Pazienza, V.; Gobbi, P.; Rocchi, M.B.L.; Sestili, P.; et al. Nine Weeks of High-Intensity Indoor Cycling Training Induced Changes in the Microbiota Composition in Non-Athlete Healthy Male College Students. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 74. [CrossRef]
172. Donati Zeppa, S.; Agostini, D.; Gervasi, M.; Annibalini, G.; Amatori, S.; Ferrini, F.; Sisti, D.; Piccoli, G.; Barbieri, E.; Sestili, P.; et al. Mutual Interactions among Exercise, Sport Supplements and Microbiota. *Nutrients* **2019**, *12*, 17. [CrossRef]
173. Mariat, D.; Firmesse, O.; Levenez, F.; Guimarăes, V.; Sokol, H.; Doré, J.; Corthier, G.; Furet, J.-P. The Firmicutes/Bacteroidetes Ratio of the Human Microbiota Changes with Age. *BMC Microbiol.* **2009**, *9*, 123. [CrossRef] [PubMed]
174. Argyridou, S.; Bernieh, D.; Henson, J.; Edwardson, C.L.; Davies, M.J.; Khunti, K.; Suzuki, T.; Yates, T. Associations between Physical Activity and Trimethylamine *N.* -Oxide in Those at Risk of Type 2 Diabetes. *BMJ Open Diabetes Res. Care* **2020**, *8*, e001359. [CrossRef] [PubMed]
175. Battillo, D.J.; Malin, S.K. Impact of Caloric Restriction and Exercise on Trimethylamine N-Oxide Metabolism in Women with Obesity. *Nutrients* **2023**, *15*, 1455. [CrossRef] [PubMed]
176. Erickson, M.; Malin, S.; Wang, Z.; Brown, J.; Hazen, S.; Kirwan, J. Effects of Lifestyle Intervention on Plasma Trimethylamine N-Oxide in Obese Adults. *Nutrients* **2019**, *11*, 179. [CrossRef]
177. Shchendrygina, A.; Rakisheva, A.; Giverts, I.; Rustamova, Y.; Soloveva, A. Effects of Glucagon-like Peptide-1 Receptor Agonists on Cardiac Function, Exercise Capacity and Quality of Life. *Card. Fail. Rev.* **2024**, *10*, e10. [CrossRef]
178. Ammar, O.F.; Sharma, K.; Liperis, G.; Fraire-Zamora, J.J.; Serdarogullari, M.; Ali, Z.E.; Ramasamy, R.; Laurentino, S.; Watkins, A.; Mincheva, M. Balancing the Scales: The Interplay of Diet, Exercise, GLP-1 Receptor Agonists, and Obesity in Shaping Male Reproductive Health. *Hum. Reprod.* **2023**, *38*, 1649–1653. [CrossRef] [PubMed]
179. Zhao, X.; Wang, M.; Wen, Z.; Lu, Z.; Cui, L.; Fu, C.; Xue, H.; Liu, Y.; Zhang, Y. GLP-1 Receptor Agonists: Beyond Their Pancreatic Effects. *Front. Endocrinol.* **2021**, *12*, 721135. [CrossRef]
180. Nejati, R.; Bijeh, N.; Rad, M.M.; Hosseini, S.R.A. The Impact of Different Modes of Exercise Training on GLP-1: A Systematic Review and Meta-Analysis Research. *Int. J. Diabetes Dev. Ctries.* **2022**, *42*, 40–48. [CrossRef]

181. Hamasaki, H. Exercise and Glucagon-like Peptide-1: Does Exercise Potentiate the Effect of Treatment? *World J. Diabetes* **2018**, *9*, 138–140. [CrossRef]
182. Sandsdal, R.M.; Juhl, C.R.; Jensen, S.B.K.; Lundgren, J.R.; Janus, C.; Blond, M.B.; Rosenkilde, M.; Bogh, A.F.; Gliemann, L.; Jensen, J.-E.B.; et al. Combination of Exercise and GLP-1 Receptor Agonist Treatment Reduces Severity of Metabolic Syndrome, Abdominal Obesity, and Inflammation: A Randomized Controlled Trial. *Cardiovasc. Diabetol.* **2023**, *22*, 41. [CrossRef]

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Twelve Months of Time-Restricted Feeding Improves Cognition and Alters Microbiome Composition Independent of Macronutrient Composition

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Abstract: Declining health, gut dysbiosis, and cognitive impairments are hallmarks of advanced age. While caloric restriction is known to robustly extend the healthspan and alter gut microbiome composition, it is difficult maintain. Time-restricted feeding or changes in dietary macronutrient composition could be feasible alternatives for enhancing late life cognitive and physical health that are easier to comply with for extended periods of time. To investigate this possibility, 8-month-old rats were placed on time-restricted feeding with a ketogenic or micronutrient- and calorically matched control diet for 13 months. A third group of rats was permitted to eat standard chow ad libitum during this time. At 22 months, all rats were tested on a biconditional association task and fecal samples were collected for microbiome composition analysis. Regardless of dietary composition, time-restricted-fed rats had better cognitive performance than ad libitum-fed rats. This observation could not be accounted for by differences in motivation, procedural or sensorimotor impairments. Additionally, there were significant differences in gut microbiome diversity and composition between all diet conditions. Allobaculum abundance was associated with cognitive task performance, indicating a link between gut health and cognitive outcomes in aged subjects. Overall, time restricted feeding had the largest influence on cognitive performance in aged rats.

Keywords: cognitive aging; dual tasking; gut–brain-axis; intermittent fasting; metabolism

1. Introduction

Two prominent hallmarks of advancing age are declining peripheral health and impaired cognitive function, which can bi-directionally influence each other [1]. Caloric restriction, which has been shown to increase lifespan in several species, has been posited to increase healthspan and cognitive function [2] as well, although the data on the latter are equivocal [3]. The difficulty of maintaining long-term caloric restriction in humans, however, limits the translational potential of this lifestyle intervention for improving cognitive and physical function in older adults. Importantly, both time-restricted feeding (which is comparable to intermittent fasting) [4] and nutritional ketosis [5] mimic several aspects of caloric restriction and may confer health benefits to aged populations while imposing less severe dietary restrictions. Diet-based interventions may also alter the gut microbiome, which could directly influence brain function through the gut–brain axis, which is comprised of a multitude of pathways and interactions between the central nervous system and the gut. Specifically, ketogenic diets have been shown to influence gut microbiome abundance and diversity [6–11], which are also altered by advanced age [12].

A previous study reported that >3 months of nutritional ketosis, when initiated in aged rats (>21 months old), resulted in improved cognitive function on a biconditional association task compared to rats that were on a standard carbohydrate-based control diet. Importantly, both diets had equivalent caloric and micronutrient content and were given with a time-restricted feeding regimen once per day. Prior to initiating experimental diets, the aged animals in this previous study had metabolic impairments, including hyperinsulinemia and excess visceral white adipose tissue that was not evident in the young animals and reversed by nutritional ketosis, but not the standard diet after 4 weeks [13]. The possibility therefore exists that diet-induced cognitive benefits of ketosis are directly related to the efficacy of reversing metabolic deficits rather than due to the elevation of ketone bodies directly improving brain function. As the long-term carbohydrate restriction that is necessary to maintain nutritional ketosis poses huge barriers for extended compliance in community dwelling older adults [14], it is critical to determine the mechanisms by which this diet confers cognitive resilience. Different dietary paradigms that may increase compliance and therefore efficacy, such as time restricted feeding rather than restriction of either calories or macronutrient content. Specifically, if dietary interventions are initiated in mid-life, prior to the development of metabolic dysfunction, does ketosis still show the same benefits on cognitive function in old age compared to a standard diet. Importantly, mid-life is a critical time point for intervention, as this is likely when early pathology associated with sporadic Alzheimer's disease and related dementias is detectable but has not yet produced cognitive impairment (e.g., [15,16]).

The goal of this study was to therefore investigate whether long-term time-restricted feeding initiated in adulthood could improve cognitive outcomes in advanced age, and the extent to which this interacts with macronutrient composition. Two groups of rats were placed on a time-restricted feeding regimen beginning at 8 months of age. These rats were given ~51 kcal of food once daily. All animals consumed the full ration of calories within 3 h, resulting in ~21 h of fasting [13]. Among the rats given time-restricted feeding, one group was fed a ketogenic diet, while the other group was fed a micronutrient and calorically equivalent control diet [17]. A third group of rats was fed ad libitum until 21 months of age, at which time they were fed standard rodent chow once daily to encourage appetitively motivated participation in cognitive testing. A previous study has reported that rats of the Fischer 344 x Brown Norway hybrid strain develop hyperinsulinemia and metabolic impairments when allowed unrestricted access to standard laboratory rodent chow from adulthood into old age [13].

In old age, all rats were tested on a biconditional associated task (BAT), which quantifies an animal's ability to cognitively multitask by simultaneously alternating between two different arms of a maze while completing a bi-conditional object discrimination. Specifically, the correct choice of the target object depends on the animal's location on the maze with each object only being rewarded in one of the two arms (Figure 1). Performance on this type of object-place paired associative learning task has repeatedly been shown to decline with age in rats [18–21], and has greater sensitivity for detecting age-related impairments than the Morris watermaze test of spatial learning and memory [19]. Critically, the BAT is more comparable to complex cognitive tasks that older human adults must complete for instrumental activities of daily living and therefore is a better behavioral metric for assessing the translational potential of novel interventions. Potential confounds due to differences in motivation, or procedural and sensorimotor impairments, were assessed with a simple object discrimination problem, in which performance is typically not impaired in aged rats [18].

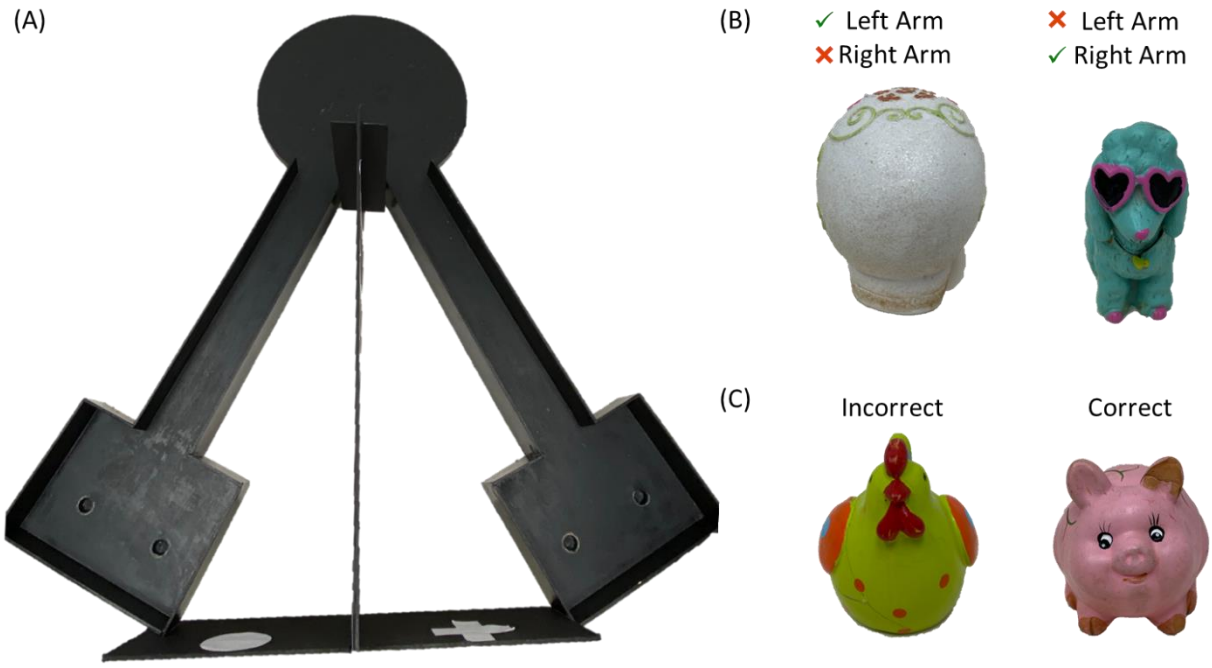

Figure 1. Behavioral testing apparatus and objects utilized for BAT and object discrimination tasks. (**A**) Birds-eye view of the testing apparatus used for alternations, BAT, and object discrimination tasks. Note during the object discrimination, only one arm of the maze was used, and rats were not required to alternate. Objects utilized during (**B**) BAT. In the left arm of the maze the white skull was rewarded when selected while the blue poodle was the correct in the right arm. Thus, the correct response updated based on spatial location. Objects used for (**C**) simple object discrimination testing. The pig was the correct choice.

There is a plethora of mechanisms by which diet interventions could improve cognition in old age. The gut–brain-axis, or interaction between gut and brain health and function, has been recently identified as a powerful player in physiological functions in a variety of conditions. Moreover, gut dysbiosis, or a perturbation in the normal composition and/or density of the gut microbiome, is rampant with advanced age [12]. Cognitive deficits have been increasingly linked to changes in the gut microbiome [22,23] as well as alterations in metabolic function [24]. Therefore, we also investigated changes in the gut microbiome across rats fed these three different diets. While it is well established that ketogenic [6–11] and other [25] diets can significantly alter microbiome composition, to our knowledge, this is the first paper to investigate gut microbiome changes in response to TRF in combination with a ketogenic diet in an animal model, and the first to relate these changes to cognitive outcomes in old age. Furthermore, potential interactions between TRF and age-related changes in gut and metabolic health have also not yet been explored.

2. Materials and Methods

2.1. Subjects and Dietary Interventions

33 aged (22 months) male Fisher 344 x Brown Norway F1 (FBN) Hybrid rats from the National Institute on Aging colony at Charles River were used in this study. All experimental procedures were performed in accordance with National Institutes of Health guidelines and were approved by Institutional Animal Care and Use Committees at the University of Florida. All rats were housed individually and maintained on a 12-h light/dark cycle with all behavioral testing occurring in the dark phase. Rats were divided into three groups: (1) fed ad libitum standard rodent chow until 21 months (n = 13), (2) fed 51 kCal of a standard diet once daily from months 8 to 21 (n = 10) and (3) fed 51 kCal of a ketogenic diet once daily from months 8 to 21 (n = 10). These group sizes were derived via power analysis utilizing a preliminary cohort of 3–4 rats per diet group through two-sample

inference-estimation of sample size [26]. At 21 months of age, all rats were further restricted (approximately 25–30 kCal/day) to encourage participation in the appetitively motivated BAT behavior. Water was provided to all rats ad libitum throughout the study.

The same ketogenic diet (KD) and micronutrient matched control diet (CD) were used as published previously [17,20,27]. An additional group of rats were fed ad libitum with standard laboratory chow (Envigo, Teklad 2918). The KD was a high fat/low carbohydrate diet (Lab Supply; 5722, Fort Worth, TX, USA) mixed with MCT oil (Neobee 895, Stephan, Northfield, Illinois) with a macronutrient profile of 76% fat, 4% carbohydrates, and 20% protein. The micronutrient-matched CD (Lab Supply; 1810727, Fort Worth, TX, USA) had a macronutrient profile of 16% fat, 65% carbohydrates, and 19% protein. Nutritional ketosis was verified by testing peripheral levels of glucose and the ketone body β-hydroxybutyrate (BHB) 1 h after feeding.

2.2. Behavioral Testing

Rats were trained on the biconditional association task (BAT) as previously published [19,21,28]. Briefly, rats were first trained to alternate between left and right arms of a V-shaped maze (see Figure 1) with a macadamia nut reward at the end of each arm. Alternation training continued until rats reached a criterion performance of $\geq$80% correct with completion of all 32 trials within 20 min. Failing to alternate was logged as an incorrect trial. Following alternation training, rats began testing on the BAT, in which a single object pair was placed over two different food wells in the choice platform at the end of both arms (Figure 1B, orb and blue poodle). One object covered a hidden food reward that the rat could retrieve for moving the correct object. Importantly, in the left arm the orb was the rewarded object while in the right arm the poodle was the rewarded object. Rats were allowed to eat the food reward (macadamia nut) if they correctly displaced the object contingent on the current location within the maze. Rats were given 32 trials per day in alternating arms with objects placed pseudorandomly on the left and right sides within a given arm. Rats were trained until a criterion performance of $\geq$80% correct for each object on 2 consecutive days. Following criterion performance on the BAT, all rats were tested on a simple object discrimination within a single arm of the maze. For this control task, object choice was not contingent upon location, and the same object was always rewarded. For both tasks involving objects, selecting the unrewarded object was logged as an incorrect trial. During this phase of testing, rats did not make any alternation errors.

2.3. Statistical Analysis

All data are expressed as group means $\pm$ standard error of the mean (SEM) unless otherwise reported. Glucose ketone index (GKI), body weights during behavior, and behavioral performance on all tasks were analyzed using a one-way ANOVA across diet groups. For all behavioral tasks, outliers were determined using the ROUT method [29] with a false discovery rate of 0.1 prior to ANOVA, and normality was determined with the omnibus K2 test. One outlier was detected in the ketogenic-fed rats during alternation training, one outlier was detected in the ketogenic-fed rats during WM/BAT training and one ab lib and two control-fed rats were outliers during the object discrimination task. Body weight throughout the duration of the study was analyzed using repeated measures-ANOVA (RM-ANOVA) across diet groups. When applicable, follow up comparisons were performed between individual groups using t-tests adjusted with Bonferroni's multiple comparisons test. Finally, to examine for potential relationships across variables, a principal component analysis (PCA) was performed with a varimax rotation. Factors with eigenvalues above 1.0 were considered meaningful and loading coefficients below 0.50 were excluded as done previously [18].

2.4. Fecal Microbiome Taxonomy

At the time of sacrifice, fecal samples were collected from 9 CD, 9 KD and 12 ad *lib-fed* rats, directly from the distal colon. Samples were immediately placed in Para-Pak (Meridian Bioscience Inc., Cincinnati, OH), frozen on dry ice and stored at -80 °C until use.

Samples were processed by the Microbiome Institutional Research Core at the University of Alabama at Birmingham using previously published methods [30–32]. Briefly, analysis of fecal microbiome was performed via 16S rRNA gene sequencing. Amplicon sequence variants (ASVs) were resolved and taxonomy was assigned using the SILVA small subunit ribosomal RNA database version 132 [33]. Alpha diversity was calculated utilizing the microbiome package in R [34], and beta diversity was calculated utilizing the Phyloseq package in R [35] via permutational multivariate analysis of variance (PERMANOVA). Analysis of Compositions of Microbiomes (ANCOM) with Bias Correction was used to test for differential using modified versions of previously published ANCOM scripts with a detection limit of 0.7 [32,36,37].

3. Results

3.1. Peripheral Effects of Feeding Paradigms

Postprandial glucose (Figure 2A) and BHB (Figure 2B) measurements were utilized to generate a glucose ketone index (GKI) for each rat as reported previously [27]. Lower GKI values indicate greater levels of ketosis. GKI values during behavioral testing indicate only rats fed the ketogenic diet were in nutritional ketosis ($F_{[2,29]} = 54.07$; $p < 0.001$; Figure 2C). There was no significant main effect of feeding method ($F_{[1,30]} = 0.76$; $p = 0.39$), as rats on time-restricted feeding with the standard diet had a significantly elevated GKI level relative to ad libitum-fed rats ($t_{[29]} = 4.18$; $p < 0.001$), but rats on time-restricted feeding with the ketogenic diet had a significantly lower GKI level relative to ad libitum-fed ($t_{[29]} = 6.87$; $p < 0.001$).

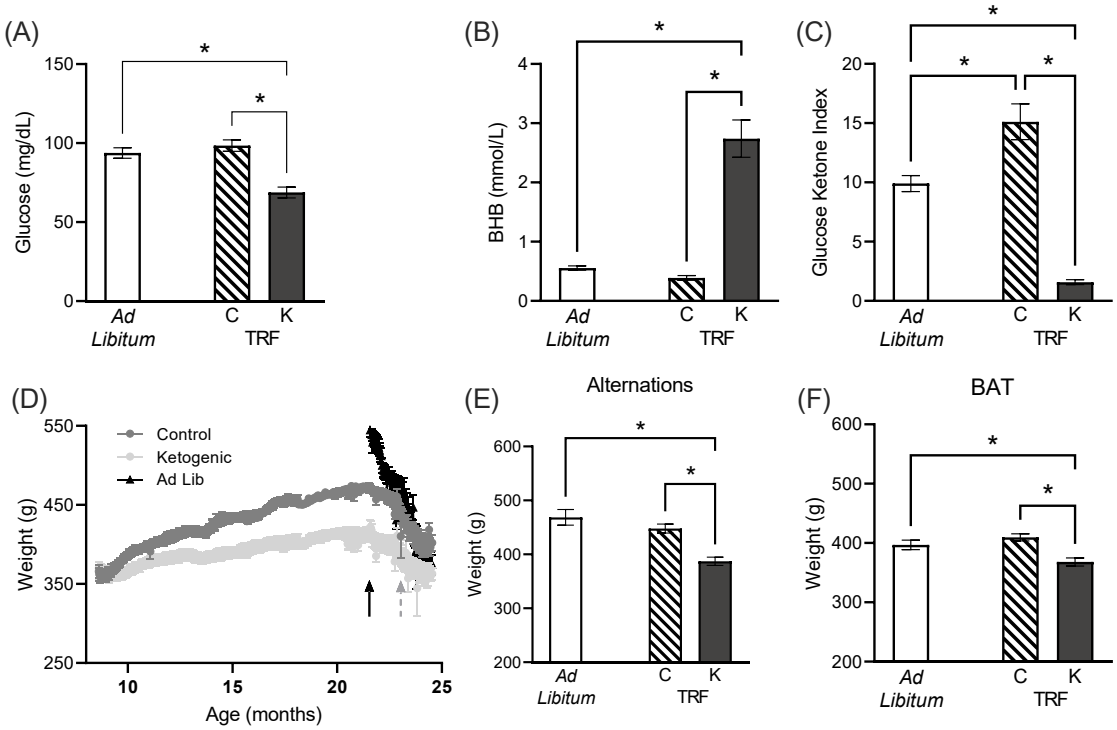

Figure 2. Postprandial glucose, BHB and GKI values and body weight. Only rats fed a ketogenic diet exhibited nutritional ketosis as evidenced by (**A**) reduced glucose, (**B**) elevated levels of the ketone body β-hydroxybutyrate and (**C**) a lower glucose ketone index (GKI). (**D**) Body weights throughout the lifespan continued to increase while fed a ketogenic or control diet via time-restricted feeding, until the onset of further dietary restriction (solid black arrow) and during BAT testing (beginning at dashed gray arrow). Body weight on the first day of (**E**) alternation training, as well as (**F**) BAT, was significantly lower in ketogenic diet-fed rats relative to both other groups. C, control-fed; K, ketogenic diet-fed; TRF, time restricted-fed; BAT, biconditional association task. All data are group means ± 1 SEM; * indicates $p < 0.05$.

Over the course of time-restricted feeding, both ketogenic and control-fed rats gained a significant amount of weight ($F_{[1,18]} = 378.50$; $p < 0.001$), indicating that they were not calorically restricted. Although caloric intake was identical, the control-fed rats gained significantly more weight that the ketogenic diet-fed rats ($F_{[1,18]} = 7.08$; $p = 0.02$). Moreover, the interaction between time and diet group was significant ($F_{[1,18]} = 67.09$; $p < 0.001$), indicating that the control-fed rats also gained weight more rapidly. Although this observation suggests that rats were not calorically restricted, weights did not reach the level of ad libitum-fed animals of the same age (Figure 2D). The control and ketogenic intermittent fasting groups reached maximal weights that were on average 472 and 421 g, respectively, compared to an average maximal weight of 547 g in the ad libitum-fed rats. This is likely because rats with unrestricted access to food overconsume, which could be related to the excessive visceral fat and metabolic impairments that have been reported for aged male rats of this strain [13,17].

Prior to beginning shaping, all rats were placed on food restriction to motivate appetitive behavior for cognitive testing. Animals were given ~25-30 Kcal/day (black arrow in Figure 2D), which initiated weight loss in all groups. Body weight on the first day of alternation and BAT testing (dashed arrow in Figure 2D) was significantly lower in ketogenic diet-fed rats than in control-fed ($t_{[30]} = 5.00$; $p = 0.004$; $t_{[30]} = 5.43$; $p = 0.002$, respectively) and ad libitum-fed rats ($t_{[30]} = 7.15$; $p < 0.001$; $t_{[30]} = 4.01$; $p = 0.02$, respectively), though the control-fed and ad libitum-fed rats did not significantly differ ($t_{[30]} = 1.83$; $p = 0.41$; $t_{[30]} = 1.76$; $p = 0.44$; respectively Figure 2D,E). The comparable body weights during behavioral testing between the time-restricted control-fed rats and rats that ate ad libitum between 8 and 21 months suggest that potential differences in behavior cannot be explained by differences in overall body condition.

3.2. Time Restricted Feeding, Regardless of Macronutrient Composition, Ameliorated Age-Related Cognitive Impairment on the Biconditional Association Task

The total number of incorrect trials across all days of training through the final day of criterion performance for all tasks were tabulated for each rat. For alternation training, neither diet group (ketogenic versus standard; $F_{[2,29]} = 1.51$; $p = 0.24$) nor feeding method (ad libitum versus TRF; $F_{[1,29]} = 0.12$; $p = 0.73$) significantly affected the number of incorrect trials required to reach criterion performance on alternations throughout the maze (Figure 3A). Aged rats typically perform comparable to young on this behavior [19]. The similar performance accuracy on alternations across diet groups indicates that despite differences in weight, rats in all diet and feeding groups were similarly motivated to retrieve the food reward.

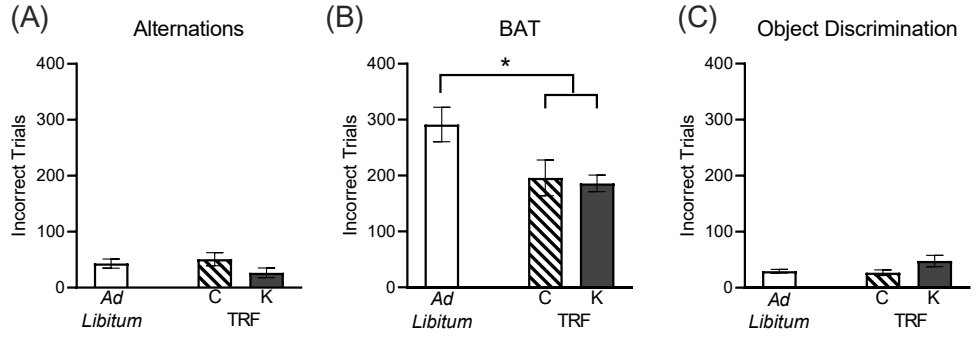

Figure 3. Behavioral performance of aged rats across diet groups and feeding methods. (**A**) There were no differences across groups in ability to alternate between the left and right arms of the maze. (**B**) Lifelong time-restricted feeding, regardless of macronutrient composition, improved ability to acquire the object-in-place rule required for criterion performance on the BAT task to a degree that was comparable to performance in young (4–8 mo) rats of the same strain. (**C**) All rats were able to perform an object discrimination task similarly, indicating no sensorimotor deficits or motivation differences across groups that would confound BAT task performance. All data are group means ± 1 SEM; * indicates $p < 0.05$.

The number of incorrect trials required to reach criterion performance on BAT testing was significantly different across the diet groups ($F_{[2,28]} = 4.48$; $p = 0.02$; Figure 3B). These results were due to the time-restricted feeding from young adulthood rather than dietary macronutrient composition, as the method of feeding also had a significant effect on performance ($F_{[1,30]} = 194.07$; $p < 0.001$). Ad libitum fed rats required significantly more trials to reach criterion performance than ketogenic diet-fed rats ($t_{[1,28]} = 2.63$; $p = 0.04$) and a strong trends towards significantly more trials than control-fed rats ($t_{[1,28]} = 2.38$; $p = 0.07$). Conversely, among the rats that were given time-restricted feeding from young adulthood into old age, there was no difference in performance between the ketogenic and control diet groups ($t_{[28]} = 0.22$; $p > 0.99$).

Data from the cohort presented here were also compared to young animals from other previously run cohorts that underwent identical BAT behavioral testing to assess how life-long TRF influenced cognitive outcomes in advanced age. While old rats made significantly more incorrect trials than young prior to reaching criterion performance on BAT testing ($F_{[1,64]} = 19.22$, $p < 0.001$), TRF-fed rats overall performed significantly better than ad lib fed rats regardless of age group ($F_{[1,64]} = 7.64$, $p < 0.01$). Moreover, when the ketogenic and control groups of TRF fed rats were compared to young rats, there were was no significant difference in performance across the three groups ($F_{[2,38]} = 1.35$, $p = 0.27$). In contrast, the ad libitum-fed aged rats made significantly more errors than the young rats ($T_{[39]} = 4.40$, $p = 0.001$). Together these data indicate that a mid-life intervention with TRF may prevent age-related cognitive deficits commonly observed on this task.

Following BAT testing, a simple object discrimination control was utilized to ensure all rats were able to discriminate between two dissimilar objects and to assess potential differences in motivation or procedural impairments (Figure 3C). There was not a significant difference in the number of incorrect trials required to reach a criterion performance on this control task across the 3 groups of rats ($F_{[2,25]} = 2.60$; $p = 0.10$), nor was there an effect of feeding method (ad libitum versus time-restricted; $F_{[1,29]} = 0.82$; $p = 0.37$). These data demonstrate all rats were able to discriminate between objects and are not visually impaired, indicating BAT task performance was not hindered by physical or sensory deficits, but rather was likely to manifest from cognitive differences across diet groups that altered the rate at which animals could learn the object-in-place rule.

3.3. Time Restricted Feeding and the Ketogenic Diet Influence Gut Microbiome Composition and Beta Diversity

At the phylum taxonomic level (Figure 4A,B), two phyla were found to significantly differ across diet groups: actinobacteria ($F_{[2,27]} = 11.49$; $p < 0.001$) and deferribacteres ($F_{[2,27]} = 7.39$; $p = 0.002$). While both TRF groups had significantly reduced abundance of actinobacteria relative to the ad libitum fed group ($p < 0.01$ for both comparisons), only the ketogenic diet fed TRF group had significantly higher levels of deferribacteres than the ad libitum fed group ($t_{[27]} = 3.30$; $p = 0.006$; Figure 4C) but not the control TRF fed group ($t_{[27]} = 0.38$; $p > 0.99$). No other phyla differed by diet group or feeding paradigm ($p > 0.19$ for all comparisons), nor did the ratio of firmicutes to bacteroidetes differ ($p > 0.30$ for both comparisons; Figure 4E). In addition to broad changes at the phylum level, additional analyses (see ANCOM below) were conducted at the genus level (Supplementary Figure S1) to assess more detailed differences in gut microbiome composition.

Two common measures of alpha diversity, the Inverse Simpson (IS) index [40], which measures the dominances of a multispecies community [40] and Shannon's (S) index, which takes taxa richness into account [41], were utilized. There were no differences across groups in alpha diversity by either measure, whether separated by dietary macronutrient composition (S: $F_{[2,27]} = 0.37$, $p = 0.72$; IS: $F_{[2,27]} = 1.32$, $p = 0.28$; A,B) or by feeding paradigm (S: $t_{[28]} = 0.67$, $p = 0.51$; IS: $t_{[28]} = 1.58$, $p = 0.13$; Figure 5C,D).

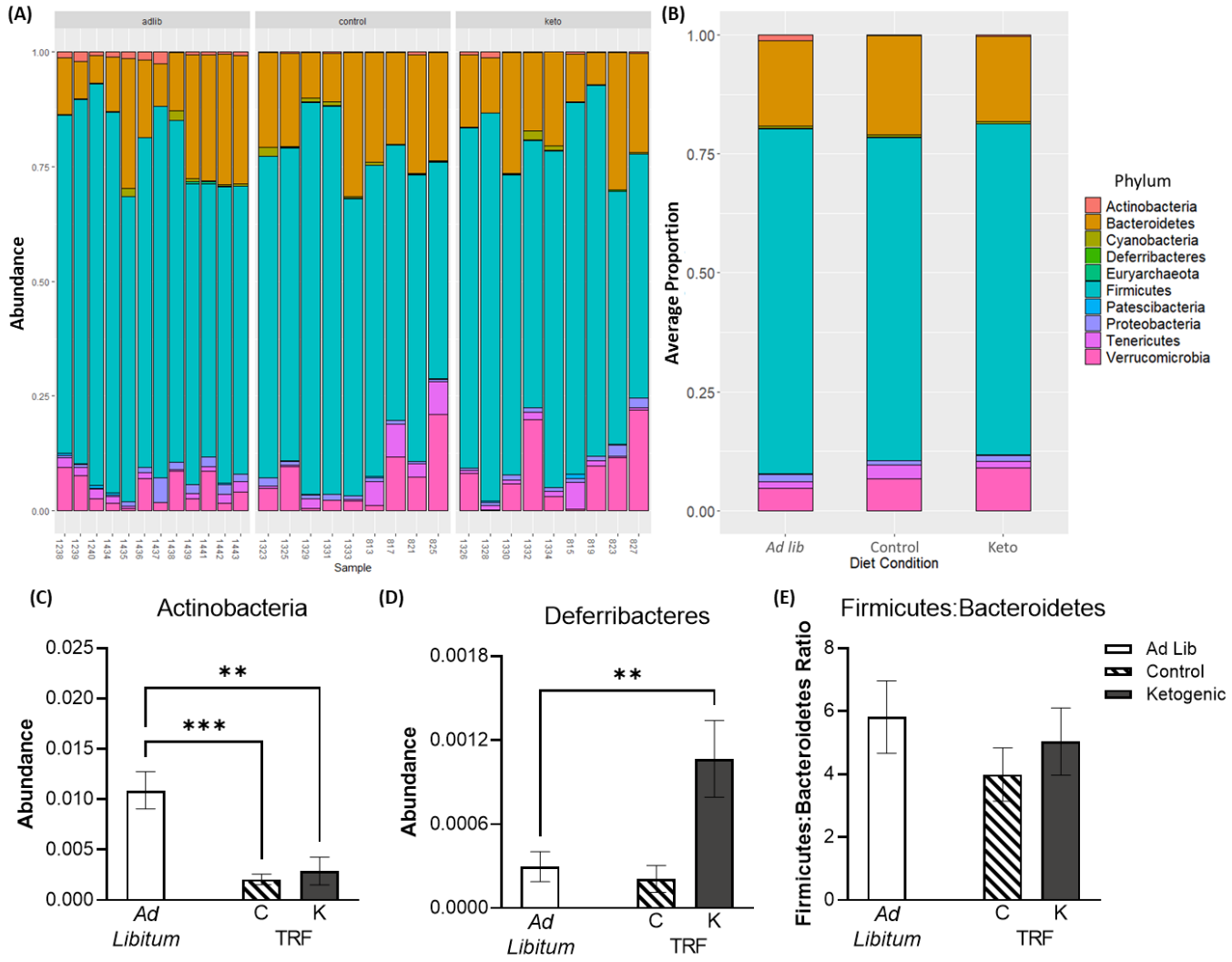

Figure 4. Relative abundance at the phylum taxonomic level by (**A**) subject and (**B**) diet condition. Relative abundance of (**C**,**D**) significantly altered phyla and (**E**) the ratio of firmicutes to bacteroidetes. All data in panels (**C**–**E**) are group means ± 1 SEM; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$.

Beta diversity was calculated using Bray Curtis (BC) Dissimilarity and differences across groups were assessed with a permutational multivariate analysis of variance (PERMANOVA). PERMANOVA revealed a significant effect of both diet ($F_{[2,29]} = 4.87$; $p = 0.001$) and feeding paradigm ($F_{[1,29]} = 5.81$; $p = 0.001$) on beta diversity. As beta diversity has been shown to correlate with better cognition in middle-aged adults [42], this is one potential mechanism by which intermittent fasting could improve BAT performance accuracy.

Analysis of composition of microbiomes (ANCOM) was utilized to examine taxa that had statistically different abundance between diet and feeding paradigm groups (Figure 6). Interestingly, different phyla and genera were identified by the two analyses. ANCOM across diet groups at the phylum level revealed 2 significantly different taxa, deferribacteres and Euryarchaeota. At the genus level, 4 taxa were differentially abundant across diet groups, 3 of which were in the firmicutes phyla (*Ileibacterium*, *[Ruminococcus] gauvreauii group*, and *Allobaculum*) and 1 from the tenericutes phyla (an uncultured bacterium).

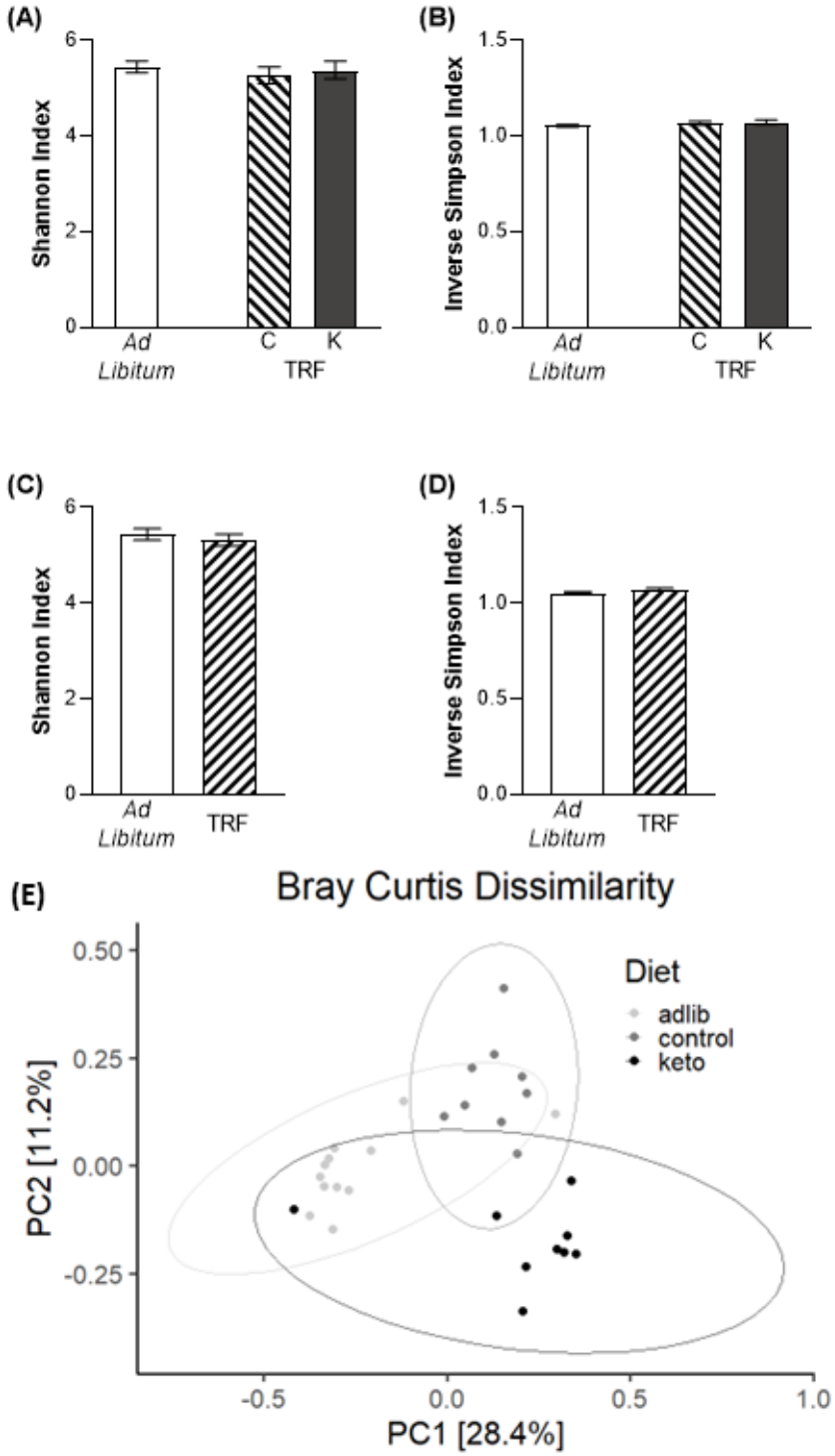

Figure 5. Beta, but not alpha, diversity differed across dietary paradigms. Neither the Shannon or Inverse Simpson indices of alpha diversity differed across (**A**,**B**) macronutrient or (**C**,**D**) feeding paradigm groups. (**E**) Conversely, beta diversity was different across both diet groups and feeding paradigms. Data in (**A**–**D**) are group means ± 1 SEM; PC = principal component.

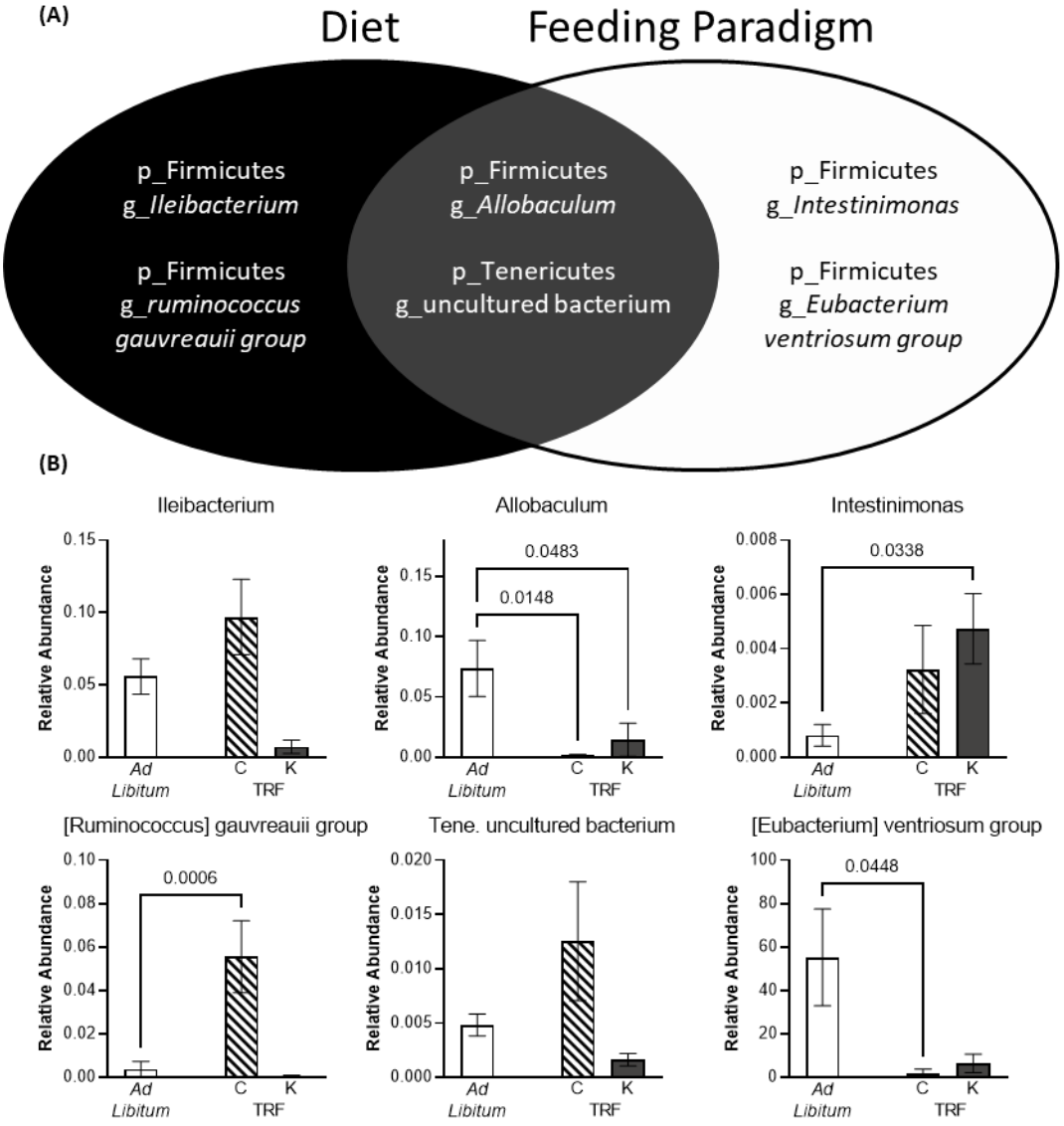

Figure 6. Significantly altered genera identified utilizing the analysis of composition of microbiomes (ANCOM) methodology. (**A**) 6 genera were identified to be significantly different across the different diet and/or feeding paradigm groups. (**B**) Relative abundance of the identified genera. All data are group means ± 1 SEM.

ANCOM across the different feeding paradigms identified only one significant difference at the phylum level, Actinobacteria. At the genus level, the same uncultured tenericutes group and *Allobaculum* from the firmicutes phyla were significantly different across feeding paradigm, along with two other firmicutes microbiota *(Intestinimonas* and *[Eubacterium] ventriosum group).*

3.4. Alterations in Gut Microbiome Composition Correlate with Behavioral Performance

A principal component analysis (PCA) was used to determine if there was a relationship between the significantly altered gut microbiota identified by ANCOM (see above) with behavioral performance and biomarkers of ketosis (Figure 7). Three components had eigenvalues > 1, which combined accounted for 61.60% of the variance.

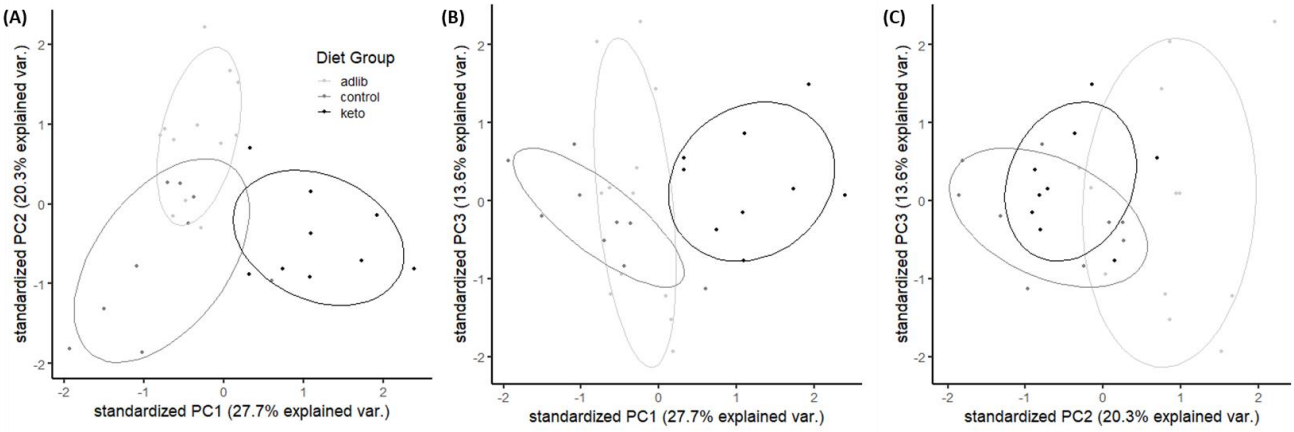

Figure 7. Principal component analysis on measures of nutritional ketosis, cognitive performance and microbiome composition. (**A**) Components 1 and 2, (**B**) components 1 and 3 (**C**) and components 2 and 3 are shown as a function of diet group (light gray = ad libitum fed rats, medium gray = control TRF fed rats and black = ketogenic diet TRF fed rats).

The first component, which accounts for 27.72% of the total variance with an Eigenvalue of 2.77, loaded negatively with circulating ketones (−0.87) and negatively with circulating glucose (0.82). Thus, component 1 largely represents dietary ketosis during behavioral task performance. Moreover, this component loaded negatively with the genera *Intestinimonas* (−0.66) and positively with *Ileibacterium* (0.61) and *Ruminococcus gauvreauii group* abundance (0.52), demonstrating a relationship between abundance of these genera with a ketogenic diet. The second component, which accounts for 20.31% of the total variance with an Eigenvalue of 2.03, positively correlates with the number of errors made on BAT prior to reaching criterion (0.73) and *Allobaculum* abundance (0.79). Thus, this component suggests that worse cognition was associated with greater abundance of a specific genera that is modified by both diet composition and feeding paradigm. The third component, which accounts for 13.58% of the total variance with an Eigenvalue of 1.36, positively loaded with *Eubacterium ventriosum group* (0.71) and the uncultured tenericutes bacterium (0.77), and negatively with *Ruminococcus gauvreauii group* abundance (−0.50).

4. Discussion

The data presented here show that time-restricted feeding initiated in mature adults, prior to the onset of age-related metabolic impairments, can influence cognitive outcomes in advanced age. Specifically, rats fed once daily between the ages of 8 and 21 months with either a ketogenic or a standard control diet performed better on a cognitive biconditional association task (BAT) compared to rats that were fed ad libitum during that same time frame. This observation could not be accounted for by differences in body weight or sensorimotor impairments. It is important to note than although rats were fed once daily, and thus prevented from obesogenic overconsumption typically observed with ad libitum feeding [43], these rats were not calorically restricted and continued to show modest increases in weight throughout their lives.

Furthermore, we observed that nutritional ketosis, which has been shown to improve metabolic health in aged rats [13,17], did not confer an additive benefit to time-restricted feeding with a standard control diet in regard to multitasking performance on a biconditional association task. Shorter term ketogenic diets initiated in old age (21 months), which is an age at which rats on lifelong ad libitum feeding have age-related declines in metabolic function, have demonstrated improved cognitive outcomes on a similar task as well as reduced anxiety-like behavior [20]. An important distinction between these two studies is the timing of the initiation of ketogenic diet therapy. When initiated at 8 months of age, normal rats show little metabolic dysfunction from lifelong ad libitum feeding with a standard diet. In contrast, rats that are allowed to consume food ad libitum into old age

gain excessive weight, acquire aberrant amounts of white adipose tissue, as well as show disrupted insulin signaling and a reduced ability to utilize glucose in the brain [13,17,43,44]. The current data suggest that time-restricted feeding throughout adulthood, regardless of the macronutrient composition, may be able to prevent these metabolic deficits in old age and lead to resilience against age-related cognitive decline. In contrast, when diet interventions are initiated in old age, declines in metabolic function need to be reversed. A previous study reported that time-restricted feeding with a ketogenic diet may be more effective at normalizing metabolic function in aged rats than time-restricted feeding with a standard diet. Thus, it may be critical to consider an individual's current metabolic status when designing an optimal diet-based intervention for optimizing cognitive performance. This type of precision medicine-based approach has recently been suggested as an new avenue for treating cognitive aging [45].

The limited number of studies that have investigated the potential of TRF to alter gut microbiome composition have reported conflicting results. One group found significant changes in the microbiome following TRF in young healthy male human subjects [46]. Moreover, such changes correlated with changes in metabolic markers and circadian rhythm-associated genes [47]. However, in another study of obese humans, TRF did not significantly alter microbiome abundance or diversity, despite significant weight loss [39]. Here, we found significant changes from TRF in rats, as well as from altered macronutrient composition via a KD. Specifically, 2 firmicutes genera (*Ileibacterium* and *ruminococcus gauvreauii group*) were reduced in abundance by the ketogenic diet alone. When the microbiota from TRF (both ketogenic and control) were compared to ad lib fed rats 2 firmicutes genera (*Intestinimonas* and *Eubacterium ventriosum group*) were modified. One firmicutes genus (*Allobaculum*) was reduced in the TRF rats while and 1 tenericutes genus (an uncultured bacterium) was significantly elevated in abundance in the TRF compared to ad lib fed rats.

Not only do our data indicate both dietary macronutrient composition and feeding paradigm influence microbiome composition, but also that these changes correlate with behavioral performance. Specifically, worse performance accuracy on the biconditional association task was associated with higher *Allobaculum* abundance. *Allobaculum*, which is involved in butyric acid production [48,49], increases following the consumption of a high fat diet in rodents that produces cognitive impairments [38,50,51]. While this suggest that increased levels of *Allobaculum* may be related to suboptimal health, other studies have reported that voluntary exercise can lead to enhanced abundance of *Allobaculum* [52,53]. Thus, future studies should attempt to manipulate *Allobaculum* abundance directly to examine the impact on cognitive function and metabolic health.

The current data also have important implications in mid-life food consumption patterns and later life cognition. While is it well documented that high fat/high sugar obesogenic diets are associated with worse cognition [54], these data show that over consuming even a standard diet that does not contain high fat or excessive sugar can also lead to worse to cognitive performance later in life relative to alternative feeding styles. Thus, it is conceivable that adults who overconsume throughout mid-life are at a higher risk for cognitive decline in advanced age. A 2020 report by the U.S. Department of Health and Human Services found 17.5% of individuals 45–64 had diabetes [55], demonstrating the dire necessity of interventions during this critical period to avoid further cognitive decline in geriatric populations.

Our data provide additional support for the strong link between gut and brain function, known as the gut–brain-axis. However, what remains unclear is how alterations in diet, such as those performed in this study, are able to influence cognitive function. One potential explanation is that alterations in gut microbiome composition are capable of improving other aspects of systemic health, including restoring insulin-related signaling and preventing or decreasing inflammation. Moreover, changing the gut microbiome composition can significantly influence metabolite production and bioavailability, which can influence neurobiological processes. For example, one group found that diabetes-induced cognitive impairments were ameliorated following both fasting-induced changes in gut microbiome

composition and direct application of affected metabolites [56]. Changes such as these then allow for the restoration of, or prevention of decline in, neurobiological function through related avenues, including nutrient availability and decreased inflammatory processes.

A second way by which gut health and gut microbiome composition influence cognitive outcomes is through a more direct impact on neurobiological function. This can take place through a variety of means, including altered levels of neurotransmitters produced by gut microbes and direct interaction of these microprobes and their metabolites with the enteric nervous system. To test this theory, experiments directly influencing these particular aspects of gut and nervous system function can help elucidate specific pathways to utilize as therapeutic targets. This can include things like a vagotomy, or the removal of part of the vagus nerve, to significantly sever the connection between gut function and the nervous system prior to dietary intervention. Alternatively, to decrease peripheral health-improving effects of dieting, such as weight loss or improved insulin resistance, fecal matter transplants from specific populations can supplemented to study more direct effects of gut microbiome population contributions to cognitive outcomes.

Moreover, these potential avenues through which gut composition and function influence neurobiological function may be overlapping and synergistic. It is likely that changes in one organ system has profound impacts on other organ systems, such as central nervous system function. Additionally, while the work presented here utilizes a well-published behavioral task that is sensitive to early cognitive decline, further cognitive characterization would complement our findings and enhance our ability to link peripheral function with cognitive performance.

5. Conclusions

Our data strongly suggest that interactions with peripheral and systemic functioning are important aspects to consider with interventional therapies targeting age-related cognitive decline through peripheral means, like through diet or orally ingested compounds. Thus, these data are in support of utilizing the gut microbiome as modifiable therapeutic target for alleviating cognitive dysfunction, which may occur through altered dietary macronutrient consumption, altered feeding patterns or other supplements such as pre- or probiotics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu14193977/s1, Figure S1: Relative abundance at the genus taxonomic level.

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References

1. Gold, P.E.; Korol, D.L. Forgetfulness during Aging: An Integrated Biology. *Neurobiol. Learn. Mem.* **2014**, *112*, 130–138. [CrossRef] [PubMed]
2. Trepanowski, J.F.; Canale, R.E.; Marshall, K.E.; Kabir, M.M.; Bloomer, R.J. Impact of Caloric and Dietary Restriction Regimens on Markers of Health and Longevity in Humans and Animals: A Summary of Available Findings. *Nutr. J.* **2011**, *10*. [CrossRef] [PubMed]
3. Carter, C.S.; Leeuwenburgh, C.; Daniels, M.; Foster, T.C. Influence of Calorie Restriction on Measures of Age-Related Cognitive Decline: Role of Increased Physical Activity. *J. Gerontol. A. Biol. Sci. Med. Sci.* **2009**, *64A*, 850–859. [CrossRef] [PubMed]
4. Lee, C.; Longo, V. Dietary Restriction with and without Caloric Restriction for Healthy Aging. *F1000Research* **2016**, *5*, 117. [CrossRef]
5. Klement, R.J. Mimicking Caloric Restriction: What about Macronutrient Manipulation? A Response to Meynet and Ricci. *Trends Mol. Med.* **2014**, *20*, 471–472. [CrossRef]
6. Newell, C.; Bomhof, M.R.; Reimer, R.A.; Hittel, D.S.; Rho, J.M.; Shearer, J. Ketogenic Diet Modifies the Gut Microbiota in a Murine Model of Autism Spectrum Disorder. *Mol. Autism* **2016**, *7*, 37. [CrossRef]
7. Swidsinski, A.; Dörffel, Y.; Loening-Baucke, V.; Gille, C.; Göktas, Ö.; Reißhauer, A.; Neuhaus, J.; Weylandt, K.-H.; Guschin, A.; Bock, M. Reduced Mass and Diversity of the Colonic Microbiome in Patients with Multiple Sclerosis and Their Improvement with Ketogenic Diet. *Front. Microbiol.* **2017**, *8*, 1141. [CrossRef]
8. Olson, C.A.; Vuong, H.E.; Yano, J.M.; Liang, Q.Y.; Nusbaum, D.J.; Hsiao, E.Y. The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. *Cell* **2018**, *173*, 1728–1741.e13. [CrossRef]
9. Gutiérrez-Repiso, C.; Hernández-García, C.; García-Almeida, J.M.; Bellido, D.; Martín-Núñez, G.M.; Sánchez-Alcoholado, L.; Alcaide-Torres, J.; Sajoux, I.; Tinahones, F.J.; Moreno-Indias, I. Effect of Synbiotic Supplementation in a Very-Low-Calorie Ketogenic Diet on Weight Loss Achievement and Gut Microbiota: A Randomized Controlled Pilot Study. *Mol. Nutr. Food Res.* **2019**, *63*, 1900167. [CrossRef]
10. Le, H.H.; Johnson, E.L. Going Keto? Say BHB-Ye Bye to Your Gut Bifidobacteria. *Cell Host Microbe* **2020**, *28*, 3–5. [CrossRef]
11. Yuan, W.; Lu, W.; Wang, H.; Wu, W.; Zhou, Q.; Chen, Y.; Lee, Y.K.; Zhao, J.; Zhang, H.; Chen, W. A Multiphase Dietetic Protocol Incorporating an Improved Ketogenic Diet Enhances Weight Loss and Alters the Gut Microbiome of Obese People. *Int. J. Food Sci. Nutr.* **2022**, *73*, 238–250. [CrossRef] [PubMed]
12. Xu, C.; Zhu, H.; Qiu, P. Aging Progression of Human Gut Microbiota. *BMC Microbiol.* **2019**, *19*, 236. [CrossRef] [PubMed]
13. Hernandez, A.R.; Truckenbrod, L.M.; Federico, Q.P.; Campos, K.T.; Moon, B.M.; Ferekides, N.; Hoppe, M.; D'Agostino, D.; Burke, S.N. Metabolic Switching Is Impaired by Aging and Facilitated by Ketosis Independent of Glycogen. *Biochemistry* **2019**, *12*, 7963. [CrossRef]
14. Kumar, N.K.; Merrill, J.D.; Carlson, S.; German, J.; Yancy, W.S. Adherence to Low-Carbohydrate Diets in Patients with Diabetes: A Narrative Review. *Diabetes Metab. Syndr. Obes. Targets Ther.* **2022**, *15*, 477–498. [CrossRef]
15. Braak, H.; Del Tredici, K. The Preclinical Phase of the Pathological Process Underlying Sporadic Alzheimer's Disease. *Brain* **2015**, *138*, 2814–2833. [CrossRef] [PubMed]
16. Reiman, E.M.; Chen, K.; Alexander, G.E.; Caselli, R.J.; Bandy, D.; Osborne, D.; Saunders, A.M.; Hardy, J. Functional Brain Abnormalities in Young Adults at Genetic Risk for Late-Onset Alzheimer's Dementia. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 284–289. [CrossRef] [PubMed]
17. Hernandez, A.R.; Hernandez, C.M.; Campos, K.T.; Truckenbrod, L.M.; Sakarya, Y.; McQuail, J.A.; Carter, C.S.; Bizon, J.L.; Maurer, A.P.; Burke, S.N. The Antiepileptic Ketogenic Diet Alters Hippocampal Transporter Levels and Reduces Adiposity in Aged Rats. *J. Gerontol. Ser. A* **2018**, *73*, 450–458. [CrossRef]
18. Hernandez, A.R.; Truckenbrod, L.M.; Campos, K.T.; Williams, S.A.; Burke, S.N. Sex Differences in Age-Related Impairments Vary across Cognitive and Physical Assessments in Rats. *Behav. Neurosci.* **2020**, *134*, 69–81. [CrossRef]
19. Hernandez, A.R.; Maurer, A.P.; Reasor, J.E.; Turner, S.M.; Barthle, S.E.; Johnson, S.A.; Burke, S.N. Age-Related Impairments in Object-Place Associations Are Not Due to Hippocampal Dysfunction. *Behav. Neurosci.* **2015**, *129*, 599–610. [CrossRef]
20. Hernandez, A.R.; Hernandez, C.M.; Campos, K.; Truckenbrod, L.; Federico, Q.; Moon, B.; McQuail, J.A.; Maurer, A.P.; Bizon, J.L.; Burke, S.N. A Ketogenic Diet Improves Cognition and Has Biochemical Effects in Prefrontal Cortex That Are Dissociable From Hippocampus. *Front. Aging Neurosci.* **2018**, *10*, 391. [CrossRef] [PubMed]
21. Hernandez, A.R.; Reasor, J.E.; Truckenbrod, L.M.; Campos, K.T.; Federico, Q.P.; Fertal, K.E.; Lubke, K.N.; Johnson, S.A.; Clark, B.J.; Maurer, A.P.; et al. Dissociable Effects of Advanced Age on Prefrontal Cortical and Medial Temporal Lobe Ensemble Activity. *Neurobiol. Aging* **2018**, *70*, 217–232. [CrossRef] [PubMed]
22. Davidson, G.L.; Cooke, A.C.; Johnson, C.N.; Quinn, J.L. The Gut Microbiome as a Driver of Individual Variation in Cognition and Functional Behaviour. *Philos. Trans. R. Soc. B Biol. Sci.* **2018**, *373*, 20170286. [CrossRef]
23. Gao, W.; Baumgartel, K.L.; Alexander, S.A. The Gut Microbiome as a Component of the Gut–Brain Axis in Cognitive Health. *Biol. Res. Nurs.* **2020**, *22*, 485–494. [CrossRef]
24. Sanz, Y.; Olivares, M.; Moya-Pérez, Á.; Agostoni, C. Understanding the Role of Gut Microbiome in Metabolic Disease Risk. *Pediatr. Res.* **2015**, *77*, 236–244. [CrossRef] [PubMed]
25. David, L.A.; Maurice, C.F.; Carmody, R.N.; Gootenberg, D.B.; Button, J.E.; Wolfe, B.E.; Ling, A.V.; Devlin, A.S.; Varma, Y.; Fischbach, M.A.; et al. Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. *Nature* **2014**, *505*, 559–563. [CrossRef]

26. Rosner, B. *Fundamentals of Biostatistics*, 8th ed.; Cengage Learning: Boston, MA, USA, 2016; ISBN 978-1-305-26892-0.
27. Hernandez, A.R.; Hernandez, C.M.; Truckenbrod, L.M.; Campos, K.T.; McQuail, J.A.; Bizon, J.L.; Burke, S.N. Age and Ketogenic Diet Have Dissociable Effects on Synapse-Related Gene Expression Between Hippocampal Subregions. *Front. Aging Neurosci.* **2019**, *11*, 239. [CrossRef] [PubMed]
28. Hernandez, A.R.; Reasor, J.E.; Truckenbrod, L.M.; Lubke, K.N.; Johnson, S.A.; Bizon, J.L.; Maurer, A.P.; Burke, S.N. Medial Prefrontal-Perirhinal Cortical Communication Is Necessary for Flexible Response Selection. *Neurobiol. Learn. Mem.* **2017**, *137*, 36–47. [CrossRef] [PubMed]
29. Motulsky, H.J.; Brown, R.E. Detecting Outliers When Fitting Data with Nonlinear Regression–a New Method Based on Robust Nonlinear Regression and the False Discovery Rate. *BMC Bioinformatics* **2006**, *7*, 123. [CrossRef] [PubMed]
30. Kumar, R.; Eipers, P.; Little, R.B.; Crowley, M.; Crossman, D.K.; Lefkowitz, E.J.; Morrow, C.D. Getting Started with Microbiome Analysis: Sample Acquisition to Bioinformatics. *Curr. Protoc. Hum. Genet.* **2014**, *82*, 18.8.1–18.8.29. [CrossRef] [PubMed]
31. Buford, T.W.; Carter, C.S.; VanDerPol, W.J.; Chen, D.; Lefkowitz, E.J.; Eipers, P.; Morrow, C.D.; Bamman, M.M. Composition and Richness of the Serum Microbiome Differ by Age and Link to Systemic Inflammation. *GeroScience* **2018**, *40*, 257–268. [CrossRef]
32. Hernandez, A.R.; Kemp, K.M.; Burke, S.N.; Buford, T.W.; Carter, C.S. Influence of Aging, Macronutrient Composition and Time-Restricted Feeding on the Fischer344 x Brown Norway Rat Gut Microbiota. *Nutrients* **2022**, *14*, 1758. [CrossRef]
33. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* **2012**, *41*, D590–D596. [CrossRef]
34. Shetty, S.A.; Lahti, L. Microbiome Data Science. *J. Biosci.* **2019**, *44*, 115. [CrossRef] [PubMed]
35. McMurdie, P.J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef] [PubMed]
36. Mandal, S.; Van Treuren, W.; White, R.A.; Eggesbø, M.; Knight, R.; Peddada, S.D. Analysis of Composition of Microbiomes: A Novel Method for Studying Microbial Composition. *Microb. Ecol. Health Dis.* **2015**, *26*, 27663. [CrossRef] [PubMed]
37. Kemp, K.M.; Colson, J.; Lorenz, R.G.; Maynard, C.L.; Pollock, J.S. Early Life Stress in Mice Alters Gut Microbiota Independent of Maternal Microbiota Inheritance. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2021**, *320*, R663–R674. [CrossRef] [PubMed]
38. Jena, P.K.; Sheng, L.; Nguyen, M.; Di Lucente, J.; Hu, Y.; Li, Y.; Maezawa, I.; Jin, L.-W.; Wan, Y.-J.Y. Dysregulated Bile Acid Receptor-Mediated Signaling and IL-17A Induction Are Implicated in Diet-Associated Hepatic Health and Cognitive Function. *Biomark. Res.* **2020**, *8*, 59. [CrossRef] [PubMed]
39. Gabel, K.; Marcell, J.; Cares, K.; Kalam, F.; Cienfuegos, S.; Ezpeleta, M.; Varady, K.A. Effect of Time Restricted Feeding on the Gut Microbiome in Adults with Obesity: A Pilot Study. *Nutr. Health* **2020**, *26*, 79–85. [CrossRef]
40. Simpson, E.H. Measurement of Diversity. *Nature* **1949**, *163*, 688. [CrossRef]
41. Routledge, R.D. Diversity Indices: Which Ones Are Admissible? *J. Theor. Biol.* **1979**, *76*, 503–515. [CrossRef]
42. Meyer, K.; Lulla, A.; Debroy, K.; Shikany, J.M.; Yaffe, K.; Meirelles, O.; Launer, L.J. Association of the Gut Microbiota With Cognitive Function in Midlife. *JAMA Netw. Open* **2022**, *5*, e2143941. [CrossRef]
43. Martin, B.; Ji, S.; Maudsley, S.; Mattson, M.P. "Control" Laboratory Rodents Are Metabolically Morbid: Why It Matters. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6127–6133. [CrossRef] [PubMed]
44. Gold, P.E. Glucose and Age-Related Changes in Memory. *Neurobiol. Aging* **2005**, *26*, 60–64. [CrossRef] [PubMed]
45. Ryan, L.; Hay, M.; Huentelman, M.J.; Duarte, A.; Rundek, T.; Levin, B.; Soldan, A.; Pettigrew, C.; Mehl, M.R.; Barnes, C.A. Precision Aging: Applying Precision Medicine to the Field of Cognitive Aging. *Front. Aging Neurosci.* **2019**, *11*, 128. [CrossRef]
46. Zeb, F.; Wu, X.; Chen, L.; Fatima, S.; Haq, I.-U.; Chen, A.; Xu, C.; Jianglei, R.; Feng, Q.; Li, M. Time-Restricted Feeding Is Associated with Changes in Human Gut Microbiota Related to Nutrient Intake. *Nutrition* **2020**, *78*, 110797. [CrossRef] [PubMed]
47. Zeb, F.; Wu, X.; Chen, L.; Fatima, S.; Haq, I.-U.; Chen, A.; Majeed, F.; Feng, Q.; Li, M. Effect of Time-Restricted Feeding on Metabolic Risk and Circadian Rhythm Associated with Gut Microbiome in Healthy Males. *Br. J. Nutr.* **2020**, *123*, 1216–1226. [CrossRef] [PubMed]
48. Janssen, A.W.F.; Katiraei, S.; Bartosinska, B.; Eberhard, D.; Willems van Dijk, K.; Kersten, S. Loss of Angiopoietin-like 4 (ANGPTL4) in Mice with Diet-Induced Obesity Uncouples Visceral Obesity from Glucose Intolerance Partly via the Gut Microbiota. *Diabetologia* **2018**, *61*, 1447–1458. [CrossRef]
49. Li, S.; Qi, Y.; Ren, D.; Qu, D.; Sun, Y. The Structure Features and Improving Effects of Polysaccharide from Astragalus Membranaceus on Antibiotic-Associated Diarrhea. *Antibiot. Basel Switz.* **2019**, *9*, 8. [CrossRef] [PubMed]
50. Zheng, Z.; Lyu, W.; Ren, Y.; Li, X.; Zhao, S.; Yang, H.; Xiao, Y. Allobaculum Involves in the Modulation of Intestinal ANGPTLT4 Expression in Mice Treated by High-Fat Diet. *Front. Nutr.* **2021**, *8*, 242. [CrossRef] [PubMed]
51. Jiao, X.; Wang, Y.; Lin, Y.; Lang, Y.; Li, E.; Zhang, X.; Zhang, Q.; Feng, Y.; Meng, X.; Li, B. Blueberry Polyphenols Extract as a Potential Prebiotic with Anti-Obesity Effects on C57BL/6 J Mice by Modulating the Gut Microbiota. *J. Nutr. Biochem.* **2019**, *64*, 88–100. [CrossRef]
52. Wang, G.; Zhou, H.-H.; Luo, L.; Qin, L.-Q.; Yin, J.; Yu, Z.; Zhang, L.; Wan, Z. Voluntary Wheel Running Is Capable of Improving Cognitive Function Only in the Young but Not the Middle-Aged Male APPSwe/PS1De9 Mice. *Neurochem. Int.* **2021**, *145*, 105010. [CrossRef] [PubMed]
53. Luo, L.; Li, R.; Wang, G.; Chen, J.; Chen, L.; Qin, L.-Q.; Yu, Z.; Wan, Z. Age-Dependent Effects of a High-Fat Diet Combined with Dietary Advanced Glycation End Products on Cognitive Function and Protection with Voluntary Exercise. *Food Funct.* **2022**, *13*, 4445–4458. [CrossRef] [PubMed]

54. Beilharz, J.; Maniam, J.; Morris, M. Diet-Induced Cognitive Deficits: The Role of Fat and Sugar, Potential Mechanisms and Nutritional Interventions. *Nutrients* **2015**, *7*, 6719–6738. [CrossRef] [PubMed]
55. U.S Department of Health and Human Services. National Diabetes Statistics Report 2020. Available online: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://oversight.house.gov/sites/democrats.oversight.house.gov/files/2022.09.14%20FINAL%20COR%20Supplemental%20Memo.pdf (accessed on 13 August 2022).
56. Liu, Z.; Dai, X.; Zhang, H.; Shi, R.; Hui, Y.; Jin, X.; Zhang, W.; Wang, L.; Wang, Q.; Wang, D.; et al. Gut Microbiota Mediates Intermittent-Fasting Alleviation of Diabetes-Induced Cognitive Impairment. *Nat. Commun.* **2020**, *11*, 855. [CrossRef]

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Comparative Analysis of Gut Microbiota from Rats Induced by Se Deficiency and T-2 Toxin

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Abstract: The aim of this study was to analyze the differences in gut microbiota between selenium deficiency and T-2 toxin intervention rats. Knee joint and fecal samples of rats were collected. The pathological characteristics of knee cartilage were observed by safranin O/fast green staining. DNA was extracted from fecal samples for PCR amplification, and 16S rDNA sequencing was performed to compare the gut microbiota of rats. At the phylum level, *Firmicutes* (81.39% vs. 77.06%) and *Bacteroidetes* (11.11% vs. 14.85%) were dominant in the Se-deficient (SD) group and T-2 exposure (T-2) groups. At the genus level, the relative abundance of *Ruminococcus_1* (12.62%) and *Ruminococcaceae_UCG-005* (10.31%) in the SD group were higher. In the T-2 group, the relative abundance of *Lactobacillus* (11.71%) and *Ruminococcaceae_UCG-005* (9.26%) were higher. At the species level, the high-quality bacteria in the SD group was *Ruminococcus_1_unclassified*, and *Ruminococcaceae_UCG-005_unclassified* in the T-2 group. *Lactobacillus_sp__L_YJ* and *Lactobacillus_crispatus* were the most significant biomarkers in the T-2 group. This study analyzed the different compositions of gut microbiota in rats induced by selenium deficiency and T-2 toxin, and revealed the changes in gut microbiota, so as to provide a certain basis for promoting the study of the pathogenesis of Kashin–Beck disease (KBD).

Keywords: Kashin–Beck disease; T-2 toxin; selenium deficiency; gut microbiota; 16S rDNA gene sequencing

1. Introduction

Kashin–Beck disease (KBD) is a chronic, degenerative and malformed osteoarticular disease that mainly affects the epiphyseal plate cartilage and articular cartilage in children during the growth and development period [1]. Many etiological studies have shown that environmental selenium deficiency and T-2 toxin in grains could be important causes of KBD [2]. Previous studies found that the selenium content in food, drinking water and

patients in KBD endemic areas was significantly lower than that in non-endemic areas [3,4]. In addition, the epidemiological survey showed that the distribution of KBD in China was an oblique band from northeast to southwest, which was highly coincident with the distribution of Se deficiency in China [5]. T-2 toxin is a tricothecene toxin produced by a variety of fusarium species [6]. In previous studies, it was suggested that T-2 toxin could cause inflammatory damage of chondrocytes and upregulate the expression of KBD susceptibility genes in chondrocytes [7,8]. The conclusions of the previous studies have proved that the imbalance of selenium nutrition caused by low selenium in the external environment and T-2 toxin poisoning in grain are likely to be important causes of the etiology and pathogenesis of KBD [9].

Gut microbiota is a microbial community inhabiting the human gut and distributed throughout the gastrointestinal tract, which is a dynamic ecosystem. The entire gut microbiota is composed of more than 1000 species of bacteria, as well as fungi, viruses, phages, parasites and archaea [10]. Under normal conditions, different types of gut microbiota in the human body exist stably in accordance with a certain proportion and quantity, which are both interdependent and restricted to each other. *Firmicutes* and *Bacteroidetes* are the most typical bacterial phyla in the healthy gut microbiota, followed by *Actinobacteria*, *Proteobacteria* and *Fusobacteria* [11,12]. At the genus level, the most typical bacterial genera are *Bacteroides*, *Faecalibacterium* and *Bifidobacterium*. The bacteria of each classification exist dynamically and stably [13]. When stimulated by the internal and external environment, the species, quantity, proportion, and metabolites of each bacterial group will change [14]. Gut microbiota plays an important role in the regulation of various diseases, including diabetes, osteoarthritis, hypertension, chronic obstructive pulmonary disease (COPD), and obesity [15–21]. The influence of gut microbiota on the above diseases is achieved through the comprehensive effect of genetic and environmental factors [22]. Relevant studies have confirmed that gut microbiota can participate in host physiological activities through its metabolites, and can migrate to subchondral bone marrow and deep chondrocytes through systemic circulation to cause cellular inflammation, thereby inducing changes in cartilage tissue and causing osteochondral diseases [23]. It has also been shown that the cartilage–gut–microbiome axis plays a role in chondrocyte differentiation, apoptosis, and injury mechanism by regulating the levels of metabolites [24]. The concept of the cartilage–gut–microbiome axis provides new ideas for the etiology and treatment of bone and cartilage diseases, such as KBD and osteoarthritis. Previously, we found a comprehensive landscape of the gut microbiota in KBD patients, and compared the gut microbiota of osteoarthritis (OA) and KBD patients, which provided strong evidence for the correlation between gut microbiota and KBD.

As the key suspected causes of KBD, mycotoxin contamination in grain and environmental Se deficiency can affect the human body through the dietary channel [1,25,26]. Studies have shown that mycotoxins in grain are absorbed into the human body mainly in the form of glucose covalent conjugates of mycotoxins, and can reach the human intestine in intact form for hydrolysis and release, causing abnormal changes in gut microbiota [27]. In addition, selenium can increase the diversity of gut microbiota in mice and participate in the composition of gut microbiota, and it also can be enriched by the gut microbiota [28,29]. These results suggest that the changes in gut microbiota in KBD patients may be closely related to the above two environmental risk factors of KBD.

In this study, to further explore whether the gut microbiota of KBD patients is altered due to Se deficiency and T-2 toxin and its relationship with cartilage damage, we established the environment risk factors rat models of KBD induced by Se deficiency and T-2 toxin with an 8-week and 4-week intervention, respectively, and the characteristics and differences of gut microbiota after intervention in rats were analyzed and compared. This study will provide an important scientific basis for clarifying that T-2 toxin and/or Se deficiency may cause chondrocytes damage and metabolic disorders in KBD through the cartilage–gut–microbiome axis.

2. Materials and Methods

2.1. Model Construction and Sample Collection

A total of 24 weaned Sprague Dawley rats (3-week-old, 30–50 g) were provided by the Experimental Animal Center of Xi'an Jiaotong University. The study protocol was approved by the Animal Ethics Committee of Xi'an Jiaotong University (No. 2018-206). The rats were reared in specific pathogen free (SPF) environment. The 24 rats were randomly divided into three groups: the normal control group (NC) was fed with control diet (selenium content: 0.18 ppm), the Se-deficient group (SD) was fed with Se-deficient diet (selenium content: 0.02 ppm), the T-2 exposure group (T-2) which fed with control diet were given T-2 toxin (200 ng/g·BW/day) by gavage [30], while the NC group and SD groups were given the same volume of 0.9% normal saline by gavage. All feeds of rats were purchased from Nantong Talofi Feed Company, Nantong, Jiangsu province, China. For intragastric administration: after the rats were fixed, the needle used for intragastric administration was inserted from the corner of the mouth, pushed gently against the roof of the mouth, and there was a feeling of hollow after entering the esophagus. Then, the needle was slowly inserted along the posterior wall of the pharynx into the esophagus, and the solution could be injected only when there was no air countercurrent in the syringe. To prevent the rats from failing to adapt to the T-2 toxin, in the T-2 group, the gavage treatment was performed at the age of 8 weeks and ended at 12 weeks, lasting a total of 4 weeks. Since the construction of the Se-deficient rat models need to last longer, the intervention of Se-deficient diet began at the age of 4 weeks and ended at 12 weeks, lasting a total of 8 weeks. The rats were fed in the mesh floor cages with 2 cages per group and 4 rats per cage, and all animals had free access to distilled water at all time. The weight of the rats was measured and recorded at a fixed time each week. During the experiment, we did not find that the food intake of rats was affected by the intervention regimen. The rats treated by Se-deficient diet had poor appetite and increased drinking water, and the rats treated by T-2 toxin were sluggish, less active, and more serious hair removal. By analyzing the weight records, it was found that the growth of rats in each group was in line with the normal development rate, and there was no statistical difference in each node. At the age of 12 weeks, fresh feces of the rats were collected with sterilized tweezers, and about 1 g (3–5 pellets) of feces were collected from each rat, of which 1–2 pellets were used for analysis and the rest for backup, all stored in the sterile EP tubes at −80 °C for follow-up experiments. At 24 h after the last gavage, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.5 mL/100 g·BW). The rats were sacrificed by blood sampling from the abdominal aorta. After the rats were killed, we detected the serum selenium content of rats by hydride generation atomic fluorescence spectrometry (HG-AFS), and the results showed that the serum selenium content of rats in the SD group was significantly lower than that in the NC group, indicating that the Se-deficient model was successfully constructed. Relevant research results have been published [31]. During the dissection of the rats, the fur and muscle were stripped with scalpel, the knee joints on both sides of the rats were removed with surgical scissors. After carefully separating the connective tissue, the knee joints of rats were placed in the 50 mL centrifuge tubes with 4% paraformaldehyde fixing solution and stored at room temperature away from light for later use.

2.2. T-2 Toxin Solution Preparation

An amount of 5 mg T-2 toxin crystals (CAS: 21259-20-1, purchased from Beijing Bairingwei Technology Co., LTD, Beijing, China) were collected in a bottle, then 2 mL absolute ethanol was added, shaken and blended using a vortex mixer, and the liquid was sucked into a 250 mL beaker. The procedure was repeated three times to ensure the complete dissolution of T-2 toxin crystals on the bottom and wall of the bottle. The 0.9% saline was used to dilute the solution in the beaker, and the T-2 toxin solution with a concentration of 20 μg/mL was obtained. The solution was divided into small aliquots and frozen at −20 °C for subsequent experiment. Before each use, the solution was dissolved at room temperature and sonicated to homogenize the solute.

2.3. Safranin O/Fast Green Staining

The fixed knee joints samples of rats were decalcified with 10% (*w*/*v*) ethylenediaminete traacetic acid (EDTA) for 4 weeks. The decalcified samples were cut to appropriate size with a surgical blade, naturally dried and then subjected to ethanol gradient dehydration, xylene transparency, and paraffin embedding. The paraffin blocks were cut into serial sections with a thickness of 4–7 μm, mounted on slides, baked at 60 °C and stored at room temperature. Sections of the knee joint were deparaffinized with xylene, hydrated with gradient ethanol, and washed with tap water. The sections were stained with hematoxylin dye for 5 min, washed with tap water to remove excess dye, and rapidly differentiated in 1% hydrochloric acid solution for several seconds. The step of fast green staining took 10 min, and the excess dye was washed off with water. Then, the slices were placed in safranin O staining solution for 45 s, washed with water and slightly soaked in differentiation solution. Finally, the sections were rapidly dehydrated in absolute ethanol, transparent through xylene, and then sealed with neutral gum for use. The stained cartilage sections were observed and the images were collected by automatic digital slicing scanner (Pannoramic desk, purchased from 3Dhistech, Budapest, Hungary).

2.4. DNA Extraction of Gut Microbiota

Fecal samples were collected from rats at 12 weeks of age, and the total DNA of the gut microbiota in the fecal samples was extracted according to the instructions of the E.Z.N.A.®Stool DNA Kit (D4015-02, Omega, Inc., Norcross, GA, USA). It has been demonstrated that the reagent, which was designed to extract DNA from trace amounts of sample, is efficient for preparing the DNA of most bacteria. Unused swabs processed through DNA extraction were utilized as sample blanks, and the absence of any DNA amplicons was confirmed in sample blanks. The quality of DNA extraction was detected by agarose gel electrophoresis, and DNA was quantified using a UV spectrophotometer. According to a modified version of the manufacturer's (Omega) instructions, the total DNA was eluted in 50 L of elution buffer and kept at −80 °C until it was measured in a PCR by LC-BIO TECHNOLOGIES (HANGZHOU) CO., LTD., Hang Zhou, Zhejiang Province, China.

2.5. 16S rDNA Gene Sequencing

The V3–V4 variable region of 16S rDNA gene was selected as the target region for PCR amplification. The upstream primer was 341F (5′-CCTACGGGNGGCWGCAG-3′) and the downstream primer was 805R (5′-GACTACHVGGGTATCTAATCC-3′). The 5′ ends of each primer were marked with a unique barcode for each sample, and the universal primers were sequenced. PCR amplification was performed after a total volume of 25 μL of the reaction mixture including 25 ng of template DNA, 12.5 μL PCR Premix, 2.5 μL of each primer, and PCR-grade water which used to adjust the volume was prepared. PCR reaction conditions were as follows: first, predenaturation at 98 °C for 30 s; and then there were 32 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s. The final extension lasted for 10 min at 72 ° C. In order to exclude the possibility of false-positive PCR results as a negative control, ultrapure water was utilized instead of sample solution throughout the DNA extraction procedure. The products of PCR were purified using AMPure XT Beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified with Qubit (Invitrogen, Carlsbad, CA, USA). The amplicon pool was used for the sequencing process, and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Illumina Library Quantification kit (Kapa Biosciences, Woburn, MA, USA), respectively, were used to measure the size and quantity of the amplicon libraries. On NovaSeq PE250 platform, the libraries were sequenced.

Samples were sequenced using the Illumina NovaSeq platform in accordance with the manufacturer's specifications. Paired-end sequences were assigned based on the unique barcode of each sample, and the barcode and primer sequence were both truncated. Paired-end reads were merged using FLASH. To produce high-quality clean labels, the raw reads

were quality filtered using fqtrim (v0.94) under specific filtering parameters. In addition, the chimeric sequences were filtered by Vsearch software (v2.3.4). After data deduplication, feature tables and feature sequences were obtained. The distances were determined for PCoA and LEfSe using the Bray–Curtis method. We used QIIME2 and R (v3.5.2) to calculate the Alpha diversity and Beta diversity and draw the pictures, respectively, reduced the number of sequences in some samples to a minimum by randomly extracting the same number of sequences, and classified bacteria using the relative abundance. The relative abundance of each sample was used to normalize the feature abundance according to SILVA classifier. Blast was used for sequence alignment, the SILVA and NT-16S databases were used for annotation of each representative sequence, and R (v3.5.2) was used for drawing pictures. LEfSe analysis was performed as the following steps: firstly, all characteristic species was detected using Kruskal–Wallis rank sum test, and species with significant differences were obtained by measuring species abundance differences among different groups. Secondly, the Wilcoxon rank sum test was used to detect all subspecies of the significantly different species obtained in the previous step to determine whether they converged to the same taxonomic level. Finally, linear discriminant analysis (LDA) was used to obtain the final differential species.

2.6. Statistical Analysis

We used SPSS 18.0 software for statistical analysis of the measured data and normality tests for continuous variables. If the data met the normal distribution, Student's *t*-test and one-way analysis of variance (ANOVA) were used to evaluate the difference between the two groups, and the LSD-*t* test was further used for comparison between the two groups. Otherwise, the data were reanalyzed after transformation, or the rank-sum test was used for comparison analysis between groups. Spearman correlation analysis was used to explore the correlation between variables. p-value < 0.05 was considered statistically significant. Statistical plots were drawn using GraphPad Prism 8.0.

3. Results

3.1. Pathological Changes in the Knee Joint of the Rats

In the NC group, the cartilage matrix was uniformly red, and the subchondral bone was blue-green, and the cartilage tissue was in sharp contrast with the bone tissue. The articular cartilage was smooth, and the chondrocytes were evenly distributed and arranged neatly. Compared with the NC group, the cartilage zone was thinner, the surface of cartilage appeared fissure, and the red part was significantly reduced in the SD and T-2 group. In addition, articular cartilage showed obvious wear and matrix loss (Figure 1).

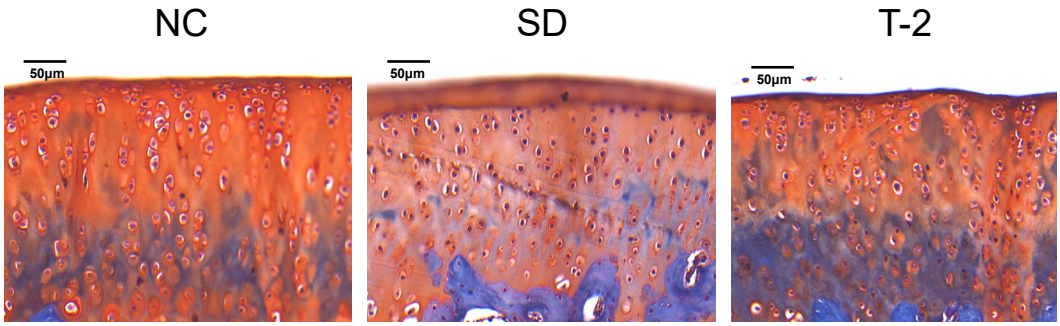

Figure 1. The articular cartilage of the rat knee joint stained with safranin fast green. NC, normal control group; SD, Se-deficient group; T-2, T-2 toxin exposure group.

3.2. Analysis of Gut Microbiota in Rats Exposed to Se Deficiency vs. Negative Control Rats

A total of 914,275 high-quality reads were obtained from 16S rDNA sequencing, with a median of 77,072.5 reads per sample (71,749 to 79,342). There were 8621 features, including 4029 in the SD group and 4592 in the NC group. The alpha diversity and beta diversity were compared between the SD and NC groups to evaluate the characteristics of gut microbiome

in rats intervened by Se deficiency. The alpha diversity was used to assess species richness and evenness of microorganisms in individual sample. In this study, observed species and Chao 1 indices were used to reflect richness, while Shannon and Simpson indices could comprehensively reflect richness and evenness in samples. The results showed that there was no significant difference in Shannon, observed species, Simpson and Chao 1 indices between the SD group and the NC group, indicating that the richness and evenness of microbial communities in the two groups did not change significantly (Figure 2a). The beta diversity was used to measure the magnitude of similarity in microbial community composition between different samples. Principal co-ordinates analysis (PCoA) was used to observe the similarities and differences between groups, and to visualize the similarity or difference of the data. In this study, the PCoA results of both weighted (Figure S1a) and unweighted UniFrac (Figure 2b) showed that there was no significant difference in beta diversity between the SD group and the NC group ($p > 0.05$), indicating that there was no significant difference in the composition and distribution of microbial communities between the two groups.

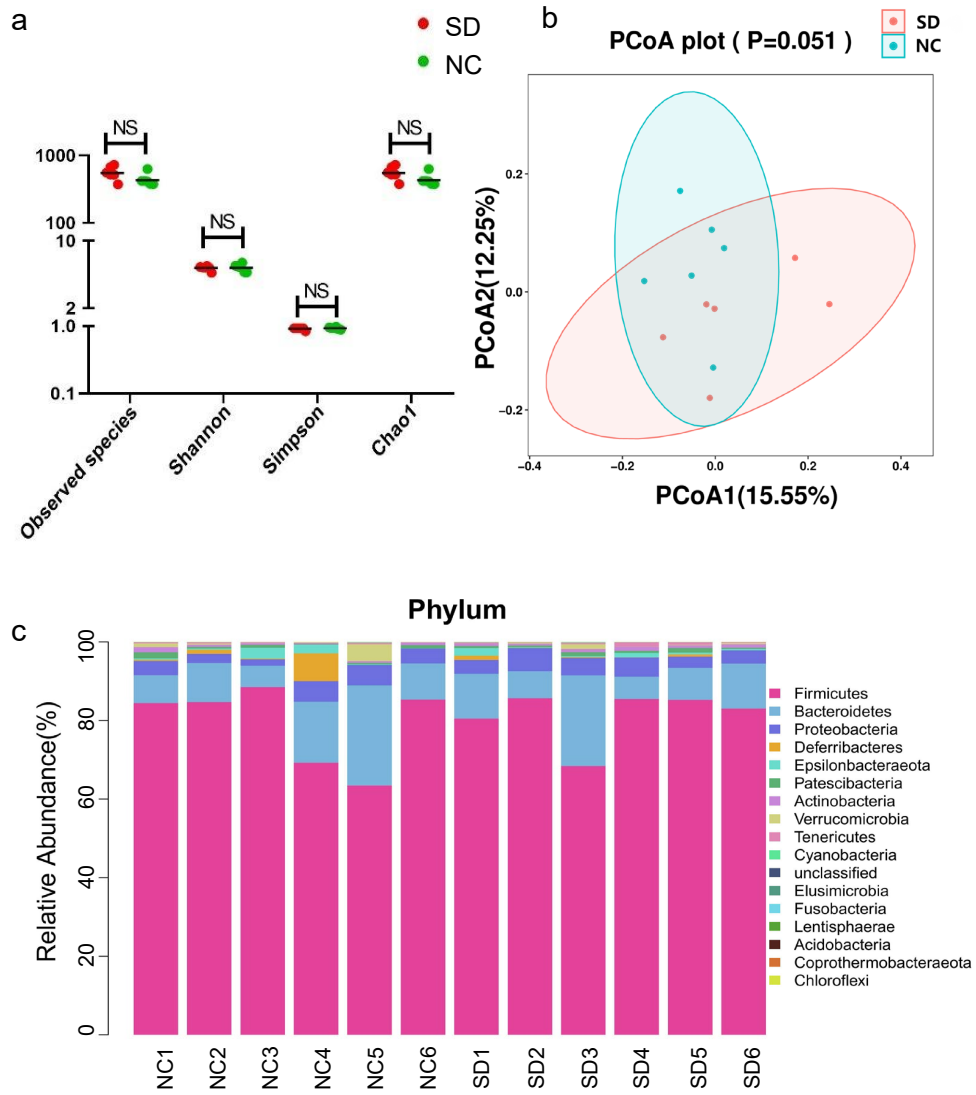

Figure 2. (**a**) Species diversity differences between the SD and NC groups were estimated by the observed species, Shannon, Simpson, and Chao1 indices. SD, the Se deficiency group; NC, the negative control group; (**b**) principal coordinate analysis (PCoA) of the gut microbiota based on the unweighted (ANOSIM, $p = 0.051$) UniFrac distance matrices for the SD and NC groups; (**c**) bar chart of species abundance at the phylum level in the SD group and NC group; $n = 6$ for the SD group and $n = 6$ for the NC group.

Microbial taxon division was performed to evaluate the relative proportions of dominant taxa at the genus level between the SD and NC groups. *Firmicutes* was the dominant phylum in the SD and NC groups, accounting for 81.39% and 79.29%, respectively. The proportion of *Bacteroidetes* in the SD group (11.11%) was lower than that in the NC group (12.12%). On the contrary, the proportion of *Proteobacteria* in SD group (4.17%) was higher than that in the NC group (3.61%) (Figure 2c). At the phylum level, *Firmicutes* was the dominant microbiome in the SD group. At the genus level, in the SD group, the relative abundance of *Ruminococcus_1* was increased and *Ruminococcaceae_UCG-005* was decreased. At the species level, the dominant microbiome of the two groups were *Ruminococcaceae_UCG-005_unclassified* (Figure 3a).

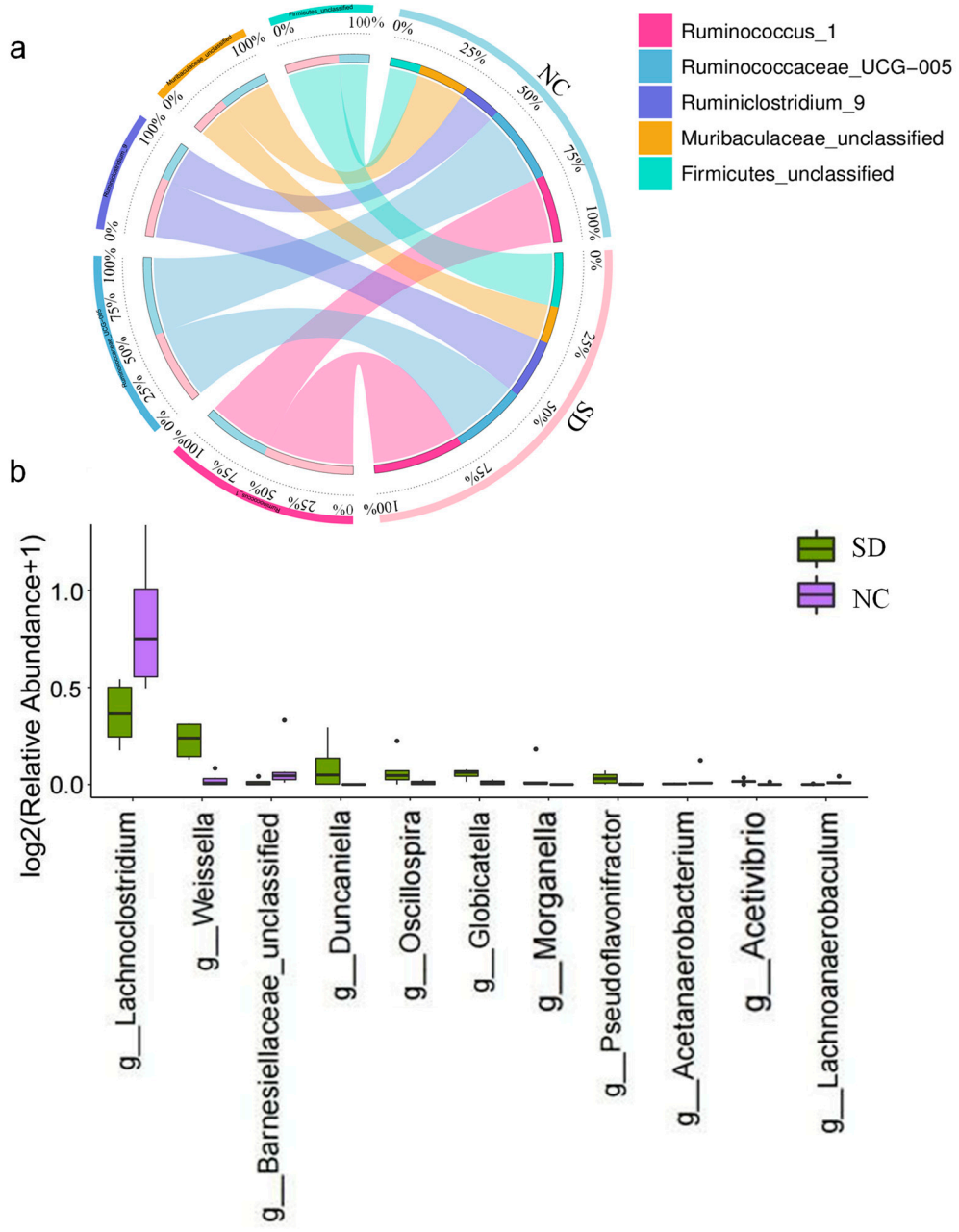

Figure 3. (**a**) Circos plot of species abundance at the genus level in the SD group and the NC group; SD, the Se deficiency group; NC, the negative control group; (**b**) Analysis of Species significant differences at the genus level in the SD group and the NC group. The black dot on each column indicates that one of the samples in the group has a higher abundance value than the other samples.

Linear discriminant analysis effect size (LEfSe) could identify two or more biomarkers and identify the microbiome with significant differences in abundance between groups. The threshold of LEfSe analysis was set as LDA value > 3, $p < 0.05$. In the comparison between the SD group and the NC group, the significant difference species with LDA value > 3 was *Lachnoclostridium* in the NC group, which was a biomarker with statistical differences between the two groups. At the genus level, *Lachnoclostridium*, *Weissella*, *Barnesiellaceae_unclassified* and *Duncaniella* had significant differences in relative abundance (Figure 3b).

In addition, species with the top 30 relative abundance at the genus level were selected. The correlation between these dominant bacterial groups was calculated based on their abundance using the SparCC method, and *p* values were obtained. The relationship pairs with the absolute value of correlation coefficient > 0.4 were selected from the results, and the correlation network diagram was drawn. Among the top 30 species of relative abundance at the genus level, there was significant positive correlation between *Ruminococcus_2* and *Ruminococcus_1*, followed by *Ruminococcus_1* and *Ruminococcaceae_UCG_014*. There was the strongest negative correlation between *Bacteroides* and *Ruminococcaceae_UCG_005* (Figure S1b). The COG database was used to annotate the gene function of the microbiota in samples, which can identify a variety of important functions, such as Na^+/alanine symporter, Transposase (or an inactivated derivative) and maltose binding periplasmic protein periplasmic protein MalE (Figure S2).

3.3. Analysis of Gut Microbiota in Rats Exposed to T-2 Toxin vs. Negative Control Rats

A total of 884,350 high-quality 16S rDNA reads were obtained by sequencing, with a median of 74,370.5 reads per sample (ranging from 67,143 to 77,629). There were 9271 features, including 5272 in the T-2 group and 3999 in the NC group. The alpha diversity and the beta diversity were compared between the T-2 group and the NC group to evaluate the characteristics of gut microbiota in rats intervened by T-2 toxin. The results showed that there was no significant difference in Shannon, observed species, Simpson and Chao 1 indices between the T-2 group and the NC group, indicating that the richness and evenness of microbial community in the two groups did not change significantly (Figure 4a). In this study, the PCoA results of unweighted UniFrac showed that the beta diversity of the T-2 group and the NC group was significantly different ($p < 0.05$) (Figure 4b), while the PCoA results of weighted UniFrac were opposite ($p > 0.05$) (Figure S3a), suggesting that there were differences in the species of the microbiota between the two groups, but there may be no significant difference in species abundance.

To evaluate the relative proportions of dominant taxa at the genus level between the T-2 group and the NC group, microbial taxa were divided. *Firmicutes* was the dominant phylum in the T-2 group and the NC group, accounting for 77.06% and 79.30%, respectively. The proportions of *Bacteroidetes* and *Proteobacteria* in the T-2 group were both higher than those in the NC group, which were 14.85% vs. 12.12% and 3.95% vs. 3.61%, respectively (Figure 4c). At the phylum level, *Firmicutes* was the dominant microbiome in the T-2 group. At the genus level, the relative abundance of *Lactobacillus* in T-2 group was the highest, while *Ruminococcaceae_UCG-005* was the most dominant in the NC group. At the species level, the dominant bacteria of the two groups were *Ruminococcaceae_UCG-005_unclassified* (Figure 5a).

In the results with LDA value > 3, the significant difference species in the T-2 group were *Lactobacillus_sp_L_YJ* and *Lactobacillus_crispatus*. However, the NC group was characterized by *Erysipelatoclostridium* and *Faecalitalea* (Figure 5b). At the genus level, the species with significant differences in relative abundance were mainly *Phascolarctobacterium*, *Erysipelatoclostridium*, *Faecalitalea* and *Veillonella* (Figure S3b). Among the top 30 species in relative abundance at the genus level, *Lactobacillus* was positively correlated with *Christensenellaceae_R_7_group* and *Muribaculaceae_unclassified*. There was a negative correlation between *Bacteroides* and *Ruminococcaceae_UCG_005* (Figure S3c).

Several important functions were identified using the COG database, such as ATPase components of ABC transporters with duplicated ATPase domains and carbamoylphosphate synthase large subunit and guanylate kinase (Figure S4).

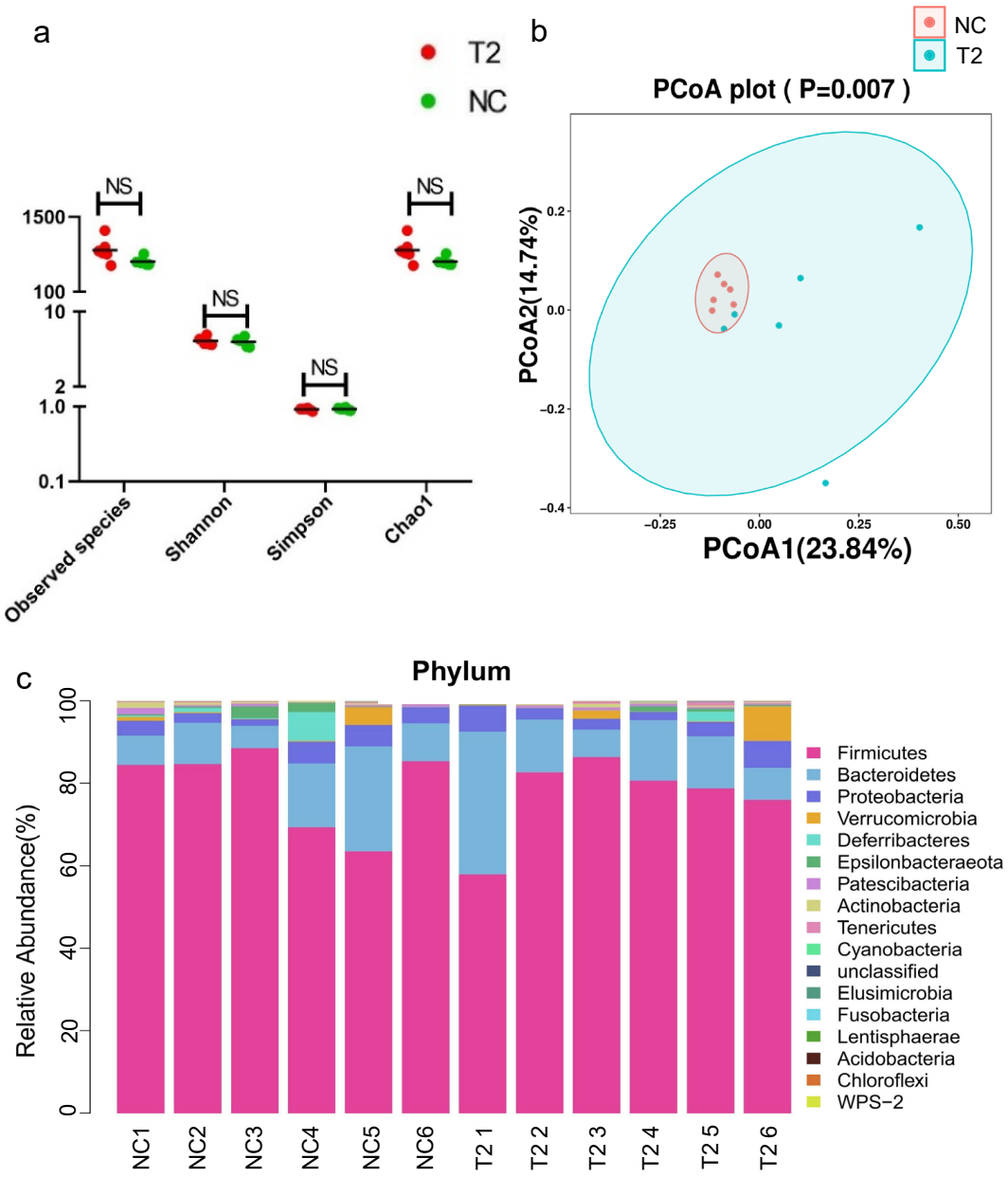

Figure 4. (**a**) Species diversity differences between the T-2 and NC groups were estimated by the observed species, Shannon, Simpson, and Chao1 indices. T-2, the T-2 toxin exposure group; NC, the negative control group; (**b**) principal coordinate analysis (PCoA) of the gut microbiota based on the unweighted (ANOSIM, $p = 0.007$) UniFrac distance matrices for the T-2 and NC groups; (**c**) bar chart of species abundance at the phylum level in the T-2 group and NC group; $n = 6$ for the T-2 group and $n = 6$ for the NC group.

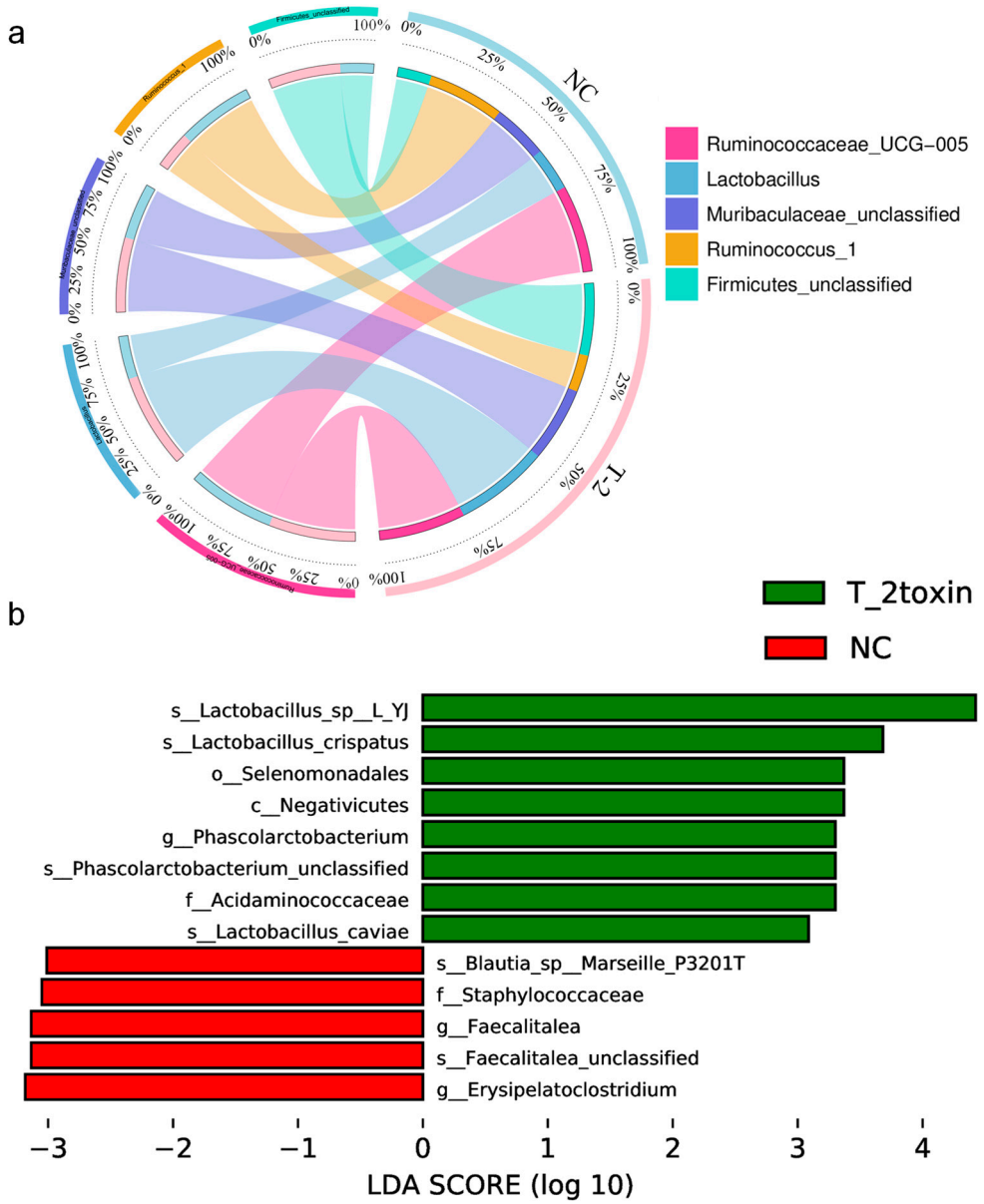

Figure 5. (**a**) Circos plot of species abundance at the genus level in the T-2 group and the NC group; T-2, the T-2 toxin exposure group; NC, the negative control group; (**b**) significantly different species with LDA > 3 in the linear discriminant analysis effect size (LEfSe); the threshold of LEfSe analysis was set as LDA > 3, $p < 0.05$.

3.4. Analysis of Gut Microbiota in Rats Exposed to Se Deficiency vs. T-2 Toxin

At the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* were the main phyla of gut microbiome in the SD and T-2 groups. *Firmicutes* had the highest relative abundance in the SD and T-2 groups, with SD group (81.39%) being higher than T-2 group (77.06%). The proportion of *Bacteroidetes* in SD group (11.11%) was lower than that in T-2 group (14.85%). On the contrary, the level of *Proteobacteria* was higher in SD group (4.17%) than in T-2 group (3.95%). In addition, *Actinobacteria* was the fourth most common bacteria in the SD group (0.70%), while it was lower in the T-2 group (0.50%). The level of *Verrucomicrobia* in the T-2 group was 1.81%, while it was only 0.36% in SD group (Table 1).

Table 1. The relative abundance of species in the SD and T-2 groups at the phylum and genus level.

	SD	**T-2**
Phylum		
Firmicutes	81.39%	77.06%
Bacteroidetes	11.11%	14.85%
Proteobacteria	4.17%	3.95%
Actinobacteria	0.70%	0.50%
Verrucomicrobia	0.36%	1.81%
Genus		
Ruminococcus_1	12.62%	4.58%
Ruminococcaceae_UCG-005	10.31%	10.75%
Ruminiclostridium_9	8.26%	4.74%
Lactobacillus	2.82%	11.71%
Muribaculaceae_unclassified	5.16%	9.26%
Firmicutes_unclassified	7.55%	8.95%

Phylum means the relative abundance of species in the SD and T-2 groups at the phylum level. **Genus** means the relative abundance of species in the SD and T-2 groups at the genus level.

At the genus level, *Ruminococcus*, *Lactobacillus*, *Firmicutes_unclassified* and *Muribaculaceae_unclassified* were the main genus in the SD group and T-2 group. In the SD group, *Ruminococcus* was the most dominant bacteria, accounting for 12.62% of *Ruminococcus_1*, 10.31% of *Ruminococcaceae_UCG-005*, and 8.26% of *Ruminiclostridium_9*, respectively. In the T-2 group, *Lactobacillus* was the most abundant genus, accounting for 11.71%. The second was *Ruminococcaceae_UCG-005* (10.75%). The third most abundant genus was *Muribaculaceae_unclassified* (9.26%). Similarly, the proportion of *Firmicutes_unclassified* was the fourth in the SD group and the T-2 group, which was 7.55% and 8.95%, respectively, showing a higher level in the T-2 group (Table 1).

In addition, at the genus level, a total of 11 species with significant differences in relative abundance in the SD group were identified: *Weissella*, *Morganella*, *Lachnoanaerobaculum*, *Barnesiellaceae_unclassified*, *Globicatella*, *Acetivibrio*, *Duncaniella*, *Lachnoclostridium*, *Acetanaerobacterium*, *Oscillospira* and *Pseudoflavonifractor*. In the T-2 group, a total of 15 species with significant differences in relative abundance were identified: *Acetanaerobacterium*, *Hydrogenoanaerobacterium*, *Rikenella*, *Weissella*, *Veillonella*, *Phocea*, *Pseudoflavonifractor*, *Aerococcus*, *DTU014_unclassified*, *Ruminococcaceae_UCG-011*, *Eubacterium_xylanophilum_group*, *Acetovibrio*, *Phascolarctobacterium*, *Erysipelatoclostridium*, and *Faecalitalea*. Among them, *Weissella*, *Acetivibrio* and *Pseudoflavonifractor* were significantly increased in SD and T-2 groups, while *Acetanaerobacterium* was significantly decreased in both groups (Table 2A).

Table 2. Comparison of the differences in the microbiota between SD group and T-2 group at the genus level.

	SD	**T-2**
(A) *		
Weissella	Up (3.45)	up (3.06)
Acetivibrio	up (2.89)	up (5.30)
Pseudoflavonifractor	up (3.49)	up (4.61)
Acetanaerobacterium	down (−2.93)	down (−Inf)
(B) **		
	Morganella (Inf)	*Veillonella* (2.03)
	Globicatella (2.46)	*Ruminococcaceae_UCG-011* (Inf)
	Duncaniella (Inf)	*Eubacterium_xylanophilum_group* (1.98)
	Oscillospira (3.05)	*Phascolarctobacterium* (0.49)

Table 2. *Cont.*

	SD	**T-2**
(C) ***		
	Lachnoanaerobaculum (−4.49)	*Hydrogenoanaerobacterium* (-Inf)
	Barnesiellaceae_unclassified (−3.11)	*Rikenella* (−3.98)
	Lachnoclostridium (−1.44)	*Phocea* (−5.03)
		Aerococcus (−4.86)
		DTU014_unclassified (−3.49)
		Erysipelatoclostridium (−1.69)
		Faecalitalea (−3.30)

* Means the same significantly different species and abundance value (Log_2FC). ** Means the significantly different species and abundance value (Log_2FC) with increased levels. *** Means the significantly different species and abundance value (Log_2FC) with decreased levels.

In the SD group, species with increased levels included *Morganella*, *Globicatella*, *Duncaniella* and *Oscillospira*; in the T-2 group, the levels of *Veillonella*, *Ruminococcaceae_UCG-011*, *Eubacterium_xylanophilum_group* and *Phascolarctobacterium* increased (Table 2B); in the SD group, the levels of *Lachnoanaerobaculum*, *Barnesiellaceae_unclassified* and *Lachnoclostridium* were decreased; in the T-2 group, the levels of *Hydroanaerobacterium*, *Rikenella*, *Phocea*, *Aerococcus*, *DTU014_unclassified*, *Erysipelatoclostridium* and *Faecalitalea* were decreased (Table 2C).

4. Discussion

Gut microbiota, composed of thousands of microbiota, strives to maintain the existence and function of intestinal mucosal barrier, participate in nutrient absorption and digestion, substance transport and metabolism, regulate human immunity, growth and development [32]. Studies on the changes in gut microbiota in osteochondral diseases have suggested that the gut microbiota of patients with osteochondral diseases is diverse, and there is a certain correlation between the pathogenesis of such diseases and gut microbiota [33]. Based on our previous study on the gut microbiota profile of KBD patients and the results of comparing the gut microbiota of OA and KBD patients, we constructed rat models of Se deficiency and T-2 toxin exposure, respectively, and conducted 16SrDNA sequencing to analyze gut microbiota, aiming to investigate whether gut microbiota affects the occurrence and development of KBD, hoping to provide inspiration for the prevention and treatment of KBD.

In our previous study, the KBD patient was found to be characterized by elevated levels of *Fusobacteria* and *Bacteroidetes*. Consistent with the 16S rDNA analysis at the genus level, most of the differentially abundant species in KBD subjects belonged to *Prevotella* according to metagenomic sequencing. This indicates a change in the composition of the gut microbiota in patients with KBD. In the results of this study, *Firmicutes* were increased in the SD group compared with the NC group, while the level of *Bacteroidetes* were decreased. In contrast, the changes in the T-2 group showed a decrease in *Firmicutes* and an increase in *Bacteroidetes*. The changes in *Proteobacteria* were consistent in the SD and T-2 groups, which were higher than those in the NC group. In addition, the beta diversity of the T-2 group was significantly different from that of the NC group ($p < 0.05$), suggesting that the species of the T-2 group was different from that of the NC group. We can speculate that both Se deficiency and T-2 toxin exposure can dysregulate the composition of the gut microbiota in KBD, and the effects they play are different.

As an important group of bacteria in the gut, *Bacteroidetes* are involved in a variety of metabolic activities and can decompose and utilize polysaccharides to help digest carbohydrates [34]. Among them, *Bacteroides* and *Prevotella* have attracted more attention from researchers. *Bacteroides* is involved in the degradation of polysaccharides and dietary fiber [35]. Studies have found that *Bacteroides* can stimulate the immune system, enhance the phagocytosis of macrophages, regulate the metabolism of the body, and induce the proliferation of probiotics to promote the health of the body [36]. *Bacteroides fragilis* has

beneficial immunomodulatory effects on the body [37]. *Prevotella* can use polysaccharides to produce metabolites such as acetic acid and succinic acid [38]. Succinic acid can help maintain the body's immunity and improve the health of the host [39]. Studies on the function of selenium point out that an adequate level of selenium is important for the initiation of immunity [40]. Se deficiency and inhibition of selenoproteins expression are associated with elevated levels of inflammatory cytokines in the gastrointestinal tract. Under Se deficiency conditions, innate and adaptive immune responses are impaired [41]. In addition, a study of T-2 toxin pointed out that T-2 toxin disrupts the gut microbiota by changing the relative abundance of species, genus, and phylum levels, and this change may be a direct effect of the toxin and its antibacterial properties, a toxic consequence of T-2 toxin on cells, or the release of antibacterial substances [42]. In this study, the level of *Bacteroidetes* in the SD group was decreased, indicating that the low selenium nutritional status caused the decrease in *Bacteroides* abundance, which led to the suppression of immune level, and then caused cartilage damage. However, the level of *Bacteroidetes* was increased in the T-2 group, which may be caused by the toxic characteristics of T-2 toxin.

Firmicutes is the largest bacterial group in the gut, most of which are Gram-positive bacteria [43]. *Lactobacillus* is the most well-known probiotic, which is colonized in the human digestive tract and widely used in the food industry, medicine and health fields. It can participate in the metabolism of a variety of amino acids, help maintain the balance of gut microbiota, improve host immunity, and maintain the health of women's vagina [44]. Studies have shown that *Lactobacillus* can ameliorate or limit inflammatory bone damage and joint dysfunction in patients with OA by reducing the expression of proinflammatory cytokines and cartilage damage [45]. Based on its anti-inflammatory effect, *Lactobacillus* has also been used as a probiotic treatment for OA for a long time [46]. In this study, compared with the NC group, the abundance of *Lactobacillus* in the T-2 group was significantly increased, and it was positively correlated with other bacteria such as *Christensenellaceae_R_7_group* and *Muribaculaceae_unclassified*. We speculated that the rise of *Lactobacillus* may be due to the lack of direct competition. These results indicated that T-2 toxin could not only unbalanced the gut microbiota of rats, but also regulate the quantity and function of other gut microbiota by affecting the level of *Lactobacillus*.

Ruminococcus is one of the earliest discovered gastric bacteria. It can degrade cellulose in the digestive tract and ferment glucose and xylose. Therefore, *Ruminococcus* can effectively break down the cell wall of plants in the digestive tract, which helps to stabilize the intestinal barrier and improve the body's immunity [47]. *Ruminococcus* can induce anti-inflammatory or pro-inflammatory responses in the host, reflecting the characteristics of strain-specific immune regulation [48]. In this study, the relative abundance of *Ruminococcus_1* and *Ruminococcaceae_UGG-005* in the SD group is higher at the genus level, and the dominant bacteria in the SD group all belong to *Ruminococcus*, indicating that exposure of Se deficiency causes the imbalance of gut microbiota in rats, and the number and proportion of *Ruminococcus* changed significantly.

The characteristic pathological changes in KBD are necrosis of deep chondrocytes in growth plate cartilage and articular cartilage. Previous studies have shown that Se supplementation has a protective effect on growth plate cartilage, alleviates the necrosis of chondrocytes in growth plate. T-2 toxins can induce oxidative stress and reduce collagen type II and chondroitin sulfate in chondrocytes; therefore, Se deficiency and T-2 toxin were considered as most important risk factor for causing and development of KBD [49]. The human gut microbiome is strongly influenced by what they have taken in, such as Se deficiency and T-2 toxin, for example, Se deficiency affects the composition and colonization of the microbiome, which may interfere with the diversity of the microbiome [50,51]. And dietary selenium supplementation in mice can optimize the composition of gut microbiome and reduce the dysfunction of gut microbiome caused by low selenium [52]. In addition, the intestinal tract plays an important role in the metabolism and absorption of T-2 toxin, leading to intestinal mucosal damage, inflammation and oxidative stress, such as necrotizing enteritis and colibacillosis in animals. Fusarium toxins, such as T-2 toxin, was

stable in gastrointestinal digestive fluid and cannot be absorbed by intestinal epithelial cells. When they come into contact with the gut microbiota, they can be efficiently hydrolyzed, allowing the metabolites to be easily absorbed from the gut [27]. According to our previous study on KBD patients, we found considerable variability in the gut microbiota, such as an increase in *Bacteroides* and a decrease in *Firmicutes*, which were similar to those in this study. At present, the mechanism of Se deficiency and fusarium toxin causing KBD has not been fully clarified. Based on the results of this study and the above discussion, we speculated that Se deficiency and T-2 toxin would affect the composition of human gut microbiota and its metabolites through dietary intake and intestinal absorption, resulting in the dysregulation of gut microbiota, and then induced joint cartilage damage through the cartilage–gut–microbiome axis, thereby triggering KBD. We believe that Se deficiency and T-2 toxin cause gut microbial dysregulation, which leads to joint damage, is one of the mechanisms triggering KBD. However, the specific mechanism is still unclear and needs further study.

5. Conclusions

In conclusion, this study comparatively analyzed the different compositions of gut microbiota in rats induced by Se deficiency and T-2 toxin, and identified and revealed the changes in the phylum, genus and species levels of gut microbiota. The results not only comprehensively reflect the situation of gut microbiota in rats with Se deficiency and T-2 toxin exposure, but also provide some clues for the role of the "cartilage–gut–microbiome" axis in the occurrence and development of KBD, and provide a scientific basis for promoting the study of the etiology and pathogenesis of KBD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15245027/s1, Figure S1: (a) the weighted PCoA results of the gut microbiota for the SD and NC groups; (b) Species correlation network in the SD group; Figure S2: COG database function annotation results between the SD and NC groups; Figure S3: (a) the weighted PCoA results of the gut microbiota for the T-2 and NC groups; (b) Analysis of Species significant differences at the genus level; (c) Species correlation network in the T-2 group; Figure S4: COG database function annotation results between the T-2 and NC groups.

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References

1. Wang, K.; Yu, J.; Liu, H.; Liu, Y.; Liu, N.; Cao, Y.; Zhang, X.; Sun, D. Endemic Kashin-Beck disease: A food-sourced osteoarthropathy. *Semin. Arthritis Rheum.* **2020**, *50*, 366–372. [CrossRef]

2. Shi, T.; Fu, X.; Wang, F.; Zhang, X.; Cai, Y.; Wu, X.; Sun, L. The WNT/β-catenin signalling pathway induces chondrocyte apoptosis in the cartilage injury caused by T-2 toxin in rats. *Toxicon* **2021**, *204*, 14–20. [CrossRef] [PubMed]
3. Fu, G.; Chen, X.; Qi, M.; Du, X.; Xia, Z.; Liu, Q.; Sun, N.; Shi, C.; Zhang, R. Status and potential diagnostic roles of essential trace elements in Kashin- Beck disease patients. *J. Trace Elem. Med. Biol.* **2022**, *69*, 126880. [CrossRef] [PubMed]
4. Zhang, S.; Li, B.; Luo, K. Differences of selenium and other trace elements abundances between the Kaschin-Beck disease area and nearby non-Kaschin-Beck disease area, Shaanxi Province, China. *Food Chem.* **2022**, *373*, 131481. [CrossRef] [PubMed]
5. Wang, J.; Wang, X.; Li, H.; Yang, L.; Li, Y.; Kong, C. Spatial distribution and determinants of health loss from Kashin-Beck disease in Bin County, Shaanxi Province, China. *BMC Public Health* **2021**, *21*, 387. [CrossRef] [PubMed]
6. Janik, E.; Niemcewicz, M.; Podogrocki, M.; Ceremuga, M.; Stela, M.; Bijak, M. T-2 Toxin-The Most Toxic Trichothecene Mycotoxin: Metabolism, Toxicity, and Decontamination Strategies. *Molecules* **2021**, *26*, 6868. [CrossRef] [PubMed]
7. Liu, J.; Wang, L.; Guo, X.; Pang, Q.; Wu, S.; Wu, C.; Xu, P.; Bai, Y. The role of mitochondria in T-2 toxin-induced human chondrocytes apoptosis. *PLoS ONE* **2014**, *9*, e108394. [CrossRef]
8. Shi, Y.; Shao, X.; Sun, M.; Ma, J.; Li, B.; Zou, N.; Li, F. MiR-140 is involved in T-2 toxin-induced matrix degradation of articular cartilage. *Toxicon* **2023**, *222*, 106987. [CrossRef] [PubMed]
9. Guo, Y.; Li, H.; Yang, L.; Li, Y.; Wei, B.; Wang, W.; Gong, H.; Guo, M.; Nima, C.; Zhao, S.; et al. Trace Element Levels in Scalp Hair of School Children in Shigatse, Tibet, an Endemic Area for Kaschin-Beck Disease (KBD). *Biol. Trace Elem. Res.* **2017**, *180*, 15–22. [CrossRef]
10. Sommer, F.; Bäckhed, F. The gut microbiota--masters of host development and physiology. *Nat. Rev. Microbiol.* **2013**, *11*, 227–238. [CrossRef]
11. Arumugam, M.; Raes, J.; Pelletier, E.; Le Paslier, D.; Yamada, T.; Mende, D.R.; Fernandes, G.R.; Tap, J.; Bruls, T.; Batto, J.M.; et al. Enterotypes of the human gut microbiome. *Nature* **2011**, *473*, 174–180. [CrossRef] [PubMed]
12. Li, Y.; Qin, C.; Dong, L.; Zhang, X.; Wu, Z.; Liu, L.; Yang, J.; Liu, L. Whole grain benefit: Synergistic effect of oat phenolic compounds and β-glucan on hyperlipidemia via gut microbiota in high-fat-diet mice. *Food Funct.* **2022**, *13*, 12686–12696. [CrossRef] [PubMed]
13. Osuna-Prieto, F.J.; Xu, H.; Ortiz-Alvarez, L.; Di, X.; Kohler, I.; Jurado-Fasoli, L.; Rubio-Lopez, J.; Plaza-Díaz, J.; Vilchez-Vargas, R.; Link, A.; et al. The relative abundance of fecal bacterial species belonging to the Firmicutes and Bacteroidetes phyla is related to plasma levels of bile acids in young adults. *Metabolomics* **2023**, *19*, 54. [CrossRef] [PubMed]
14. Dong, L.; Qin, C.; Li, Y.; Wu, Z.; Liu, L. Oat phenolic compounds regulate metabolic syndrome in high fat diet-fed mice via gut microbiota. *Food Biosci.* **2022**, *50*, 101946. [CrossRef]
15. Qin, J.; Li, R.; Raes, J.; Arumugam, M.; Burgdorf, K.S.; Manichanh, C.; Nielsen, T.; Pons, N.; Levenez, F.; Yamada, T.; et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **2010**, *464*, 59–65. [CrossRef]
16. Liang, Y.; Zhan, J.; Liu, D.; Luo, M.; Han, J.; Liu, X.; Liu, C.; Cheng, Z.; Zhou, Z.; Wang, P. Organophosphorus pesticide chlorpyrifos intake promotes obesity and insulin resistance through impacting gut and gut microbiota. *Microbiome* **2019**, *7*, 19. [CrossRef]
17. Liu, B.N.; Liu, X.T.; Liang, Z.H.; Wang, J.H. Gut microbiota in obesity. *World J. Gastroenterol.* **2021**, *27*, 3837–3850. [CrossRef]
18. Iatcu, C.O.; Steen, A.; Covasa, M. Gut Microbiota and Complications of Type-2 Diabetes. *Nutrients* **2021**, *14*, 166. [CrossRef]
19. Verhaar, B.J.H.; Prodan, A.; Nieuwdorp, M.; Muller, M. Gut Microbiota in Hypertension and Atherosclerosis: A Review. *Nutrients* **2020**, *12*, 2982. [CrossRef]
20. Lai, H.C.; Lin, T.L.; Chen, T.W.; Kuo, Y.L.; Chang, C.J.; Wu, T.R.; Shu, C.C.; Tsai, Y.H.; Swift, S.; Lu, C.C. Gut microbiota modulates COPD pathogenesis: Role of anti-inflammatory Parabacteroides goldsteinii lipopolysaccharide. *Gut* **2022**, *71*, 309–321. [CrossRef]
21. Ramires, L.C.; Santos, G.S.; Ramires, R.P.; da Fonseca, L.F.; Jeyaraman, M.; Muthu, S.; Lana, A.V.; Azzini, G.; Smith, C.S.; Lana, J.F. The Association between Gut Microbiota and Osteoarthritis: Does the Disease Begin in the Gut? *Int. J. Mol. Sci.* **2022**, *23*, 1494. [CrossRef] [PubMed]
22. Gomaa, E.Z. Human gut microbiota/microbiome in health and diseases: A review. *Antonie Van Leeuwenhoek* **2020**, *113*, 2019–2040. [CrossRef] [PubMed]
23. Jeffries, M.A.; Donica, M.; Baker, L.W.; Stevenson, M.E.; Annan, A.C.; Beth Humphrey, M.; James, J.A.; Sawalha, A.H. Genome-Wide DNA Methylation Study Identifies Significant Epigenomic Changes in Osteoarthritic Subchondral Bone and Similarity to Overlying Cartilage. *Arthritis Rheumatol.* **2016**, *68*, 1403–1414. [CrossRef] [PubMed]
24. Berthelot, J.M.; Sellam, J.; Maugars, Y.; Berenbaum, F. Cartilage-gut-microbiome axis: A new paradigm for novel therapeutic opportunities in osteoarthritis. *RMD Open* **2019**, *5*, e001037. [CrossRef] [PubMed]
25. Zou, K.; Liu, G.; Wu, T.; Du, L. Selenium for preventing Kashin-Beck osteoarthropathy in children: A meta-analysis. *Osteoarthr. Cartil.* **2009**, *17*, 144–151. [CrossRef]
26. Peng, A.; Wang, W.H.; Wang, C.X.; Wang, Z.J.; Rui, H.F.; Wang, W.Z.; Yang, Z.W. The role of humic substances in drinking water in Kashin-Beck disease in China. *Environ. Health Perspect.* **1999**, *107*, 293–296. [CrossRef] [PubMed]
27. Gratz, S.W.; Dinesh, R.; Yoshinari, T.; Holtrop, G.; Richardson, A.J.; Duncan, G.; MacDonald, S.; Lloyd, A.; Tarbin, J. Masked trichothecene and zearalenone mycotoxins withstand digestion and absorption in the upper GI tract but are efficiently hydrolyzed by human gut microbiota in vitro. *Mol. Nutr. Food Res.* **2017**, *61*, 1600680. [CrossRef]

28. Kasaikina, M.V.; Kravtsova, M.A.; Lee, B.C.; Seravalli, J.; Peterson, D.A.; Walter, J.; Legge, R.; Benson, A.K.; Hatfield, D.L.; Gladyshev, V.N. Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. *FASEB J.* **2011**, *25*, 2492–2499. [CrossRef] [PubMed]
29. Knezevic, J.; Starchl, C.; Tmava Berisha, A.; Amrein, K. Thyroid-Gut-Axis: How Does the Microbiota Influence Thyroid Function? *Nutrients* **2020**, *12*, 1769. [CrossRef]
30. Yang, H.J.; Zhang, Y.; Wang, Z.L.; Xue, S.H.; Li, S.Y.; Zhou, X.R.; Zhang, M.; Fang, Q.; Wang, W.J.; Chen, C.; et al. Increased Chondrocyte Apoptosis in Kashin-Beck Disease and Rats Induced by T-2 Toxin and Selenium Deficiency. *Biomed. Environ. Sci.* **2017**, *30*, 351–362. [CrossRef]
31. Wu, Y.; Gong, Y.; Liu, Y.; Chen, F.; Chen, S.; Zhang, F.; Wang, C.; Li, S.; Hu, M.; Huang, R.; et al. Comparative Analysis of Differentially Expressed Genes in Chondrocytes from Rats Exposed to Low Selenium and T-2 Toxin. *Biol. Trace Elem. Res.* **2023**. [CrossRef] [PubMed]
32. Adak, A.; Khan, M.R. An insight into gut microbiota and its functionalities. *Cell. Mol. Life Sci.* **2019**, *76*, 473–493. [CrossRef]
33. Liu, Y.; Ding, W.; Wang, H.L.; Dai, L.L.; Zong, W.H.; Wang, Y.Z.; Bi, J.; Han, W.; Dong, G.J. Gut microbiota and obesity-associated osteoarthritis. *Osteoarthr. Cartil.* **2019**, *27*, 1257–1265. [CrossRef] [PubMed]
34. Gibiino, G.; Lopetuso, L.R.; Scaldaferri, F.; Rizzatti, G.; Binda, C.; Gasbarrini, A. Exploring Bacteroidetes: Metabolic key points and immunological tricks of our gut commensals. *Dig. Liver Dis.* **2018**, *50*, 635–639. [CrossRef] [PubMed]
35. Martens, E.C.; Chiang, H.C.; Gordon, J.I. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* **2008**, *4*, 447–457. [CrossRef] [PubMed]
36. Wang, C.; Zhao, J.; Zhang, H.; Lee, Y.K.; Zhai, Q.; Chen, W. Roles of intestinal bacteroides in human health and diseases. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 3518–3536. [CrossRef] [PubMed]
37. Sun, F.; Zhang, Q.; Zhao, J.; Zhang, H.; Zhai, Q.; Chen, W. A potential species of next-generation probiotics? The dark and light sides of Bacteroides fragilis in health. *Food Res. Int.* **2019**, *126*, 108590. [CrossRef]
38. Fehlner-Peach, H.; Magnabosco, C.; Raghavan, V.; Scher, J.U.; Tett, A.; Cox, L.M.; Gottsegen, C.; Watters, A.; Wiltshire-Gordon, J.D.; Segata, N.; et al. Distinct Polysaccharide Utilization Profiles of Human Intestinal Prevotella copri Isolates. *Cell Host Microbe* **2019**, *26*, 680–690.e685. [CrossRef]
39. Mills, E.; O'Neill, L.A. Succinate: A metabolic signal in inflammation. *Trends Cell Biol.* **2014**, *24*, 313–320. [CrossRef]
40. Huang, Z.; Rose, A.H.; Hoffmann, P.R. The role of selenium in inflammation and immunity: From molecular mechanisms to therapeutic opportunities. *Antioxid. Redox Signal.* **2012**, *16*, 705–743. [CrossRef]
41. Avery, J.C.; Hoffmann, P.R. Selenium, Selenoproteins, and Immunity. *Nutrients* **2018**, *10*, 1203. [CrossRef] [PubMed]
42. Zhang, J.; Liu, X.; Su, Y.; Li, T. An update on T2-toxins: Metabolism, immunotoxicity mechanism and human assessment exposure of intestinal microbiota. *Heliyon* **2022**, *8*, e10012. [CrossRef] [PubMed]
43. Ahlawat, S.; Asha; Sharma, K.K. Gut-organ axis: A microbial outreach and networking. *Lett. Appl. Microbiol.* **2021**, *72*, 636–668. [CrossRef] [PubMed]
44. Chee, W.J.Y.; Chew, S.Y.; Than, L.T.L. Vaginal microbiota and the potential of Lactobacillus derivatives in maintaining vaginal health. *Microb. Cell Fact.* **2020**, *19*, 203. [CrossRef]
45. Lee, S.H.; Kwon, J.Y.; Jhun, J.; Jung, K.; Park, S.H.; Yang, C.W.; Cho, Y.; Kim, S.J.; Cho, M.L. Lactobacillus acidophilus ameliorates pain and cartilage degradation in experimental osteoarthritis. *Immunol. Lett.* **2018**, *203*, 6–14. [CrossRef] [PubMed]
46. Lei, M.; Guo, C.; Wang, D.; Zhang, C.; Hua, L. The effect of probiotic Lactobacillus casei Shirota on knee osteoarthritis: A randomised double-blind, placebo-controlled clinical trial. *Benef. Microbes* **2017**, *8*, 697–703. [CrossRef]
47. Mizrahi, I.; Wallace, R.J.; Moraïs, S. The rumen microbiome: Balancing food security and environmental impacts. *Nat. Rev. Microbiol.* **2021**, *19*, 553–566. [CrossRef]
48. Crost, E.H.; Coletto, E.; Bell, A.; Juge, N. Ruminococcus gnavus: Friend or foe for human health. *FEMS Microbiol. Rev.* **2023**, *47*, fuad014. [CrossRef]
49. Zhou, X.; Yang, H.; Guan, F.; Xue, S.; Song, D.; Chen, J.; Wang, Z. T-2 Toxin Alters the Levels of Collagen II and Its Regulatory Enzymes MMPs/TIMP-1 in a Low-Selenium Rat Model of Kashin-Beck Disease. *Biol. Trace Elem. Res.* **2016**, *169*, 237–246. [CrossRef]
50. Wu, G.D.; Chen, J.; Hoffmann, C.; Bittinger, K.; Chen, Y.Y.; Keilbaugh, S.A.; Bewtra, M.; Knights, D.; Walters, W.A.; Knight, R.; et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* **2011**, *334*, 105–108. [CrossRef]
51. Ferreira, R.L.U.; Sena-Evangelista, K.C.M.; de Azevedo, E.P.; Pinheiro, F.I.; Cobucci, R.N.; Pedrosa, L.F.C. Selenium in Human Health and Gut Microflora: Bioavailability of Selenocompounds and Relationship With Diseases. *Front. Nutr.* **2021**, *8*, 685317. [CrossRef] [PubMed]
52. Qiao, L.; Zhang, X.; Pi, S.; Chang, J.; Dou, X.; Yan, S.; Song, X.; Chen, Y.; Zeng, X.; Zhu, L.; et al. Dietary supplementation with biogenic selenium nanoparticles alleviate oxidative stress-induced intestinal barrier dysfunction. *NPJ Sci. Food* **2022**, *6*, 30. [CrossRef] [PubMed]

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The Human Gut and Dietary Salt: The *Bacteroides*/*Prevotella* Ratio as a Potential Marker of Sodium Intake and Beyond

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Abstract: The gut microbiota is a dynamic ecosystem that plays a pivotal role in maintaining host health. The perturbation of these microbes has been linked to several health conditions. Hence, they have emerged as promising targets for understanding and promoting good health. Despite the growing body of research on the role of sodium in health, its effects on the human gut microbiome remain under-explored. Here, using nutrition and metagenomics methods, we investigate the influence of dietary sodium intake and alterations of the human gut microbiota. We found that a high-sodium diet (HSD) altered the gut microbiota composition with a significant reduction in *Bacteroides* and inverse increase in *Prevotella* compared to a low-sodium diet (LSD). However, there is no clear distinction in the Firmicutes/Bacteroidetes (F/B) ratio between the two diet types. Metabolic pathway reconstruction revealed the presence of sodium reabsorption genes in the HSD, but not LSD. Since it is currently difficult in microbiome studies to confidently associate the F/B ratio with what is considered healthy (e.g., low sodium) or unhealthy (e.g., high sodium), we suggest that the use of a genus-based ratio such as the *Bacteroides*/*Prevotella* (B/P) ratio may be more beneficial for the application of microbiome studies in health.

Keywords: microbiome; gut; microbial ratios; ENaC; TspO; sodium intake; cholesterol; *Bacteroides*; *Prevotella*

1. Introduction

The gut microbiota is an intricate and dynamic ecosystem, hosting an array of microorganisms that play a pivotal role in maintaining host health [1,2]. The perturbation of these microbes is linked to several conditions, such as cardiovascular diseases [3,4], respiratory diseases [5–7], neurological diseases [8,9], gastrointestinal diseases [10,11], endocrine diseases [12,13], immunity [14], cancers [15], and the brain–gut–immune axis [16]. At the phyla level, the bacteria in the human gut are mainly composed of Firmicutes and Bacteroidetes; others are Actinobacteria, Proteobacteria, Spirochetes, Synergistetes, Verrucomicrobiota, and Fusobacteria [17–19].

The microbial definition of a healthy human gut is debated because of the variation in what constitutes a 'healthy microbiome' [11]. However, the answer is being unravelled with the advancement in high-resolution genomic studies such as metagenomics, metatranscriptomics, and metabolomics [20]. The gut microbiota is influenced in the foetus by the maternal diet, health, and exposures; at infancy by the mode of birth, birth term, and feeding; and in childhood and later life by host genetics, lifestyle, diet, age, environment, and medications [21–23]. However, microbial diversity and ecological balance is largely accepted as a measure of a 'healthy gut' [24].

High sodium intake is a global health concern, primarily because of its association with hypertension and cardiovascular diseases, and could adversely affect other targeted organs

even when there is no increase in blood pressure [25–27]. The World Health Organization recommends an intake of less than 2000 mg of sodium/day [27]. However, most people around the world still consume too much sodium, which can cause about 1.7 million deaths each year [28]. Dietary sodium is predominantly consumed from salt. The implications of sodium on the gut microbiome and the potential mechanistic pathways through which it may influence host health are not well understood. Emerging research suggests that sodium can alter the gut microbial composition, potentially affecting the host's immune response, metabolic function, and even the pathogenesis of diseases [29–31].

There are minimal human studies that evidence the influence of sodium on gut microbiota as most studies are largely from non-human guts. A high-salt/sodium diet (HSD) has been implicated in the exacerbation of colitis, characterized by a decrease in *Lactobacillus* and butyrate production [30]. Most microbiome reports on dietary salt or sodium have focused on how HSD negatively affects intestinal immunity, and exacerbates colitis, inflammatory bowel disease, and hypertension [29,31,32]. Wang et al. [33] reported that HSDs increase the abundance of *Lachnospiraceae* and *Ruminococcus* but decreased *Lactobacillus*. A pilot study using Wistar rats showed that the high-salt group had a significant reduction in *Lactobacillus* and *Prevotella* NK3B31, and a significant increase in *Alloprevotella* and *Prevotella 9* [34]. Studies by Kumar et al. [35] reported a reduction in the abundance of both Firmicutes and Bacteroidetes in the gut of high-salt-fed rats compared to low-salt guts. In another animal study, loss of *Lactobacillus* and other beneficial genera in Firmicutes was not observed in the high-salt cohort of wilding mice [36], though it was observed in conventional laboratory mice. In a related study on risk factors of hypertension, HSD led to the reduction in beneficial *Bacteroides* (not *B. fragilis*) [37]. A human study by Ferguson et al. [38] showed that HSD is associated with increases in *Prevotella*. There is a research gap in the field of human studies that investigates the impact of sodium on the gut microbiome. While there is a growing body of research on the role of sodium in systemic health, the specific effects of sodium on the gut microbiome remain inadequately explored. Existing human studies primarily focus on the cardiovascular implications of high sodium intake, neglecting the potential interactions with the gut microbial community. Understanding these interactions is crucial because emerging evidence suggests that dietary components, including sodium, can influence the composition and functionality of the gut microbiome, and influence overall health. Consequently, the aim of this study is to assess the influence of dietary sodium on the gut microbiome in human subjects.

In this study, we used human subjects. First, we collected food diaries and faecal samples and then performed a dietary analysis. Thereafter, samples were grouped into a high-sodium diet and low-sodium diet and afterwards taxonomic analyses from the high-resolution shotgun metagenome to understand the compositional uniqueness in both gut types were performed. Then, we explored the distinct abundance of *Bacteroides* and *Prevotella* within the gut ecosystem and examined their potential as indicators of dietary sodium. We also consider how the ratio of these two genera can reflect compositional changes in the gut microbiota. Finally, we use predictive functional tools to investigate the likely relationship between the microbiome and metabolic pathways relevant to sodium reabsorption and cholesterol metabolism.

2. Results

2.1. Dietary Record and Nutrient Evaluation

Each high-salt/sodium diet (HSD) and low-salt/sodium diet (LSD) group has three samples. Dietary and metagenomic results were based on these six samples (HSD—n = 3, LSD—n = 3). The average sodium intake for the HSD group was 4534 mg/day while that of LSD was 1058 mg/day. Sodium consumption in HSD is four folds higher than LSD, and two folds higher than the recommended maximum sodium intake (Supplementary S7). This allows for the comparison of the influence of dietary salt intake on the gut microbiota. The AOAC fibre intake for LSD was 17.5 $\pm$ 1.95 g/day and HSD was 16.8 $\pm$ 5.29 g/day (Supplementary S7). The mean age of the group on HSD was 30 $\pm$ 2.08 years while those

classified as LSD were 44 ± 5.24 years. All the participants were residents in England and were females except a male participant in the LSD group. A detailed nutritional analysis is available in the supplemental information (Supplementary S1).

2.2. Taxonomic Analysis of Microbial Communities in High-Salt Gut and Low-Salt Gut

In the life-level domain, there is a close proportion in the relative abundance of bacteria irrespective of diet groupings—HSD (99.6 ± 0.03) and LSD (99.2 ± 0.21) (Figure 1b). In both groups, the dominant phylum is Firmicutes (61.8 ± 1.4), followed by Bacteroidetes (24.7 ± 3.2), Actinobacteria (5.9 ± 2.4), Proteobacteria (3 ± 0.9), and Verrucomicrobia (0.7 ± 0.01) (Figure 1a). This is similar to studies by Sánchez et al. [17], Shkoporov and Hill [18], and Zhang et al. [19]. An alpha diversity analysis revealed slightly higher microbial diversity in LSD (Shannon index: 3.23) when compared to HSD (Shannon index: 3.17) (Supplementary S2a). Non-metric Multidimensional Scaling (NMDS) by a Bray–Curtis dissimilarity matrix indicated that HSD samples were more loosely grouped based on relative abundance at the genus level than LSD (Supplementary S2b). Previous studies by Ferguson et al. [38] also indicated like ours no significant difference in the alpha diversity in LSD versus HSD. Their NMDS results also mirrored ours and indicated no significant differences in bacterial clustering between LSD and HSD. This suggests individual variation in microbial communities for all dietary groups, though variation is higher in high-salt guts. The human gut microbiome is a complex and diverse community of microorganisms, and variation may be due to the individual genetic makeup, health status, age, diet, immune response, microbial interactions, environment, and lifestyle [21–23]. In both groups, the dominant genera include *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, and *Roseburia.* Figure 1c shows the top 55 genera across the groups.

By class, HSD has a higher abundance of Bifidobacteriales than LSD (Figure 2a) and this is chiefly *Bifidobacterium adolescentis* (Figure 2h). Bacteria that are cellulose-related in nomenclature constitute about 2.8% in the low-salt gut compared to 1.7% in the high-salt gut (Figure 2g). To the best of our knowledge, this is the first report about the abundance of cellulose-related bacteria in the gut in relation to dietary salt. The mean AOAC fibre consumed by an HSD recipient was 16.8 g/100 g and LSD was 17.5 g/100 g (Supplementary S7). Despite a similar overall fibre consumption pattern in both groups, these cellulose-degrading bacteria are more abundant in the low-salt gut and chiefly dominated by *Bacteroides cellulosilyticus* (Figure 2n). Other comparative taxonomy includes Lachnospiraceae, dominated by *Roseburia intestinalis* (Figure 2b,i), and Ruminococcaceae, dominated by *Faecalibacterium prausnitzii* (Figure 2c,j), *Lactobacillus* (Figure 2d,k), *Bacteroides* (Figure 2e,l), and *Prevotella* (Figure 2f,m).

2.3. Significant Microbial Shift

A linear discriminant analysis revealed that there are significant differences between HSD and LSD at the genus level. *Heliobacterium* had the highest LDA score of 7200 and positively leaned towards LSD (Figure 3a). Other significant microbes for LSD include *Leptotrichia*, *Thermoanaerobacter*, *Exiguobacterium*, *Anaerococcus*. Despite a low LDA score for *Bacteroides*, the genus had the highest relative abundance among the differential genera (10% for HSD and 23% for LSD) (Figure 3b). Because of their high abundance and differential significance in HSD and LSD, investigating their ratios or microbial shift may be important in understanding diet–microbiome functions and interactions.

The visualization in Figure 4 represents a network analysis based on the pairwise Spearman correlations between genera across the HSD and LSD conditions. The more abundant genera are *Bacteroides*, *Faecalibacterium*, *Clostridium*, *Eubacterium*, *Ruminococcus*, *Prevotella*, *Bifidobacterium*, and *Dorea*. Others are *Coprococcus*, *Parabacteroides*, *Subdoligranum*, *Butyrivibrio*. Hence, microbial shifts in any of these abundant genera could be investigated in regards to the dietary intake and functional microbiome. From the analysis, *Bacteroides* and *Prevotella* have a negative correlative relationship, implying that as the abundance of one genus increases in one dietary type (e.g., *Bacteroides* in LSD), the abundance of the other

genus tends to decrease (e.g., *Prevotella*). Bacteroidia (class) and Bacteroidaceae (family) have the highest mean differential abundance of 3.6% and 11.9%, respectively (Figure 3c,d).

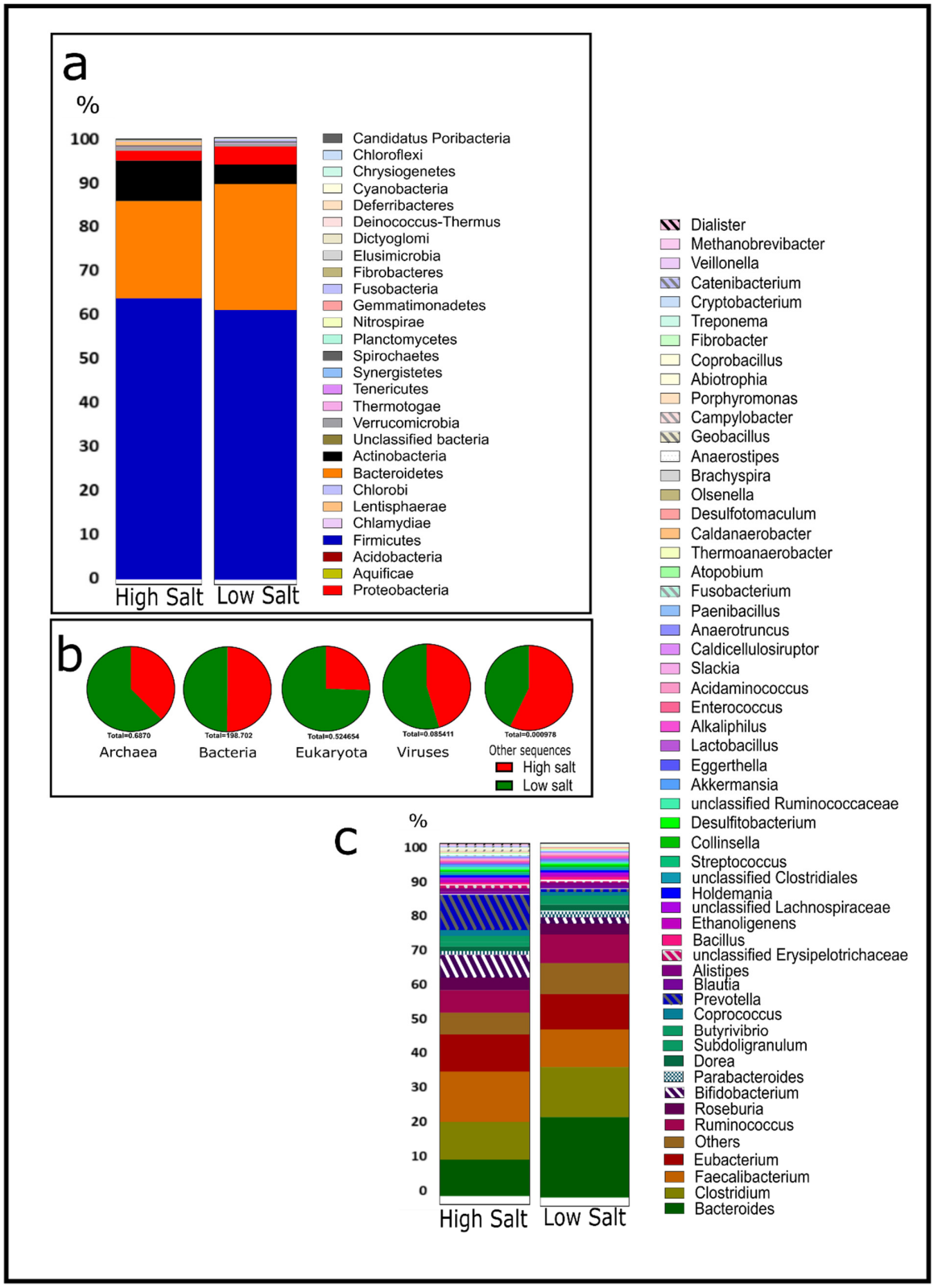

Figure 1. Metagenomic microbial community analysis of the human guts by salt intake classification. (**a**) Relative abundance at phylum level. (**b**) Proportions of the domains. (**c**) Relative abundance of top fifty-five genera. HSD, $n = 3$; LSD, $n = 3$.

Microbiome studies currently use a shift in the Bacteroidetes/Firmicute ratio as a measure of dissimilar conditions (e.g., healthy vs. disease states) [39–41]. However, Figure 5c indicates that there is no clear difference between high salt consumption and low salt consumption based on phyla ratios. Both HSD and LSD show similar 2-fold variance for the F/B ratio. Interestingly, there is significant large variation between these two cohorts when a genus-based *Bacteroides*/*Prevotella* (B/P) ratio is applied even when two different taxonomy platforms are compared (Figure 5a,b). This suggests that low dietary salt intake correlates with increased *Bacteroides* and reduced *Prevotella*. In short, analyses of results from both taxonomic tools indicate that there are low chances (1.1–2.2%) of randomly observing interaction in an experiment of this size. Further supportive statistics for genus-based ratios is available in the supplemental information (Supplementary S3).

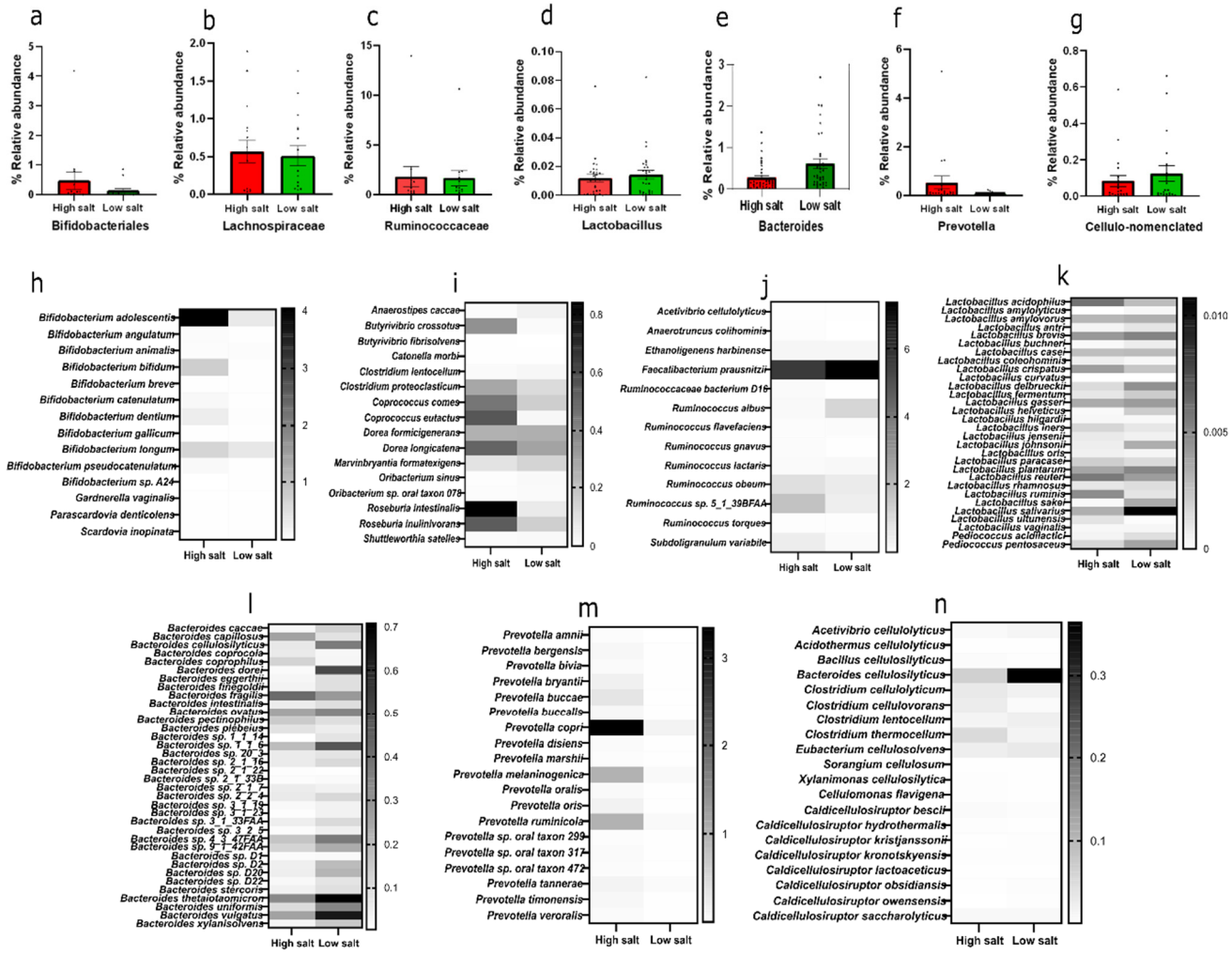

Figure 2. Relative abundance of selected commensal microbes. Top row—relative abundance of (**a**) Bifidobacteriales, (**b**) Lachnospiraceae, (**c**) Ruminococcaceae, (**d**) Lactobacillus, (**e**) *Bacteroides*, (**f**) *Prevotella*, (**g**) cellulo-related. Middle and bottom row—heatmaps of members (**h**) Bifidobacteriales species, (**i**) Lachnospiraceae species, (**j**) Ruminococcaceae species, (**k**) *Lactobacillus* species, (**l**) *Bacteroides* species, (**m**) *Prevotella* species, (**n**) cellulo-related species. Calculated as relative abundance with standard error of means within the bacteria domain. HSD, $n = 3$; LSD, $n = 3$.

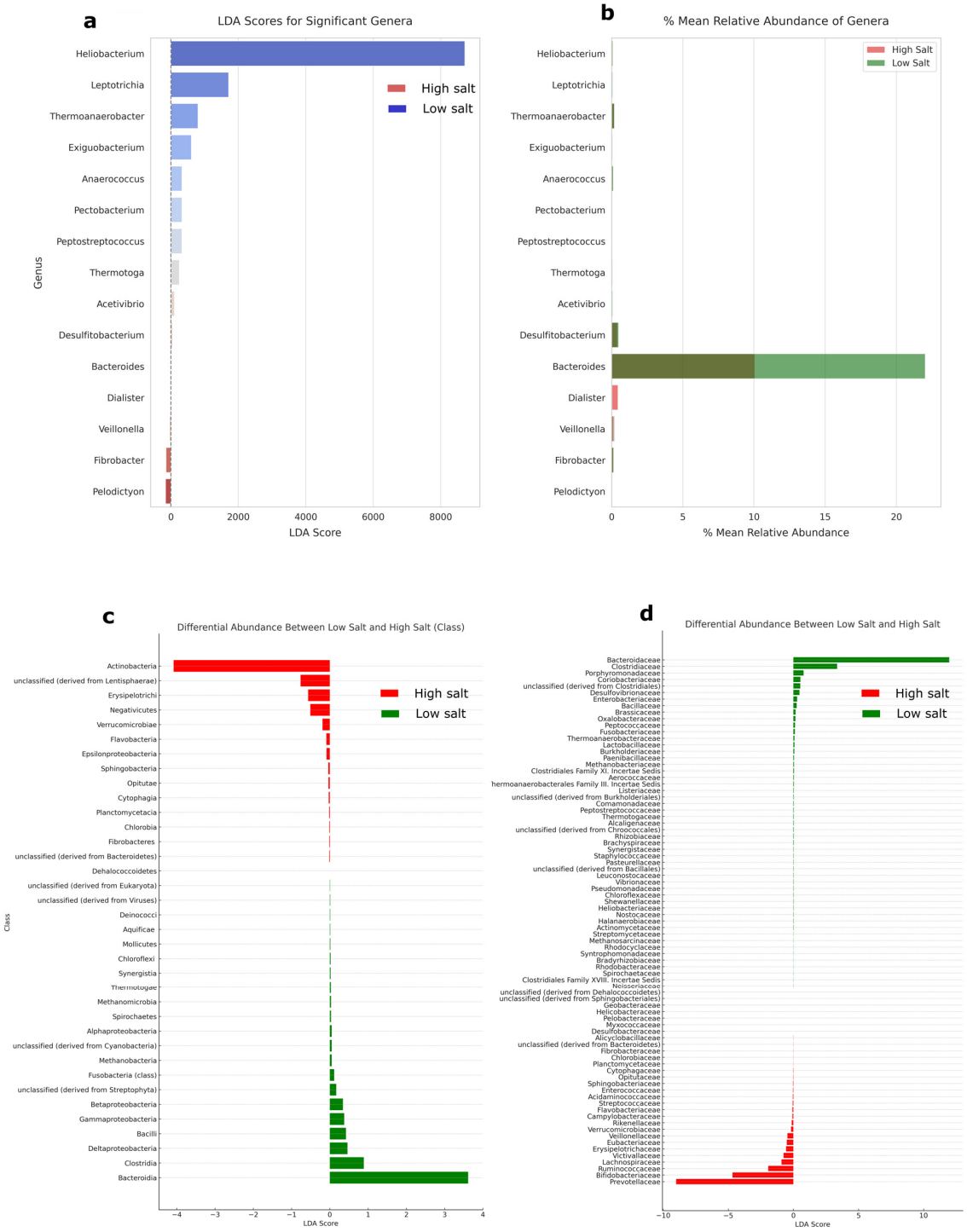

Figure 3. Linear discriminant analysis. (**a**) Kruskal–Wallis test was performed to identify genera with statistically significant differences in abundance between the high- and low-salt groups ($p = 0.05$). (**b**) Relative abundance of significantly differential genera. (**c**) Mean differential abundance between HSD and LSD at class level. The total class number was 126, and classes with less than 0.05% abundance were filtered out before calculation of differential abundance with significance across the two groups. (**d**) Mean differential abundance between HSD and LSD at family level. The total family classification was 416, and families with less than 0.05% abundance were filtered out before calculation of differential abundance with significance across the two groups. Further results on genus-level p values for Shapiro–Wilk test and Krustal–Wallis test are available in Supplementary S5. HSD, $n = 3$; LSD, $n = 3$.

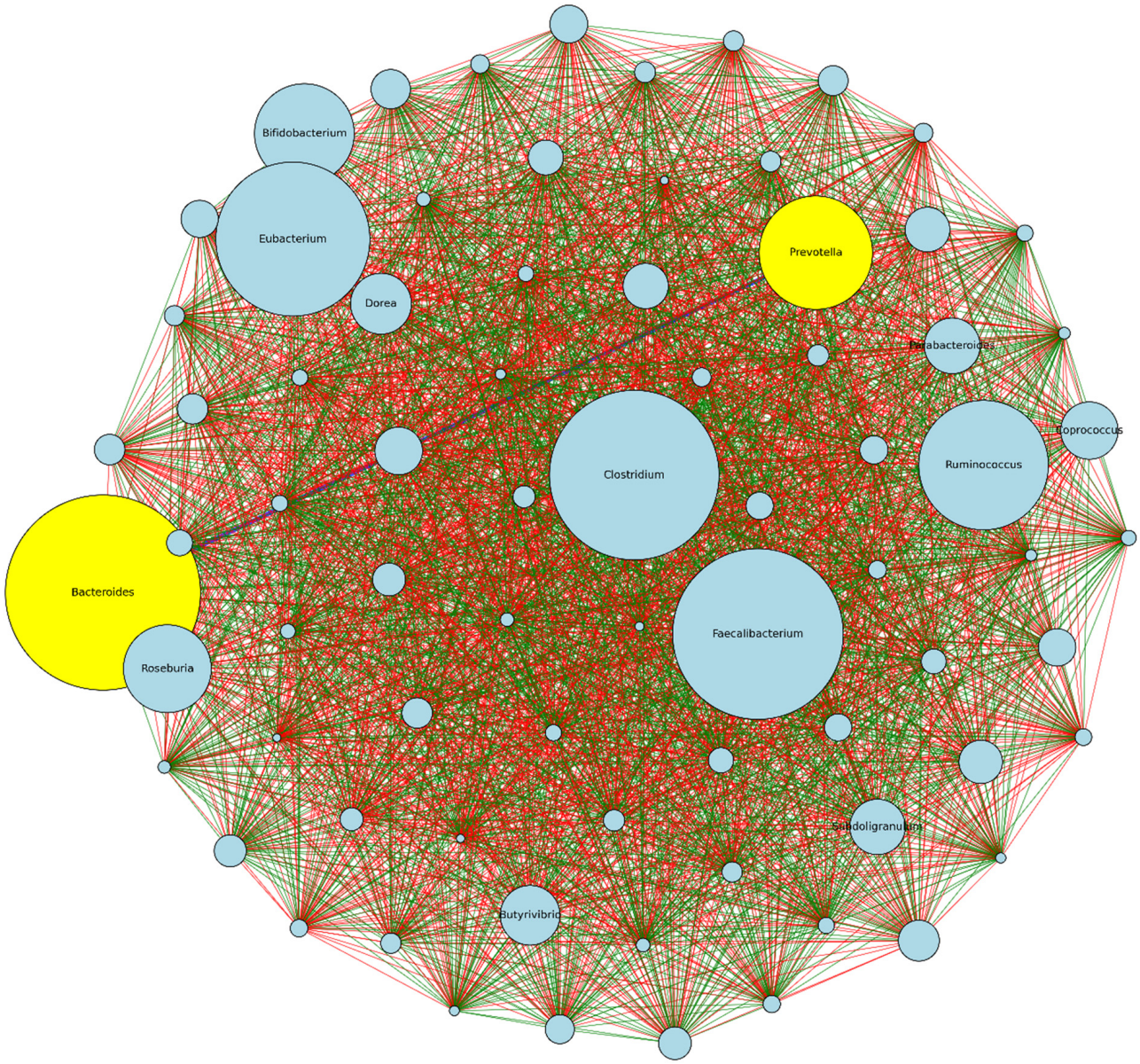

Figure 4. Correlation and network analysis of genera. Each node (circle) represents a genus, and the size of the node corresponds to the average abundance of the genus across both dietary classifications. The lines (edges) connecting the nodes represent the strength and direction of the correlation between the connected genera. Green edges (-) indicate a positive correlation. Red edges (-) signify a negative correlation. HSD, $n = 3$; LSD, $n = 3$.

There is significant ecological interaction and change in the *Bacteroides*/*Prevotella* ratio by dietary sodium intake. Hence, the suggestion here is that the *Bacteroides*/*Prevotella* ratio should be checked alongside the Bacteroidetes/Firmicutes ratio. Since the consumption of high sodium is known to be unhealthy [27,28], this work proposed that the *Bacteroides*/*Prevotella* ratio could be used complimentarily with the Bacteroidetes/Firmicutes ratio in microbiome studies and investigating a 'healthy gut'. There is a higher *Bacteroides*/*Prevotella* ratio in a low-sodium gut, which is at least 15-fold. However, a validation analysis would require larger sample sizes and studies performed in various physiological states to elucidate if the *Bacteroides*/*Prevotella* ratio will be a more clinically and statistically accurate ratio rather than the phyla-based Bacteroidetes/Firmicutes ratio for a compositional change marker and the determination of health indices [42].

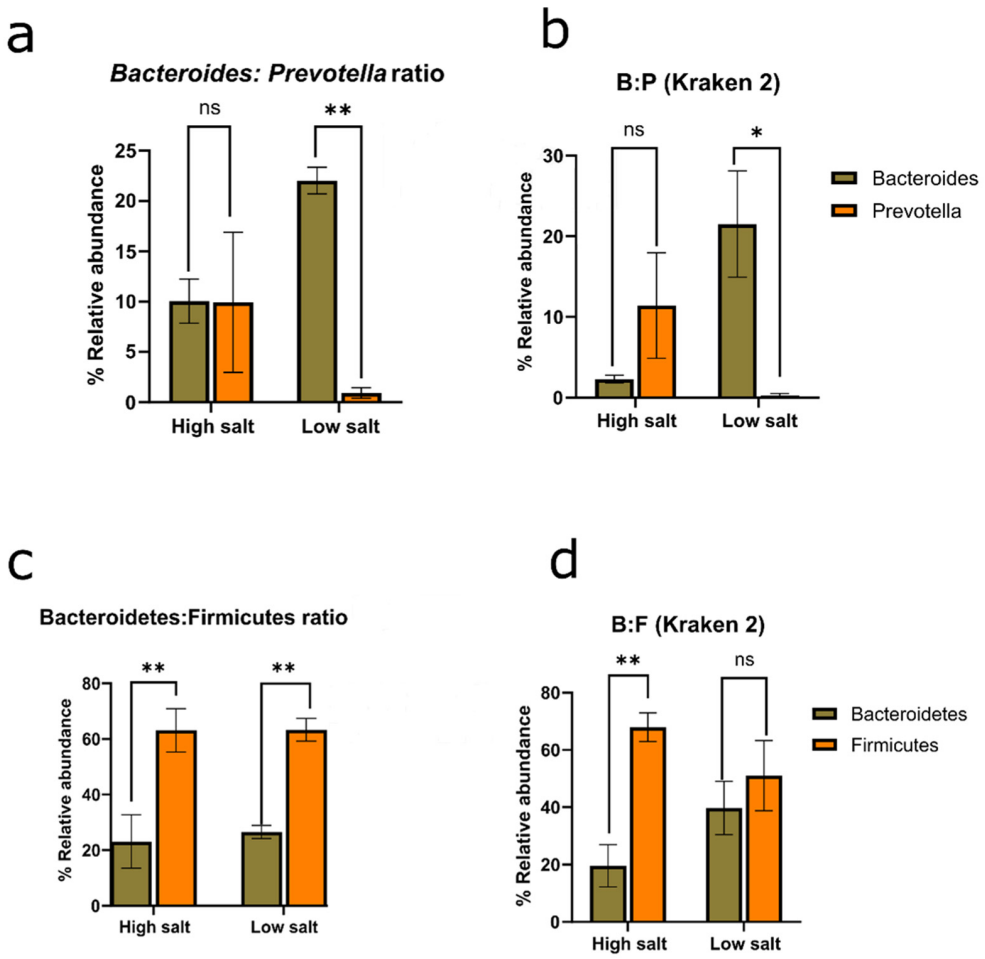

Figure 5. Microbial ratios. (**a**) *Bacteroides*/*Prevotella* ratio based on MG-RAST. Interaction p value = 0.0114. (**b**) *Bacteroides*/*Prevotella* ratio based on Kraken 2 taxonomic reports. Interaction p value = 0.0224. (**c**) Bacteroidetes/Firmicutes ratio based on MG-RAST. Interaction p value = 0.8143. (**d**) Bacteroidetes/Firmicutes ratio based on Kraken 2 taxonomic reports. Interaction p value = 0.0709. ANOVA was corrected for multiple comparisons with Šídák's multiple comparisons test. p values were interpreted: >0.1234 (not significant or ns), <0.0332 (*), <0.0021 (**). HSD, n = 3; LSD, n = 3.

2.4. Functional Predictions

The average predicted gene length for both HSD and LSD was 146bp. There was a higher sequence count in LSD (464,813) than HSD (106,752). Similarly, the base pair count was higher in LSD (45,143,166) than HSD (15,645,478) (Supplementary S6). Functional prediction showed that uncharacterised genes were present in the epithelial sodium channel of high-sodium guts but not low-sodium guts (Figure 6). To the best of our knowledge, this is the first microbiome report to find a significant link between high sodium intake and the SCNN1B and SCNN1G gene of the epithelial sodium channel (ENaC). The sequences found in ENaC do not match with any protein in the protein data bank (PDB), Swiss Prot, reference protein (RefSeq), and environmental metagenomic protein (env_nr) (Table 1). The SCNN1G gene (gamma subunit) and SCNN1B (beta subunit) are two of three genes that create the ENaC channel, with the last being SCNN1A (alpha subunit) [43,44]. When compared to the canonical sequence P30536-1 in UniProt using Clustal 2.1 alignment, the *Brotolimicola acetigignens* TspO isoform from our study has been found to have the higher similarity (35.76%) than one of the three computational isoforms B1AH88-1 (31.63%). *Alistipes onderdonkii*, Blautia, *Phocaeicola dorei*, *Phocaeicola vulgatus*, *Brotolimicola acetigignens* were linked to TspO in the cholesterol metabolic pathway (Figure 7) and their corresponding computational structures (Supplementary S8).

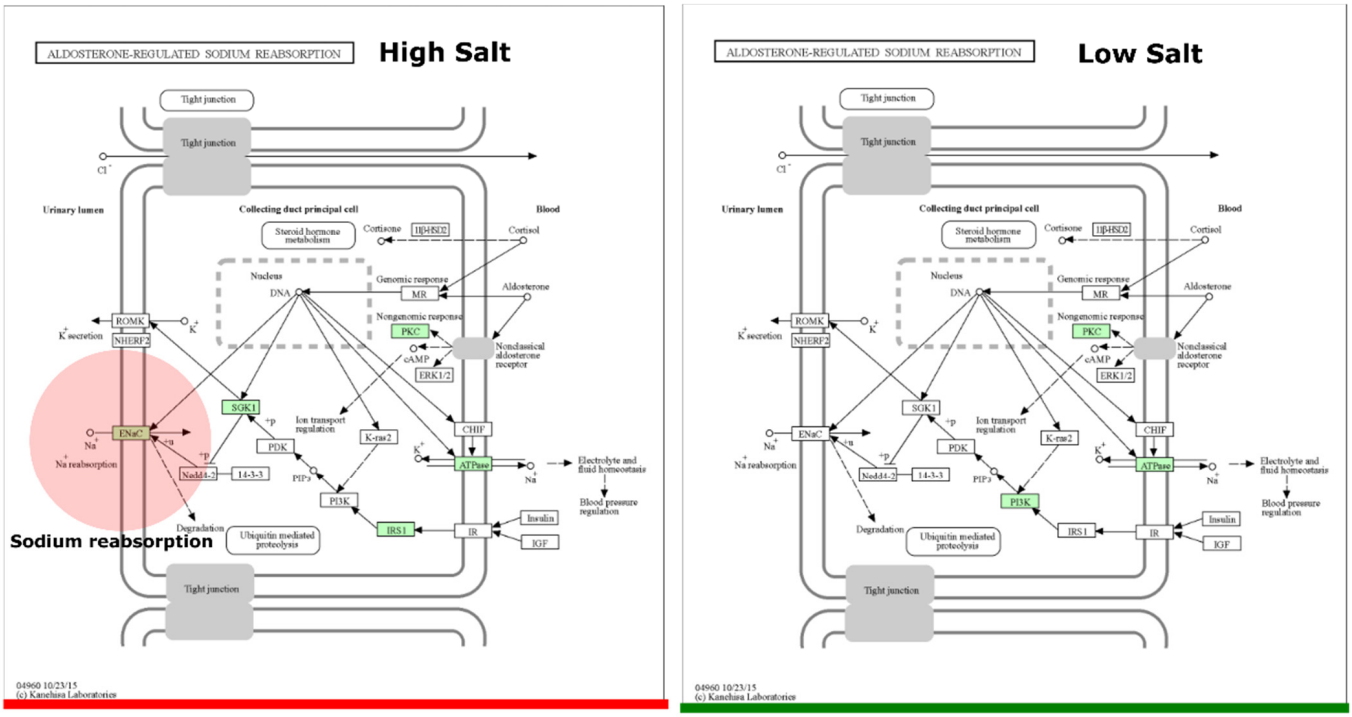

Figure 6. Aldosterone-regulated sodium reabsorption pathway. The ortholog of interest is the epithelial sodium channel (ENaC). Copyright permission obtained for the use of KEGG output figures. HSD, $n = 3$; LSD, $n = 3$.

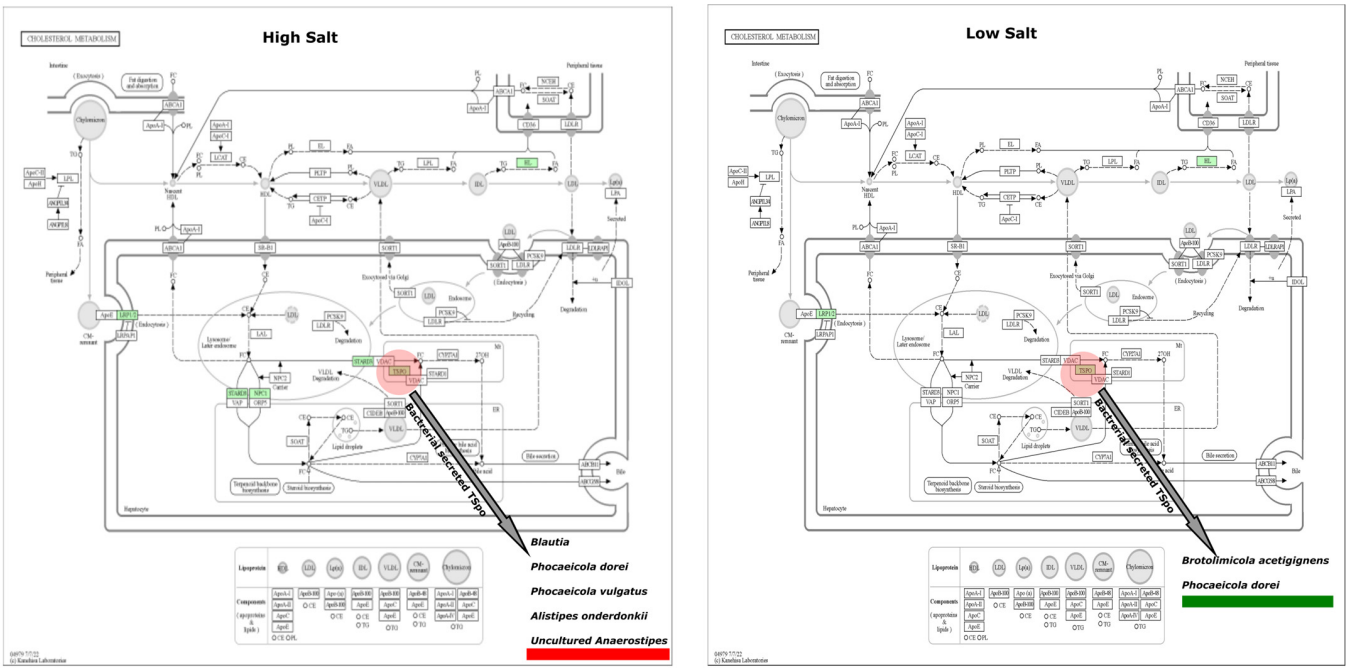

Figure 7. Cholesterol metabolism pathway. The ortholog of interest is the translocator protein (TspO). Copyright permission obtained for the use of KEGG output figures. Reconstruction of all good-quality, complete genomes of *Alistipes onderdonkii* ($n = 7$), *Blautia* ($n = 31$), *Phocaeicola dorei* ($n = 17$), and *Phocaeicola vulgatus* ($n = 10$) available in the BV-BRC/PATRIC also confirms the presence of the TspOs in the genome of organisms above. *Brotolimicola acetigignens* belongs to a new genus with no complete genome [45]. HSD, $n = 3$; LSD, $n = 3$.

Table 1. Genes found in the aldosterone-regulated sodium reabsorption and cholesterol pathways. HSD, $n = 3$; LSD, $n = 3$.

Pathway	Kegg Orthology	PDB	Swiss Prot	Metagenomic (env_nr)	RefSeq (ref-seq_protein)	Closest Neighbour in RefSeq (% Ident)	Gene Length (aa)	Gut Type
Cholesterol metabolism	TspO (K05770)	Yes	Yes	Yes	Yes	*Brotolimicola acetigignens* (100)	161	Low sodium
		Yes	Yes	Yes	Yes	*Phocaeicola dorei* (100)	154	Low sodium
		Yes	Yes	Yes	Yes	*Phocaeicola dorei* (100)	79	High sodium
		Yes	Yes	Yes	Yes	*Blautia* (100)	150	High sodium
		Yes	Yes	Yes	Yes	*Phocaeicola vulgatus* (99.35)	154	High sodium
		Yes	Yes	Yes	Yes	*Alistipes onderdonkii* (93.39)	158	High sodium
		Yes	Yes	Yes	Yes	Uncultured *Anaerostipes* sp. (70.20)	169	High sodium
Aldosterone-regulated sodium reabsorption	ENaC SCNN1B (K04825)	No	No	No	No		196	High sodium
	ENaC SCNN1G (K04827)	No	No	No	No		135	High sodium
		No	No	No	No		63	High sodium

3. Discussion

Based on previous extensive assessment of microbiota studies, other studies have shown that it is difficult to associate the Firmicutes/Bacteroidetes ratio with a determined health status [40,46]. A clear suggestion was to explore compositional changes at the family, genus, or species level, which might be more relevant than the phyla-based Firmicutes/Bacteroidetes ratio [40,47].

Hence, our study suggests the use of *Bacteroides*/*Prevotella* as a compositional change marker. It is well established that *Bacteroides* and *Prevotella* are the two most abundant genera in the Bacteroidetes irrespective of heathy or disease states in breast cancer [48], blastocystis infection [49], HIV infection [50], urolithin metabotype cardiovascular risk [51], and diabetes [52].

The dominance of these genera in the phylum and indeed the gut microbiome makes them ideal to explore for microbial changes in various physiological states. For instance, studies by An and colleagues [48] reported that the F/B ratio was three times lower in patients with breast cancer than in healthy controls. Indeed, there was about a threefold increase in Bacteroidetes in the disease cohort compared to the heathy cohort; however, their work showed that there were only minor changes in the Firmicutes. In the healthy cohort, mean relative abundance was Firmicutes at 33% and Bacteroidetes at 7% compared to cancer (Firmicutes at 30%, Bacteroidetes at 16%). From the results in their work, a distinct microbial shift may be better reported if the genus-based ratio is applied—healthy (*Bacteroides* at 33%, *Prevotella* at 30%) and cancer (*Bacteroides* at 72%, *Prevotella* at 10%). Nonetheless, not all studies on microbial ratios present genus-level data. For instance, studies by Koliada et al. [39] reported that obese adults have a significantly higher level of Firmicutes and lower level of Bacteroidetes compared to healthy-weight and lean adults. Further inference may have been obtained if results on genera abundance were presented.

Previous works by Gabrielli et al. [49] support our position for the use of the genus-based ratio. In their work, the microbial relative abundance of the blastocystis free group of patients was *Bacteroides* at 32% and *Prevotella* at 2% compared to blastocystis carriers (*Bacteroides* at 1%, *Prevotella* at 11%). *Bacteroides* and *Prevotella* were found to be major bacteria clusters in their investigation on intestinal disorders. Even at the family level, their work showed an inverse ratio of Bacteroidaceae and Prevotellaceae in the control vs. infected. In a viral infection study, the faecal microbiota of untreated individuals with chronic HIV infection exhibited a significantly higher abundance of *Prevotella* compared to HIV-negative individuals (control). Inversely, HIV-negative individuals had increased *Bacteroides* compared to those infected [50]. In a diabetes study, the LDA analysis shows that *Bacteroides* (differential for control) and *Prevotella* (differential for type 2 diabetes) were the two most significantly differential genera [52]. Additionally, these two genera were found to be significantly pronounced in association studies of host factors and microbiome diversity within different compositional clusters [53]. *Prevotella*- and *Bacteroides*-rich compositions were found to be relatively non-overlapping.

Other studies on enterotype detection using cross-national clusters showed that *Bacteroides* and *Prevotella* are the two top genera that drive variation in the human gut [54]. Multispecies research across cows, dogs, deer, geese, humans, pigs, horses, chickens, and seagulls also indicated the usefulness of the *Bacteroides*/*Prevotella* ratio [55]. In their studies, a cluster analysis of *Bacteroides*–*Prevotella* community profiles indicates that *Bacteroides*–*Prevotella* populations from samples of the same host species are much closer to each other than to samples from different source species. Aside from the 16s rRNA bacterial community and metagenome sequencing, which covers genus-level composition, investigation using qPCR and RT-PCR may benefit from genomic targeting of *Bacteroides* and *Prevotella* for elucidating who is there in various physiological environments.

To explore what they (microbes) can do, we applied functional tools with a focus on the epithelial sodium channel (ENaC) in the sodium reabsorption pathway and translocator proteins (TspOs) in the cholesterol metabolism pathway because of their relevance to sodium intake. TspOs, also known as tryptophan-rich sensory proteins, are the mito-

chondrial benzodiazepine receptor (MBR) family of transmembrane protein present in prokaryotes and eukaryotes [1,56,57]. Mammalian TspO is both a biomarker and therapeutic target, and the roles of these proteins are of research interest [58–60]. The knowledge on the similarity between bacterial TspO and the mammalian homologues is a growing area. Yeliseev et al. [61] reported 30% sequence similarity and with advancement in genomic tools, improved structural and functional similarity has been inferred [59,62,63]. However, experiments on *Escherichia coli* and *Saccharomyces cerevisiae* showed the absence of TspO, indicating that it is an unimportant protein for biological activity in some organisms [64]. In our metagenome-based work, metabolic pathway reconstruction found TspOs relating to *Phocaeicola dorei*, *Blautia*, *Brotolimicola acetigignens*, *Phocaeicola vulgatus*, *Alistipes onderdonkii*, and uncultured *Anaerostipes*. Most of these were from guts fed with high-sodium food (72%) compared to low-sodium guts (28%). However, the experimental validation of the involvement of these proteins within the metabolic pathway remains to be elucidated and is proposed as a direction for subsequent research endeavours. This will help answer the question of what the microbes and their bioproducts are actually doing.

Three-decade studies on TspO proteins show that their expression and functions relate to stress-induced changes including exogenous stress such as salt [63,65]. Here, the presence of the TspO in both high-sodium and low-sodium guts may suggest a relationship that is not limited to high salt stress alone. It is beyond the remit of this study to determine if the bacterial TspO displayed function as a sensor (e.g., for high salt or sodium), or as a translocator and transporter (e.g., for cholesterol). Despite the current understanding on the TspO relationship with stress-induced situations, it is unclear how TspO itself functions [10,63].

Meanwhile, the presence of sodium reabsorption genes in the ENaC of HSD (but not LSD) may suggest that there might be a response or adaptation mechanism as a result of high sodium in the gut. ENaC is crucial for sodium absorption in various tissues, including the kidneys and gastrointestinal tract [66,67]. An increased presence or activity of SCNN1B and SCNN1G could potentially reflect the body's effort to maintain sodium homeostasis in the face of high dietary sodium [68,69]. The lack of these ENaC genes in LSD (but not HSD) could be part of a compensatory mechanism that is less active or unnecessary when the sodium intake is lower [70]. Bacterial proteases such as those from *Pseudomonas aeruginosa* and *Serratia marcescens* are capable of activating the ENaC signalling pathway [71–74]. However, the precise mechanism of ENaC modulation by proteases has not been fully elucidated as the responsible protease(s) for the proteolytic activation of ENaC is yet to be identified in vivo [75]. Notwithstanding, reconstructed metabolic profiles in this study indicate that high sodium intake activates ENaC at the apical membrane via SCNN1B and SCNN1G genes. Previous studies have shown that HSDs increase ENaC activity and sodium absorption, contributing to hypertension via enhanced sodium entry in the kidneys and immune system [13,76–81].

There is still limited knowledge of the role of bacterial TspO in different physiological conditions—when, how, and with whom do they function as a sensor, a translocator, or a transporter. Moreover, on the microbial compositional changes observed here, they are not an indication of any disease predisposition or causality. Rather, they are observations on the relevance of genus-based analyses. The genus-based *Bacteroides*/*Prevotella* ratio may be beneficial for microbiome studies. Future microbiome studies will require correlation and regression analyses of the differentially significant genera alongside various indices within a structured causal methodology such as longitudinal studies, randomised control trials, Mendelian randomization, and structural equation modelling.

4. Methods

4.1. Study Participants

All participants gave written informed consent before starting this study. Stool samples for this study were collected between January 2018 and December 2018. Ethical approval was granted by the School of Applied Sciences Research Integrity and Ethics

Committee, University of Huddersfield (SAS-REIC-17-2711-1). Faecal samples were collected from individuals who responded to the research adverts, which were placed on notice boards within the university, SU shops, and specialised food group. Individuals who were in any of these categories as under 18 years, self-reporting as sick, pregnant, not comfortable with collecting their faeces, or using antibiotics within the past three months before the commencement of this study were excluded from this study. An additional inclusion criterion is that participants should demographically be residents in England. The included participants were initially classified as a vegetarian/vegan/raw cohort and Western/omnivore cohort. Participants could withdraw from this study any time.

4.2. Food Diary and Dietary Analysis

Participants were given instructions on how to complete a one-week food diary with detailed daily records of all foods and drinks consumed. After the completion of the food diary on day seven, participants hygienically self-collected faecal samples in pre-supplied DNA/RNA shield tubes [82]. Food diaries, collection tubes, and accompanying quick instructions were single-blindly sent to participants and received without names. On arrival, food diaries and samples were further coded with a laboratory ID to eliminate traceability of samples to participants. Dietary information was examined for completeness, and calculations were made for the average daily consumption of total energy, carbohydrates (including sugars), fats (including saturated fats), protein, fibre, vitamins (including vitamin A, thiamin, vitamin C), minerals (including iron, calcium, chloride, and sodium).

Nutrimen, a professional dietary analysis, was used to analyse the food diaries [83]. This platform allows for a quick look at pre-populated nutritional values according to portions. It is based on a data source from Public Health England's McCance and Widdowson's The Composition of Foods Integrated Datasets (CoFIDs) and Food Standards Agency food portion sizes [84–86]. In rare situations where particular foods are not available in the Nutrimen database, they were manually entered using the nutritional values on product labels, which were checked in grocery stores or websites. After analyses, the samples were appropriately classified into the low-salt diet (LSD) cohort (<2500 mg/day of salt) and high-salt diet (HSD) cohort (>7000 mg/day of salt). The corresponding sodium is LSD at <1500 mg/day and HSD at >2700 mg/day. This reclassification fit into the joint WHO/FAO international recommendations and most country-specific recommendations, which range between 4000 and 5000 mg/day of salt, estimated at <2000 mg/day of sodium [23,27]. Three samples from each cohort were used.

4.3. DNA Extraction

Upon arrival, the samples were processed within 24 h. Where brief storage was necessary before processing, the DNA/RNA shield tubes were stored in a fridge. After processing, the remaining samples were immediately destroyed by submerging in a hypochlorite disinfectant and discarded in compliance with Human Tissue Act 2004. Genomic DNA was extracted and purified using Zymo Quick-DNA Faecal/Soil microbe kits (Zymo Research, Irvine, CA, USA), an ultra-high-density BashingBeads™ fracture resistant that omits or reduces the use of organic denaturants and proteinases [87]. Briefly, about 150 mg of the faecal sample was added to the BashingBead™ lysis tube and buffer. Combined chemical and mechanical lysis was then achieved by vortexing at 5 m/s, for 1 min in 5 cycles with a 30 s interval at 25 °C using Bead Blaster 24 (Benchmark Scientific, Sayreville, NJ, USA). The lysed DNA was separated from the cell debris by centrifugation (10,000× *g*). Thereafter, series of filtration lysis, DNA prewashing, gDNA washing, and DNA elution steps were performed. Finally, the eluted DNA was measured using a Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were stored at −20 °C for metagenomic sequencing downstream applications.

4.4. Sequencing, Quality Checks, and Reads' Assembling

The extracted DNA passed through quality control (QC) using microfluidics and lab-on-a-chip technology—a Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared using a Nextera DNA Flex library preparation kit (Illumina Inc., San Diego, CA, USA). Next, the libraries were normalized, pooled, and paired-end sequenced for system-specific cycles using the Illumina systems (Supplementary S4). Thereafter, a Next-Generation Sequence by shotgun metagenomics was performed on Illumina Hiseq and Novaseq systems (Illumina Inc., San Diego, CA, USA) by Molecular Research LP (Mr DNA, Shallowater, TX, USA). The sequencer produced 2 × 150-bp-paired sequences at 10 million reads per sample. The generated sequencing reads were quality-checked with FASTQC v0.11.8 [88]. After pre-processing stages of trimming, filtering, and removing contaminants, all the samples had high-quality reads. The FASTQ files were assembled into contigs on the command line with St. Petersburg genome assembler toolkits (SPAdes v3.13.1) set with multiple k-mer lengths of 21, 33, 55, 77 [89–91]. The assembled contigs were processed for taxonomic classification on MG-RAST [92,93] and Kraken 2 [94], and also used as base files for microbial metabolic pathway analyses on Kyoto Encyclopaedia of Genes and Genomes (KEGG) [43,44].

4.5. MG RAST Taxonomic Annotation

The Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) bioinformatic pipeline offers automated quality control, annotation, comparative analyses, and archiving services [92,93,95]. The assembled FASTA files were uploaded to the server. This was followed by the detection and removal of adapter sequences using a bit-masked k-difference matching algorithm, Skewer [96]. The backend pipelines and processes include screening and removal of *Homo sapiens* host-specific species sequences using DNA-level matching with bowie [97,98]. Thereafter, the sequence files were trimmed by removing low-quality sequences using a modified DynamicTrim [99]. The lowest phred score that was to be counted as a high-quality base was set at a default of 15 and sequences were trimmed to contain at most many sequences below 5. Duplicate Read Inferred Sequencing Error Estimation (DRISEE) was used to assess sequencing quality and noise within the samples [100]. All sequences passed the pre-processing filtration step as checked with FASTQ-MCF. All samples passed the dereplication checks. Next, SortMeRNA, an RNA genecalling tool, was used to search all sequences for potential rRNA genes with a cut-off of 70% identity to the ribosomal sequences from a reduced version of M5RNA [101]. Sequences were clustered with CD-HIT software V4.6.8 using 97% identity for same species clustering [102]. An RNA similarity search was then performed in BLAT, a BLAST-like alignment tool [103]. The parameters include a default e-value of 5, minimum alignment length of 15 bases, minimum abundance adjusted to 2 and searched against representative hits in the RefSeq database (release version 203) [104,105]. Detailed TSV files downloaded were used for the calculation of relative abundance and statistical analyses. Except otherwise stated, all taxonomic reports presented use this pipeline.

4.6. Microbial Shift Analyses

A linear discriminant analysis (LDA) was employed to identify bacterial genera that are differentially abundant between high and low sodium intakes. Data were filtered to exclude genera with relative abundances below 0.01%. Initially, the Shapiro–Wilk test was employed to assess the normality of the data distribution. Given the non-normal distribution observed, a non-parametric approach was adopted. The Kruskal–Wallis test was applied to identify genera that exhibited statistically significant differences in abundance between the high- and low-sodium groups ($p = 0.05$). LDA scores were calculated to assess the effect size of differences alongside mean relative abundance providing a comprehensive view on microbial shifts of significance.

4.7. Galaxy–Kraken 2 Taxonomic Analyses

For further understanding of the genus-based ratios and pipeline comparison, Kraken 2 (Version 2.1.1) on the Galaxy bioinformatic pipeline was used [106]. The assembled contig FASTA files were inputted, and default parameters include the confident score threshold (0.0), minimum base quality (0), and minimum hit groups adjusted to 2. The database used was Prebuilt Refseq indexes: Standard-16 (Standard with DB capped at 16 GB) (Version: 2022-06-07—Downloaded: 2023-08-17T071759Z). For all samples, Kraken report outputs that assign taxonomic labels to sequencing reads were generated. The reports were viewed and converted into tabular files for the calculation of relative abundance, statistical analyses, and visualization.

4.8. Gene Predictions and Metabolic Annotations

To predict genes within the metagenomic sequences derived from stool samples, we employed Prokka v1.13.3, a rapid prokaryotic genome annotation tool [107]. This command-line tool is adept at recognizing coding sequences (CDSs), rRNA, tRNA, and other non-coding RNA molecules, as well as some miscellaneous features, using an amalgamation of various bioinformatics tools. The earlier assembled contigs also served as the input for Prokka. Each sample was processed individually using the default Prokka parameters, which entail a suite of annotation tools such as Prodigal for CDS prediction, Aragorn for tRNA detection, and Barrnap for rRNA identification. Prokka utilized its default database stack, starting with the manually curated Swiss-Prot, followed by RefSeq, and finally relying on computationally predicted annotations from Pfam, TIGRFAMs, and other sources if no match was found in the primary databases.

The output files generated served as input for the KEGG database, a resourceful tool for predictive inference on the functional microbiome [43]. Specifically, the predicted genes were analysed in metagenome-designed GHOSTKOALA for genes that correspond to prokaryotes at the genus level, eukaryotes at the family level, and viruses [44]. Thereafter, pathways were reconstructed for aldosterone-regulated sodium reabsorption (map04960) to understand a potential microbiological link between the amount of sodium intake and sodium reabsorption [108,109]. Likewise, associated cholesterol metabolism (map04979) was reconstructed [110,111]. Finally, the genes found in the biological pathway were pooled together by cohorts and reconstructed again to increase the specificity. The genes and proteins underwent a search against four protein databases (Protein Data Bank, SwissProt, Metagenomic Protein, and RefSeq) for similarity with experimental structures. Finally, those found with significant similarity were reported.

4.9. Biostatistics and Visualization

Where relevant, data generated in various genomic pipelines used were pre-processed in Ms Excel, with further statistics and visualization carried out using Graphpad 8, Python v3.12.0 (pandas, scipy, numpy, matplotlib, seaborn), and R v4.3.0 (ggplot2 v3.3.6, phyloseq, tibble, dplyr, ggforce, tidyverse, igraph, gggraph, Hmisc, vegan, BiocManager).

5. Conclusions

In conclusion, a high-sodium diet altered the composition of the human gut microbiota with a significant reduction in *Bacteroides* and inverse increase in *Prevotella*. Since it is currently difficult in many microbiome studies to confidently associate the Firmicutes/Bacteroidetes (F/B) ratio with what is considered healthy (e.g., low sodium) or unhealthy (e.g., high sodium), we proposed the use of a genus-based ratio such as the *Bacteroides*/*Prevotella* (B/P) ratio for investigating compositional dynamics of gut microbiota.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16070942/s1, Supplementary S1: Nutrients analysis. Supplementary S2: Alpha and beta diversity. Supplementary S3: Firmicutes/Bacteroidetes ratio. Supplementary S4:

Sequencing librariese. Supplementary S5: Shapiro Wilk Krustal. Supplementary S6: Predicted genes statistics. Supplementary S7: Nutrition statistics. Supplementary S8: Bacterial translocator proteins.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

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References

1. Fan, Y.; Pedersen, O. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* **2021**, *19*, 55–71. [CrossRef]
2. Lynch, S.V.; Pedersen, O. The Human Intestinal Microbiome in Health and Disease. *N. Engl. J. Med.* **2016**, *375*, 2369–2379. [CrossRef]
3. Masenga, S.K.; Hamooya, B.; Hangoma, J.; Hayumbu, V.; Ertuglu, L.A.; Ishimwe, J.; Rahman, S.; Saleem, M.; Laffer, C.L.; Elijovich, F.; et al. Recent advances in modulation of cardiovascular diseases by the gut microbiota. *J. Hum. Hypertens.* **2022**, *36*, 952–959. [CrossRef]
4. Tang, W.H.; Kitai, T.; Hazen, S.L. Gut Microbiota in Cardiovascular Health and Disease. *Circ. Res.* **2017**, *120*, 1183–1196. [CrossRef]
5. Chunxi, L.; Haiyue, L.; Yanxia, L.; Jianbing, P.; Jin, S. The Gut Microbiota and Respiratory Diseases: New Evidence. *J. Immunol. Res.* **2020**, *2020*, 2340670. [CrossRef]
6. Marsland, B.J.; Trompette, A.; Gollwitzer, E.S. The Gut-Lung Axis in Respiratory Disease. *Ann. Am. Thorac. Soc.* **2015**, *12* (Suppl. S2), S150–S156. [CrossRef]
7. Natalini, J.G.; Singh, S.; Segal, L.N. The dynamic lung microbiome in health and disease. *Nat. Rev. Microbiol.* **2023**, *21*, 222–235. [CrossRef]
8. Drljača, J.; Milošević, N.; Milanović, M.; Abenavoli, L.; Milić, N. When the microbiome helps the brain-current evidence. *CNS Neurosci. Ther.* **2023**, *29*, 43–58. [CrossRef]
9. Matheson, J.-A.T.; Holsinger, R.M.D. The Role of Fecal Microbiota Transplantation in the Treatment of Neurodegenerative Diseases: A Review. *Int. J. Mol. Sci.* **2023**, *24*, 1001. [CrossRef]
10. Leneveu-Jenvrin, C.; Bouffartigues, E.; Maillot, O.; Cornelis, P.; Feuilloley, M.G.; Connil, N.; Chevalier, S. Expression of the translocator protein (TSPO) from *Pseudomonas fluorescens* Pf0-1 requires the stress regulatory sigma factors AlgU and RpoH. *Front. Microbiol.* **2015**, *6*, 1023. [CrossRef]
11. Lloyd-Price, J.; Abu-Ali, G.; Huttenhower, C. The healthy human microbiome. *Genome Med.* **2016**, *8*, 51. [CrossRef]
12. Tremaroli, V.; Bäckhed, F. Functional interactions between the gut microbiota and host metabolism. *Nature* **2012**, *489*, 242–249. [CrossRef]
13. Wu, Z.; Tian, E.; Chen, Y.; Dong, Z.; Peng, Q. Gut microbiota and its roles in the pathogenesis and therapy of endocrine system diseases. *Microbiol. Res.* **2023**, *268*, 127291. [CrossRef]
14. Zheng, D.; Liwinski, T.; Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Res.* **2020**, *30*, 492–506. [CrossRef]
15. Schwabe, R.; Jobin, C. The microbiome and cancer. *Nat. Rev. Cancer* **2013**, *13*, 800–812. [CrossRef]
16. Ratsika, A.; Codagnone, M.C.; O'Mahony, S.; Stanton, C.; Cryan, J.F. Priming for Life: Early Life Nutrition and the Microbiota-Gut-Brain Axis. *Nutrients* **2021**, *13*, 423. [CrossRef]
17. Sánchez, B.; Delgado, S.; Blanco-Míguez, A.; Lourenço, A.; Gueimonde, M.; Margolles, A. Probiotics, gut microbiota, and their influence on host health and disease. *Mol. Nutr. Food Res.* **2017**, *61*, 1600240. [CrossRef]

18. Shkoporov, A.N.; Hill, C. Bacteriophages of the Human Gut: The "Known Unknown" of the Microbiome. *Cell Host Microbe* **2019**, *25*, 195–209. [CrossRef]
19. Zhang, F.; Aschenbrenner, D.; Yoo, J.Y.; Zuo, T. The gut mycobiome in health, disease, and clinical applications in association with the gut bacterial microbiome assembly. *Lancet Microbe* **2022**, *3*, e969–e983. [CrossRef]
20. McBurney, M.I.; Davis, C.; Fraser, C.M.; Schneeman, B.O.; Huttenhower, C.; Verbeke, K.; Walter, J.; Latulippe, M.E. Establishing What Constitutes a Healthy Human Gut Microbiome: State of the Science, Regulatory Considerations, and Future Directions. *J. Nutr.* **2019**, *149*, 1882–1895. [CrossRef]
21. Kumbhare, S.V.; Patangia, D.V.V.; Patil, R.H.; Shouche, Y.S.; Patil, N.P. Factors influencing the gut microbiome in children: From infancy to childhood. *J. Biosci.* **2019**, *44*, 49. [CrossRef]
22. The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* **2012**, *486*, 207–214. [CrossRef]
23. World Health Organization/Food and Agriculture Organisation. Diet, nutrition and the prevention of chronic diseases: Report of a joint WHO/FAO expert consultation. In *Public Health Nutrition*; 7(1a) Supplement 1001; WHO Technical Report Series; World Health Organization: Geneva, Switzerland, 2002; Volume 916.
24. Valdes, A.M.; Walter, J.; Segal, E.; Spector, T.D. Role of the gut microbiota in nutrition and health. *BMJ* **2018**, *361*, k2179. [CrossRef]
25. Marques, F.; Mackay, C.; Kaye, D. Beyond gut feelings: How the gut microbiota regulates blood pressure. *Nat. Rev. Cardiol.* **2018**, *15*, 20–32. [CrossRef]
26. Robinson, A.T.; Edwards, D.G.; Farquhar, W.B. The Influence of Dietary Salt Beyond Blood Pressure. *Curr. Hypertens. Rep.* **2019**, *21*, 42. [CrossRef]
27. World Health Organization. *Guideline: Sodium Intake for Adults and Children*; World Health Organization: Geneva, Switzerland, 2012. Available online: https://www.ncbi.nlm.nih.gov/books/NBK133309/ (accessed on 22 January 2024).
28. World Health Organization. Healthy Diet. 2018. Available online: https://www.who.int/news-room/fact-sheets/detail/healthy-diet (accessed on 22 January 2024).
29. Jama, H.A.; Marques, F.Z. Don't take it with a pinch of salt: How sodium increases blood pressure via the gut microbiota. *Circ. Res.* **2020**, *126*, 854–856. [CrossRef]
30. Miranda, P.M.; De Palma, G.; Serkis, V.; Lu, J.; Louis-Auguste, M.P.; McCarville, J.L.; Verdu, E.F.; Collins, S.M.; Bercik, P. High salt diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production. *Microbiome* **2018**, *6*, 57. [CrossRef]
31. Qi, J.; Wang, J.; Zhang, Y.; Long, H.; Dong, L.; Wan, P.; Zuo, Z.; Chen, W.; Song, Z. High-Salt-Diet (HSD) aggravates the progression of Inflammatory Bowel Disease (IBD) via regulating epithelial necroptosis. *Mol. Biomed.* **2023**, *4*, 28. [CrossRef]
32. Wei, Y.; Lu, C.; Chen, J.; Cui, G.; Wang, L.; Yu, T.; Yang, Y.; Wu, W.; Ding, Y.; Li, L.; et al. High salt diet stimulates gut Th17 response and exacerbates TNBS-induced colitis in mice. *Oncotarget* **2017**, *8*, 70–82. [CrossRef]
33. Wang, C.; Huang, Z.; Yu, K.; Ding, R.; Ye, K.; Dai, C.; Xu, X.; Zhou, G.; Li, C. High-Salt Diet Has a Certain Impact on Protein Digestion and Gut Microbiota: A Sequencing and Proteome Combined Study. *Front. Microbiol.* **2017**, *8*, 1838. [CrossRef]
34. Dong, Z.; Liu, Y.; Pan, H.; Wang, H.; Wang, X.; Xu, X.; Xiao, K.; Liu, M.; Xu, Z.; Li, L.; et al. The Effects of High-Salt Gastric Intake on the Composition of the Intestinal Microbiota in Wistar Rats. *Med. Sci. Monit. Int. Med. J. Exp. Clin. Res.* **2020**, *26*, e922160. [CrossRef] [PubMed]
35. Kumar, S.; Perumal, N.; Yadav, P.K.; Pandey, R.P.; Chang, C.M.; Raj, V.S. Amoxicillin impact on pathophysiology induced by short term high salt diet in mice. *Sci. Rep.* **2022**, *12*, 19351. [CrossRef] [PubMed]
36. Cardilli, A.; Hamad, I.; Dyczko, A.; Thijs, S.; Vangronsveld, J.; Müller, D.N.; Rosshart, S.P.; Kleinewietfeld, M. Impact of High Salt-Intake on a Natural Gut Ecosystem in Wildling Mice. *Nutrients* **2023**, *15*, 1565. [CrossRef] [PubMed]
37. Yan, X.; Jin, J.; Su, X.; Yin, X.; Gao, J.; Wang, X.; Zhang, S.; Bu, P.; Wang, M.; Zhang, Y.; et al. Intestinal Flora Modulates Blood Pressure by Regulating the Synthesis of Intestinal-Derived Corticosterone in High Salt-Induced Hypertension. *Circ. Res.* **2020**, *126*, 839–853. [CrossRef] [PubMed]
38. Ferguson, J.F.; Aden, L.A.; Barbaro, N.R.; Van Beusecum, J.P.; Xiao, L.; Simmons, A.J.; Warden, C.; Pasic, L.; Himmel, L.E.; Washington, M.K.; et al. High dietary salt-induced dendritic cell activation underlies microbial dysbiosis-associated hypertension. *JCI Insight* **2019**, *4*, e126241. [CrossRef] [PubMed]
39. Koliada, A.; Syzenko, G.; Moseiko, V.; Budovska, L.; Puchkov, K.; Perederiy, V.; Gavalko, Y.; Dorofeyev, A.; Romanenko, M.; Tkach, S.; et al. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol.* **2017**, *17*, 120. [CrossRef] [PubMed]
40. Magne, F.; Gotteland, M.; Gauthier, L.; Zazueta, A.; Pesoa, S.; Navarrete, P.; Balamurugan, R. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? *Nutrients* **2020**, *12*, 1474. [CrossRef]
41. Yañez, C.M.; Hernández, A.M.; Sandoval, A.M.; Domínguez, M.A.M.; Muñiz, S.A.Z.; Gómez, J.O.G. Prevalence of Blastocystis and its association with Firmicutes/Bacteroidetes ratio in clinically healthy and metabolically ill subjects. *BMC Microbiol.* **2021**, *21*, 339. [CrossRef]
42. Mars, R.A.T.; Frith, M.; Kashyap, P.C. Functional Gastrointestinal Disorders and the Microbiome-What Is the Best Strategy for Moving Microbiome-based Therapies for Functional Gastrointestinal Disorders into the Clinic? *Gastroenterology* **2021**, *160*, 538–555. [CrossRef]
43. Kanehisa, M.; Goto, S.; Sato, Y.; Kawashima, M.; Furumichi, M.; Tanabe, M. Data, information, knowledge and principle: Back to metabolism in KEGG. *Nucleic Acids Res.* **2014**, *42*, D199–D205. [CrossRef]

44. Kanehisa, M.; Sato, Y.; Morishima, K. BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J. Mol. Biol.* **2016**, *428*, 726–731. [CrossRef] [PubMed]
45. Hitch, T.C.A.; Riedel, T.; Oren, A.; Overmann, J.; Lawley, T.D.; Clavel, T. Automated analysis of genomic sequences facilitates high-throughput and comprehensive description of bacteria. *ISME Commun.* **2021**, *1*, 16. [CrossRef] [PubMed]
46. Kusnadi, Y.; Saleh, M.I.; Ali, Z.; Hermansyah, H.; Murti, K.; Hafy, Z.; Yuristo, E. Firmicutes/Bacteroidetes Ratio of Gut Microbiota and Its Relationships with Clinical Parameters of Type 2 Diabetes Mellitus: A Systematic Review. *Open Access Maced. J. Med. Sci.* **2023**, *11*, 67–72. [CrossRef]
47. Aguirre, M.; Venema, K. Does the Gut Microbiota Contribute to Obesity? Going beyond the Gut Feeling. *Microorganisms* **2015**, *3*, 213–235. [CrossRef] [PubMed]
48. An, J.; Kwon, H.; Kim, Y.J. The Firmicutes/Bacteroidetes Ratio as a Risk Factor of Breast Cancer. *J. Clin. Med.* **2023**, *12*, 2216. [CrossRef]
49. Gabrielli, S.; Furzi, F.; Fontanelli Sulekova, L.; Taliani, G.; Mattiucci, S. Occurrence of Blastocystis-subtypes in patients from Italy revealed association of ST3 with a healthy gut microbiota. *Parasite Epidemiol. Control* **2020**, *9*, e00134. [CrossRef]
50. Lozupone, C.A.; Li, M.; Campbell, T.B.; Flores, S.C.; Linderman, D.; Gebert, M.J.; Knight, R.; Fontenot, A.P.; Palmer, B.E. Alterations in the gut microbiota associated with HIV-1 infection. *Cell Host Microbe* **2013**, *14*, 329–339. [CrossRef]
51. Romo-Vaquero, M.; Cortés-Martín, A.; Loria-Kohen, V.; Ramírez-de-Molina, A.; García-Mantrana, I.; Collado, M.C.; Espín, J.C.; Selma, M.V. Deciphering the Human Gut Microbiome of Urolithin Metabotypes: Association with Enterotypes and Potential Cardiometabolic Health Implications. *Mol. Nutr. Food Res.* **2019**, *63*, e1800958. [CrossRef]
52. Wang, J.; Li, W.; Wang, C.; Wang, L.; He, T.; Hu, H.; Song, J.; Cui, C.; Qiao, J.; Qing, L.; et al. Enterotype Bacteroides Is Associated with a High Risk in Patients with Diabetes: A Pilot Study. *J. Diabetes Res.* **2020**, *2020*, 6047145. [CrossRef]
53. Manor, O.; Dai, C.L.; Kornilov, S.A.; Smith, B.; Price, N.D.; Lovejoy, J.C.; Gibbons, S.M.; Magis, A.T. Health and disease markers correlate with gut microbiome composition across thousands of people. *Nat. Commun.* **2020**, *11*, 5206. [CrossRef]
54. Arumugam, M.; Raes, J.; Pelletier, E.; Le Paslier, D.; Yamada, T.; Mende, D.R.; Fernandes, G.R.; Tap, J.; Bruls, T.; Batto, J.M.; et al. Enterotypes of the human gut microbiome. *Nature* **2011**, *473*, 174–180. [CrossRef] [PubMed]
55. Fogarty, L.R.; Voytek, M.A. Comparison of bacteroides-prevotella 16S rRNA genetic markers for fecal samples from different animal species. *Appl. Environ. Microbiol.* **2005**, *71*, 5999–6007. [CrossRef] [PubMed]
56. Crooke, E.; Wickner, W. Bacterial Protein Translocation. In *Membrane Biogenesis*; Op den Kamp, J.A.F., Ed.; NATO ASI Series; Springer: Berlin/Heidelberg, Germany, 1988; Volume 16. [CrossRef]
57. McEnery, M.W.; Snowman, A.M.; Trifiletti, R.R.; Snyder, S.H. Isolation of the mitochondrial benzodiazepine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3170–3174. [CrossRef] [PubMed]
58. Fan, J.; Lindemann, P.; Feuilloley, M.G.; Papadopoulos, V. Structural and functional evolution of the translocator protein (18 kDa). *Curr. Mol. Med.* **2012**, *12*, 369–386. [CrossRef] [PubMed]
59. Lee, Y.; Park, Y.; Nam, H.; Lee, J.W.; Yu, S.W. Translocator protein (TSPO): The new story of the old protein in neuroinflammation. *BMB Rep.* **2020**, *53*, 20–27. [CrossRef]
60. Papadopoulos, V.; Amri, H.; Li, H.; Yao, Z.; Brown, R.C.; Vidic, B.; Culty, M. Structure, function and regulation of the mitochondrial peripheral-type benzodiazepine receptor. *Therapie* **2001**, *56*, 549–556.
61. Yeliseev, A.A.; Krueger, K.E.; Kaplan, S. A mammalian mitochondrial drug receptor functions as a bacterial "oxygen" sensor. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5101–5106. [CrossRef]
62. Chapalain, A.; Chevalier, S.; Orange, N.; Murillo, L.; Papadopoulos, V.; Feuilloley, M.G. Bacterial ortholog of mammalian translocator protein (TSPO) with virulence regulating activity. *PLoS ONE* **2009**, *4*, e6096. [CrossRef]
63. Hiser, C.; Montgomery, B.L.; Ferguson-Miller, S. TSPO protein binding partners in bacteria, animals, and plants. *J. Bioenerg. Biomembr.* **2021**, *53*, 463–487. [CrossRef]
64. Tu, L.N.; Zhao, A.H.; Stocco, D.M.; Selvaraj, V. PK11195 effect on steroidogenesis is not mediated through the translocator protein (TSPO). *Endocrinology* **2015**, *156*, 1033–1039. [CrossRef]
65. Leneveu-Jenvrin, C.; Connil, N.; Bouffartigues, E.; Papadopoulos, V.; Feuilloley, M.G.; Chevalier, S. Structure-to-function relationships of bacterial translocator protein (TSPO): A focus on *Pseudomonas*. *Front. Microbiol.* **2014**, *5*, 631. [CrossRef]
66. Hanukoglu, I. Epithelial Sodium Channel (ENaC) in GtoPdb v.2023.1. IUPHAR/BPS Guide to Pharmacology CITE. Available online: http://journals.ed.ac.uk/gtopdb-cite/article/view/8741 (accessed on 22 January 2024).
67. Hanukoglu, I.; Hanukoglu, A. Epithelial sodium channel (ENaC) family: Phylogeny, structure-function, tissue distribution, and associated inherited diseases. *Gene* **2016**, *579*, 95–132. [CrossRef] [PubMed]
68. Ohland, C.L.; Jobin, C. Microbial activities and intestinal homeostasis: A delicate balance between health and disease. *Cell. Mol. Gastroenterol. Hepatol.* **2015**, *1*, 28–40. [CrossRef] [PubMed]
69. Reihill, J.A.; Walker, B.; Hamilton, R.A.; Ferguson, T.E.; Elborn, J.S.; Stutts, M.J.; Harvey, B.J.; Saint-Criq, V.; Hendrick, S.M.; Martin, S.L. Inhibition of Protease-Epithelial Sodium Channel Signaling Improves Mucociliary Function in Cystic Fibrosis Airways. *Am. J. Respir. Crit. Care Med.* **2016**, *194*, 701–710. [CrossRef] [PubMed]
70. Sanada, H.; Jones, J.E.; Jose, P.A. Genetics of salt-sensitive hypertension. *Curr. Hypertens. Rep.* **2011**, *13*, 55–66. [CrossRef]
71. Butterworth, M.B.; Zhang, L.; Heidrich, E.M.; Myerburg, M.M.; Thibodeau, P.H. Activation of the epithelial sodium channel (ENaC) by the alkaline protease from Pseudomonas aeruginosa. *J. Biol. Chem.* **2012**, *287*, 32556–32565. [CrossRef]

72. Butterworth, M.B.; Zhang, L.; Liu, X.; Shanks, R.M.; Thibodeau, P.H. Modulation of the epithelial sodium channel (ENaC) by bacterial metalloproteases and protease inhibitors. *PLoS ONE* **2014**, *9*, e100313. [CrossRef] [PubMed]
73. Edwinson, A.L.; Grover, M. Measurement of novel intestinal secretory and barrier pathways and effects of proteases. *Neurogastroenterol. Motil.* **2019**, *31*, e13547. [CrossRef]
74. Kleyman, T.R.; Eaton, D.C. Regulating ENaC's gate. American journal of physiology. *Am. J. Physiol. Physiol.* **2020**, *318*, C150–C162. [CrossRef]
75. Anand, D.; Hummler, E.; Rickman, O.J. ENaC activation by proteases. *Acta Physiol.* **2022**, *235*, e13811. [CrossRef]
76. Mattson, D.L.; Dasinger, J.H.; Abais-Battad, J.M. Amplification of Salt-Sensitive Hypertension and Kidney Damage by Immune Mechanisms. *Am. J. Hypertens.* **2021**, *34*, 3–14. [CrossRef]
77. Barbaro, N.R.; Foss, J.D.; Kryshtal, D.O.; Tsyba, N.; Kumaresan, S.; Xiao, L.; Mernaugh, R.L.; Itani, H.A.; Loperena, R.; Chen, W.; et al. Dendritic Cell Amiloride-Sensitive Channels Mediate Sodium-Induced Inflammation and Hypertension. *Cell Rep.* **2017**, *21*, 1009–1020. [CrossRef]
78. Huang, B.S.; Van Vliet, B.N.; Leenen, F.H. Increases in CSF [Na+] precede the increases in blood pressure in Dahl S rats and SHR on a high-salt diet. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *287*, H1160–H1166. [CrossRef]
79. Noreng, S.; Bharadwaj, A.; Posert, R.; Yoshioka, C.; Baconguis, I. Structure of the human epithelial sodium channel by cryo-electron microscopy. *eLife* **2018**, *7*, e39340. [CrossRef]
80. Sun, Y.; Zhang, J.N.; Zhao, D.; Wang, Q.S.; Gu, Y.C.; Ma, H.P.; Zhang, Z.R. Role of the epithelial sodium channel in salt-sensitive hypertension. *Acta Pharmacol. Sin.* **2011**, *32*, 789–797. [CrossRef]
81. Mutchler, S.M.; Kirabo, A.; Kleyman, T.R. Epithelial Sodium Channel and Salt-Sensitive Hypertension. *Hypertension* **2021**, *77*, 759–767. [CrossRef] [PubMed]
82. Zymo Research 2019. DNA/RNA Shield. Available online: https://www.zymoresearch.com/collections/dna-rna-shield (accessed on 22 January 2024).
83. Nutrimen. Nutrimen: Professional Dietary Analysis. 2018. Available online: https://www.nutrimen.co.uk/ (accessed on 22 January 2024).
84. McCance, R.A.; Widdowson, E.M. *The Composition of Foods*, 3rd ed.; Her Majesty's Stationery Office: London, UK, 1960.
85. Public Health England. McCance and Widdowson's the Composition of Foods Integrated Dataset 2021: User Guide. 2021. Available online: https://www.gov.uk/government/publications/composition-of-foods-integrated-dataset-cofid (accessed on 4 January 2024).
86. Roe, M.; Pinchein, H.; Church, S.; Finglas, P. McCance and Widdowson's The composition of foods seventh summary edition and updated composition of foods integrated dataset. *Nutr. Bull.* **2015**, *40*, 36–39. [CrossRef]
87. Zymo Research 2019. Quick-DNA Fecal/Soil Microbe Kits. Available online: https://zymoresearch.eu/collections/quick-dna-fecal-soil-microbe-kits (accessed on 22 January 2024).
88. Wingett, S.W.; Andrews, S. FastQ Screen: A tool for multi-genome mapping and quality control. *F1000Research* **2018**, *7*, 1338. [CrossRef] [PubMed]
89. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol. J. Comput. Mol. Cell Biol.* **2012**, *19*, 455–477. [CrossRef] [PubMed]
90. Korobeynikov. SPAdes. 2020. Available online: https://github.com/ablab/spades/#assembling-long-illumina-paired-reads-2x150-and-2x250 (accessed on 22 January 2024).
91. Prjibelski, A.; Antipov, D.; Meleshko, D.; Lapidus, A.; Korobeynikov, A. Using SPAdes De Novo Assembler. *Curr. Protoc. Bioinform.* **2020**, *70*, e102. [CrossRef] [PubMed]
92. Meyer, F.; Paarmann, D.; D'Souza, M.; Olson, R.; Glass, E.M.; Kubal, M.; Paczian, T.; Rodriguez, A.; Stevens, R.; Wilke, A.; et al. The metagenomics RAST server—A public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinform.* **2008**, *9*, 386. [CrossRef]
93. Wilke, A.; Bischof, J.; Harrison, T.; Brettin, T.; D'Souza, M.; Gerlach, W.; Matthews, H.; Paczian, T.; Wilkening, J.; Glass, E.M.; et al. A RESTful API for accessing microbial community data for MG-RAST. *PLoS Comput. Biol.* **2015**, *11*, e1004008. [CrossRef]
94. Wood, D.E.; Lu, J.; Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **2019**, *20*, 257. [CrossRef]
95. Meyer, F.; Bagchi, S.; Chaterji, S.; Gerlach, W.; Grama, A.; Harrison, T.; Paczian, T.; Trimble, W.L.; Wilke, A. MG-RAST version 4-lessons learned from a decade of low-budget ultra-high-throughput metagenome analysis. *Brief. Bioinform.* **2019**, *20*, 1151–1159. [CrossRef]
96. Jiang, H.; Lei, R.; Ding, S.W.; Zhu, S. Skewer: A fast and accurate adapter trimmer for next-generation sequencing paired-end reads. *BMC Bioinform.* **2014**, *15*, 182. [CrossRef] [PubMed]
97. Langmead, B.; Salzberg, S. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357–359. [CrossRef] [PubMed]
98. Langmead, B.; Trapnell, C.; Pop, M.; Salzberg, S.L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **2009**, *10*, R25. [CrossRef] [PubMed]
99. Cox, M.P.; Peterson, D.A.; Biggs, P.J. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinform.* **2010**, *11*, 485. [CrossRef] [PubMed]
100. Keegan, K.P.; Trimble, W.L.; Wilkening, J.; Wilke, A.; Harrison, T.; D'Souza, M.; Meyer, F. A platform-independent method for detecting errors in metagenomic sequencing data: DRISEE. *PLoS Comput. Biol.* **2012**, *8*, e1002541. [CrossRef] [PubMed]

101. Kopylova, E.; Noé, L.; Touzet, H. SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* **2012**, *28*, 3211–3217. [CrossRef] [PubMed]
102. Fu, L.; Niu, B.; Zhu, Z.; Wu, S.; Li, W. CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics* **2012**, *28*, 3150–3152. [CrossRef] [PubMed]
103. Kent, W.J. BLAT—The BLAST-like alignment tool. *Genome Res.* **2002**, *12*, 656–664. [CrossRef] [PubMed]
104. O'Leary, N.A.; Wright, M.W.; Brister, J.R.; Ciufo, S.; Haddad, D.; McVeigh, R.; Rajput, B.; Robbertse, B.; Smith-White, B.; Ako-Adjei, D.; et al. Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res.* **2016**, *44*, D733–D745. [CrossRef] [PubMed]
105. RefSeq. Index of Refseq Release, Release-Catalog and Archive. 2021. Available online: https://ftp.ncbi.nlm.nih.gov/refseq/release/release-catalog/archive/ (accessed on 22 January 2024).
106. Galaxy Community; Afgan, E.; Nekrutenko, A.; Blankenberg, D.; Goecks, J.; Schatz, M.C.; E Ostrovsky, A.; Mahmoud, A.; Lonie, A.J.; Syme, A.; et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Res.* **2022**, *50*, W345–W351. [CrossRef]
107. Seemann, T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* **2014**, *30*, 2068–2069. [CrossRef] [PubMed]
108. Lee, I.H.; Campbell, C.R.; Cook, D.I.; Dinudom, A. Regulation of epithelial Na+ channels by aldosterone: Role of Sgk1. *Clin. Exp. Pharmacol. Physiol.* **2008**, *35*, 235–241. [CrossRef] [PubMed]
109. Pearce, D.; Kleyman, T.R. Salt, sodium channels, and SGK1. *J. Clin. Investig.* **2007**, *117*, 592–595. [CrossRef]
110. Kingwell, B.A.; Chapman, M.J.; Kontush, A.; Miller, N.E. HDL-targeted therapies: Progress, failures and future. *Nat. Rev. Drug Discov.* **2014**, *13*, 445–464. [CrossRef]
111. Wang, S.; Smith, J.D. ABCA1 and nascent HDL biogenesis. *BioFactors* **2014**, *40*, 547–554. [CrossRef]

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Co-Supplementation of Baobab Fiber and Arabic Gum Synergistically Modulates the In Vitro Human Gut Microbiome Revealing Complementary and Promising Prebiotic Properties

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Abstract: Arabic gum, a high molecular weight heteropolysaccharide, is a promising prebiotic candidate as its fermentation occurs more distally in the colon, which is the region where most chronic colonic diseases originate. Baobab fiber could be complementary due to its relatively simple structure, facilitating breakdown in the proximal colon. Therefore, the current study aimed to gain insight into how the human gut microbiota was affected in response to long-term baobab fiber and Arabic gum supplementation when tested individually or as a combination of both, allowing the identification of potential complementary and/or synergetic effects. The validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), an in vitro gut model simulating the entire human gastrointestinal tract, was used. The microbial metabolic activity was examined, and quantitative 16S-targeted Illumina sequencing was used to monitor the gut microbial composition. Moreover, the effect on the gut microbial metabolome was quantitatively analyzed. Repeated administration of baobab fiber, Arabic gum, and their combination had a significant effect on the metabolic activity, diversity index, and community composition of the microbiome present in the simulated proximal and distal colon with specific impacts on *Bifidobacteriaceae* and *Faecalibacterium prausnitzii*. Despite the lower dosage strategy (2.5 g/day), co-supplementation of both compounds resulted in some specific synergistic prebiotic effects, including a biological activity throughout the entire colon, SCFA synthesis including a synergy on propionate, specifically increasing abundance of *Akkermansiaceae* and *Christensenellaceae* in the distal colon region, and enhancing levels of spermidine and other metabolites of interest (such as serotonin and ProBetaine).

Keywords: gut microbiome; prebiotics; baobab fiber; Arabic gum; SHIME® technology; *Christensenellaceae*; *Akkermansiaceae*

1. Introduction

The human microbiota contains up to 10^{14} bacterial cells, which is ten times higher than the number of eukaryotic cells present in the human body. These bacterial cells are able to colonize every body part exposed to the external environment. However, the most heavily colonized organ is the gastrointestinal tract, i.e., 70% of all microbes reside in the intestinal region [1]. These gut microbiota have been reported to have a strong effect on human health and disease. Their presence prevents, amongst others, colonization of the gut by pathogenic species such as *Salmonella* spp. (e.g., by means of nutrient competition or secretion of bacteriocins) [2]. In addition, certain metabolites produced by the intestinal microbiome serve as an important energy source for the host [3]. Butyrate is, for example, recognized as one of the main energy sources for the gut epithelium [4]. The composition and activity of the microbial community have also been related to metabolic syndrome,

a group of risk factors including obesity [5]. Gut microbiota can also act as important regulators of the immune system by the production of anti-inflammatory compounds [2]. For example, previous studies reported that the production of butyrate by bacterial species such as *Faecalibacterium prausnitzii* can prevent intestinal inflammation [6]. Finally, the enzymatic activity of some microbial groups proved to result in the conversion of certain food constituents into potentially toxic or carcinogenic compounds related to the occurrence of colorectal cancer (CRC) [7].

The beneficial health effects associated with gut microbiota can be stimulated by the consumption of prebiotics, which have been defined as substrates that are selectively utilized by host microorganisms, conferring a health benefit [8]. The most commonly used prebiotics include fructo-oligosaccharides, inulin, lactulose, and galacto-oligosaccharides [9]. These prebiotics are not digested by the enzymes produced by the human host and end up in the colonic region, where they can be fermented by the gut microbiota [10]. As a result, the composition of the microbial community will be altered due to (i) the difference in fermentation capacity of the different bacterial species, (ii) the antimicrobial activity of some specifically generated metabolites, mainly short-chain fatty acids (SCFA), and (iii) the acid tolerance of the different species [11,12]. These compositional changes can, in turn, also result in a decreased production of certain toxic/carcinogenic compounds such as ammonia [13].

Since most chronic colonic diseases, such as ulcerative colitis and CRC, mainly originate in the distal colon, there is a need for (combinations of) prebiotics that exert biological activity in this specific part of the colon or, preferably, throughout the entire colonic region. However, currently used prebiotics are predominantly fermented in the proximal colon due to their colonization by microbiota with a high saccharolytic potential [14]. The fermentation of Arabic gum (AG), a water-soluble heteropolysaccharide with high molecular weight, is believed to occur mainly in the distal colon due to its rather complex structure and could, therefore, be a promising prebiotic candidate [15–17]. This distal fermentation profile was indeed observed by Daguet et al. [18,19] upon long-term administration of AG in an in vitro gut model using fecal inocula of IBS (irritable bowel syndrome) and IBD (irritable bowel disease) donors, respectively. The study of Cherbut et al. [20] also provided evidence for a high digestive tolerance of AG in healthy individuals, i.e., no intestinal symptoms such as flatulence, bloating, abdominal cramps, and diarrhea were reported when dosing up to 30 g/day. Moreover, this repeated intake of AG resulted in increased total lactic acid-producing bacteria and bifidobacteria levels in fecal stools [20]. Similar results were observed in the in vivo study of Calame et al. [21], i.e., a daily dosage of 10 g AG/day increased the amount of bifidobacteria and lactobacilli, which are well recognized for their beneficial health effects related to (i) an increased resistance towards pathogenic colonization and (ii) an increased production of certain SCFA [22]. Other health-promoting effects that have been reported following repeated administration of AG were (i) stimulation of the integrity of the gut wall barrier [18,19], (ii) modulation of gut inflammation [18,19], (iii) transit modulation [23], (iv) kidney health [24], and (v) increased immunity [18,19]. The prebiotic effect of baobab fiber (BF), on the other hand, has not been extensively studied before. Studies investigating its potential health-promoting effects reported strong antioxidative properties linked with its high vitamin C content [25,26]. In addition, preliminary studies revealed metabolic health improvement and gut transit modulation [27–29]. Moreover, the study of Foltz et al. [30] revealed a promising prebiotic potential of baobab fruit pulp powder using an in vitro (short-term) fermentation model, i.e., administration of the test product resulted in a stimulated production of acetate, propionate, lactate, and butyrate (to a lower extent) by the gut microbial community of three healthy adult human donors. Finally, an additional benefit of BF might be its potential complementarity with AG, i.e., the relatively simple structure of BF might facilitate breakdown in the proximal part of the colon, leading to a combined prebiotic effect exerted throughout the entire colonic region [31].

In order to systematically examine the prebiotic properties of test substrates on the intestinal microbial community, in vitro approaches are widely used. These in vitro studies range from short-term single-stage colonic incubations (e.g., [32]) to dynamic gut models (e.g., [33,34]), with the latter being able to simulate long-term administration studies under highly controlled and representative intestinal conditions. To mimic the intestinal microbiome as closely as possible, these in vitro gut models rely on fecal inocula from human individuals. While it is generally estimated that the gut microbial community of one individual contains 500 to 1000 different species [35], the collective human microbiome is composed of more than 35,000 bacterial species, which stresses interindividual differences [36]. Therefore, these differences need to be taken into account in in vitro studies, as they can affect the response to dietary interventions. In order to elucidate the potential prebiotic effects of these interventions in sufficient detail, one relies on highly advanced techniques such as 16S rRNA gene sequencing and targeted metabolomic analysis to investigate the effect on microbial metabolic activity and community composition following long-term administration.

Within this study, the main objective was to gain insight into how the human gut microbiota was affected in response to long-term BF and AG supplementation when tested individually or as a combination of both prebiotic compounds in order to investigate potential complementary and/or synergetic effects. For this purpose, the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME®, ProDigest BV and Ghent University, Ghent, Belgium), an in vitro gut model simulating the entire human gastrointestinal tract, was used.

2. Materials and Methods

2.1. Experimental Design

In the first part of this study, a donor pre-screening test was performed by means of short-term fecal batch fermentations. Three different donors were tested, and it was examined whether the two test products (i.e., AG and BF) affected the metabolic activity of the gut microbiota. The test products were assessed individually and as a combination of both. Moreover, a low and high dosage strategy was investigated. For the combined treatment, in particular, a fiber ratio of 1:1 was used. Samples were taken at different time points, and the metabolic activity was assessed by measuring the pH and the concentration of (i) SCFA, (ii) lactate, (iii) branched SCFA (bCFA), and (iv) ammonium. All conditions were tested in technical replications.

In the second part of this study, a long-term SHIME® experiment was performed using a TripleSHIME® configuration with one donor and one test dose for each individual or combined treatment procedure. The donor was selected based on the results of the short-term fecal batch fermentations. Samples were again collected at different time points in order to assess the effect of the repeated administration of the test products on the metabolic activity and the composition of the gut microbiota. In order to monitor the metabolic activity, the concentrations of (i) SCFA, (ii) lactate, (iii) bSCFA, and (iv) ammonium were determined. For the composition of the gut microbiota, the novel quantitative 16S-targeted Illumina sequencing approach of Vandeputte et al. [37] was used. Moreover, the effect on the gut microbial metabolome was quantitatively analyzed by means of targeted ultra-high-performance liquid chromatography coupled with high-resolution Orbitrap mass spectrometry (UHPLC-LC-MLS/MS).

2.2. Chemicals and Test Products

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). AG (Inavea pure acacia™) was provided by Nexira (Rouen, France). It is an exudate from the acacia tree, characterized by its content of 90% soluble fiber composed of arabinogalactans hydrocolloid. Monomeric sugar distribution is about 40% galactose, 26% arabinose, 14% rhamnose, and 15% glucuronic acid [38]. The ramified, complex, and heterogeneous polysaccharidic structure is partially linked to low amounts of proteins

(about 2%) with a global molecular weight distributed from 20 kDa to 6000 kDa [15,17]. This structure specificity confers resilience to the fiber, a progressive fermentation profile, and high tolerability in the gut [20]. BF was also provided by Nexira and consists of a powder obtained from the baobab fruit pulp. Fibers of the baobab fruit are mainly insoluble (about 64%), and the main classifications contained are cellulose, fructo-oligosaccharides, and pectin (internal analysis). Polysaccharide structure has been described to be a rather small polysaccharide (Mw 53 kDa) in comparison with AG, and it is composed of fructose and glucose [31]. During the short-term fecal batch fermentations, the test products were investigated at two different dosages, i.e., 2.5 g/L and 5 g/L total fiber, with a fiber ratio of 1:1 being applied for the combined treatment. For the long-term SHIME® experiment, only one individual and one combined treatment dose were examined. For the individual treatment, on the one hand, an in vitro daily dosage of 5 g/d BF or AG was applied, corresponding to an in vivo human dose of 10 g/d. For the combined treatment, on the other hand, the in vitro dosage was equal to 2.5 g/d (i.e., 1.25 g/d BF + 1.25 g/d AG), thus corresponding to 5 g/d (i.e., 2.5 g/d BF + 2.5 g/d AG) in vivo. The lower combined test dose, as compared to the individual treatment, was selected in order to examine whether significant effects (as compared to the control period) could still be obtained while optimizing the combined treatment dose.

2.3. Donor Pre-Screening via Short-Term Fecal Batch Fermentations

A donor pre-screening test was performed by means of a short-term fecal batch fermentation experiment simulating the conditions of the proximal large intestine. The appropriate amount of test product was dissolved in water and dosed aseptically to reactors containing 63 mL sugar-depleted nutritional medium (5.2 g/L K_2HPO_4 (Chem-lab NV, Zedelgem, Belgium; CL00.1155.1000), 16.3 g/L KH_2PO_4 (Chem-lab NV; CL00.1146.1000), 2.0 g/L $NaHCO_3$ (Chem-lab NV; CL00.1432.1000), 2.0 g/L yeast extract (Oxoid, Cheshire, UK; LP0021B), 2.0 g/L special peptone (Oxoid; LP0072B), 1.0 g/L mucin (Carl Roth, Karlsruhe, Germany; 8494.7), 0.5 g/L L-cystein (Sigma-Aldrich; 2430-100 GM), and 2.0 mL/L Tween80 (P4780-100 ML)) to obtain a (total) fiber concentration of 2.5 or 5 g/L. Non-sterile anaerobic fecal slurries were prepared from freshly collected feces of three healthy human donors (i.e., donor A, donor B, and donor C) and inoculated at 10% (*v*/*v*) into the respective reactors, resulting in a total reactor volume of 70 mL. A blank was included for each donor to monitor the background activity of the community. For this purpose, only sugar-depleted nutritional medium was provided without the addition of AG and/or BF. All reactors were anaerobically incubated in a shaking incubator (90 rpm) at 37 °C for 48 h. Two technical replicates were performed for each treatment (and blank) procedure. As a consequence, 14 incubation experiments were performed for each donor or 42 experiments in total (i.e., 7 test conditions × 2 replicates × 3 donors, with blank, 2.5 and 5 g/L baobab fiber (BF), 2.5 and 5 g/L Arabic gum (AG), and 2.5 and 5 g/L BF + AG as test conditions).

2.4. Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

The reactor setup used to simulate the human gastrointestinal tract was derived from the SHIME® (ProDigest BV and Ghent University, Ghent, Belgium) as described by Molly et al. [33]. However, the SHIME® setup was adapted from a single SHIME® configuration (including one SHIME® arm) to a TripleSHIME® configuration (including three SHIME® arms) in order to allow a parallel comparison between the three different treatment conditions. Each arm of the TripleSHIME® configuration consisted of a succession of three reactors, with each of them representing a different region of the human gastrointestinal tract. The first reactor was used to simulate the upper gastrointestinal tract, followed by the subsequent simulation of the gastric and small intestinal phases. The second and third reactors were used to simulate the proximal colon (PC) and the distal colon (DC), respectively. The PC reactor, on the one hand, was operated at pH 5.6–5.9 and had a retention time of 20 h. The DC reactor, on the other hand, was operated at pH 6.6–6.9, and the retention time

was set at 32 h. Inoculum preparation, temperature settings, feeding regime, and reactor feed composition were adopted from Possemiers et al. [39]. Therefore, the authors refer to this specific study for more detailed information concerning these experimental aspects.

Upon inoculation of the PC and DC reactors with the fecal inoculum from the selected donor, a two-week stabilization period was initiated. This stabilization period allows the fecal microbiota to differentiate in the colonic reactors depending on the local environmental conditions (e.g., difference in pH). Subsequently, the baseline microbial community composition and activity were determined in the PC and DC reactors during a two-week control period, during which stability and reproducibility of the model were confirmed, reaching an average of 93.1% and 91.0%, respectively. Finally, a three-week treatment period of repeated daily administration of the test product(s) was incorporated to examine the effect of the treatment on the activity and the composition of the gut microbiota. The test products (i.e., AG and/or BF) were added to the SHIME nutritional medium and administered during each feeding cycle to obtain the corresponding daily doses. Thus, the test products were first transferred to the upper gastrointestinal tract region before reaching the colon vessels. It should be noted that SHIME nutritional medium (containing 1.2 g/L Arabic gum, 2.0 g/L pectin, 0.5 g/L xylan, 4 g/L starch, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L special peptone, 3 g/L mucin, and 0.5 g/L L-Cystein, ProDigest BV) was administered during both the stabilization and control period in order to allow differentiation of the gut microbial community within the proximal and distal colonic region, similar as what would occur in an in vivo situation. However, the SHIME nutritional medium was supplemented with AG and/or BF during the treatment period in order to determine whether AG and/or BF had an effect on the gut microbial activity and composition. Thus, the results of the treatment period were always compared to the results of the corresponding control period.

2.5. Analysis of the Microbial Metabolic Activity

For the short-term fecal batch fermentation experiments, the metabolic activity of the microbial community present in each reactor was examined at three different time points, i.e., following 0, 24, and 48 h of incubation. In order to characterize the metabolic activity, pH values were measured using a Senseline pH meter F410 (ProSense, Oosterhout, The Netherlands). Moreover, the concentration of different metabolites was assessed. The concentration of SCFA, including acetate, propionate, butyrate, and bCFA (i.e., the sum of isobutyrate, isovalerate, and isocaproate), was determined according to the procedure of De Weirdt et al. [40]. Moreover, also the total concentration of all identified linear and branched SCFA was calculated, i.e., by making the sum of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate, and caproate concentrations. The lactate levels were assessed using a commercially available enzymatic assay kit (R-Biopharm Nederland B.V., Arnhem, The Netherlands; E8240) according to the manufacturer's instructions. Finally, ammonium levels were determined, as previously reported by Duysburgh et al. [41]. Ideally, administration of the fibers should result in an alteration of the environmental pH and an increased production of lactate and SCFA, with propionate and butyrate being the most important metabolites due to their health-promoting effect.

During the long-term SHIME® experiment, the effect of the test products on the metabolic activity of the gut microbiota was also assessed based on the concentration of (i) SCFA (acetate, propionate, butyrate, bCFA, and total SCFA), (ii) lactate, and (iii) ammonium at different time points. For each colonic reactor (i.e., PC and DC), samples were collected three times per week during the entire control and treatment period.

2.6. Microbial Community Analysis through Quantitative 16S-Targeted Illumina Sequencing

In order to determine the microbial community composition in the long-term SHIME® experiment, samples were collected three times per week during the final week of the control and treatment period from the PC and DC reactors. To obtain proportional abundances at different phylogenetic levels (phylum, family, and OTU), 16S-targeted Illumina sequencing (LGC Genomics GmbH, Berlin, Germany) was applied using the procedure of

Props et al. [42]. Briefly, library preparation and sequencing were performed on an Illumina Miseq platform with v3 chemistry. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified using primers 341F and 785Rmod (5′-CCT ACG GGN GGG WGC AG-3′ and 5′-GAC TAC HVG GGT ATC TAA KCC-3′, respectively). As described in Schloss and Westcott [43] and Kozich et al. [44], the 16S-targeted sequencing analysis was adapted from the MiSeq protocol for read assembly and clean-up using the mothur software (v. 1.39.5). The different steps applied within this procedure were the following: (i) assembling of reads into contigs, (ii) alignment-based quality filtering performed by alignment to the mothur-reconstructed SILVA SEED alignment (v. 123), (iii) removal of chimeras, (iv) assignment of taxonomy via a naive Bayesian classifier [45] and RDP release 14 [46], and (v) clustering of contigs into OTUs at 97% sequence similarity. In addition, sequences classified as Eukaryota, Archaea, Chloroplasts, Mitochondria, and non-classified sequences were removed. For each OTU, representative sequences were selected as the most abundant sequence within that OTU. Furthermore, the obtained proportional abundances were used to calculate the reciprocal Simpson diversity index. This value is a measure of both the diversity and the evenness of the population and was calculated by the formula presented in Equation (1).

$$Reciprocal\ Simpson\ Diversity\ Index = \frac{1}{\sum_{i=1}^{n} proportional\ abundance_i^2} \quad (1)$$

with n = the total number of OTUs detected in one sample.

From each colonic reactor, samples were also collected three times per week during the final control and treatment week for enumeration of the bacterial cells via flow cytometry. A ten-fold dilution series was initially prepared in a phosphate-buffered saline (PBS) solution. Assessment of the viable, non-viable, and total population of the microbial community was performed by staining the appropriate dilutions with SYTO 24 (0.1 mM; Ex/Em: 480/500 nm; Life Technologies Europe B.V., Bleiswijk, The Netherlands; S7559) and propidium iodide (0.2 mM; Ex/Em: 490/635 nm; Fisher Scientific B.V., Merelbeke, Belgium; 11599296). Samples were analyzed on a BD FACSVerse (BD Biosciences, Erembodegem, Belgium). The samples were run using a high flow rate, and bacterial cells were separated from medium debris and signal noise by applying a threshold level of 200 on the SYTO channel. Proper parent and daughter gates were set to determine all populations. The data were analyzed using the FlowJo software (version 10.5.2), and the results were obtained as log (cells/mL).

By combining the proportional abundance values obtained by the 16S-targeted Illumina sequencing method and the total amount of bacterial cells determined through flow cytometry, quantitative abundances of the different taxonomic entities inside the reactors could be determined. As a result, a potential community shift following the administration of the test products could be mapped in large detail.

2.7. Evolution of the Gut Microbiome Activity Using Targeted Metabolomics

The dynamics of the metabolic patterns of the gut microbiome were analyzed during the last week of both the control and treatment period (three samples/week) of the long-term SHIME® experiment, and this was for both colonic regions and for each test condition. The metabolomic profile was quantitatively analyzed (Biocrates Life Sciences AG, Innsbruck, Austria) by means of targeted ultra-high-performance liquid chromatography coupled with high-resolution Orbitrap mass spectrometry (UHPLC-LC-MLS/MS). Moreover, the Biocrates MxP® Quant 500 kit (Biocrates, Innsbruck, Austria; 21094.12) was applied according to the manufacturer's instructions in order to analyze approximately 500 microbiome-related metabolites. A custom-made UHPLC column, based on a ZORBAX Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA), and a Triple Quad mass spectrometer Xevo® TQ-S (Waters, Milford, MA, USA) were used, and each identified metabolite was categorized into a different metabolite category as summarized in Table S1.

2.8. Data Processing and Statistical Analysis

Principle component analysis (PCA) was applied as a tool to aid the selection of the most appropriate donor following the short-term fecal batch fermentations. The metabolic activity results obtained from the incubations (i.e., pH, acetate, propionate, butyrate, bCFA, and total SCFA) were thereto normalized and uploaded to an online software program, i.e., ClustVis 1.0. The normalized results were included for all donors (i.e., donor A, donor B, and donor C) and all test conditions (i.e., blank, 2.5 or 5 g/L AG, 2.5 or 5 g/L BF, and 2.5 or 5 g/L BF + AG). Relative changes between 0 and 48 h of incubation were applied, and the normalized values were also calculated for an 'average donor' in order to identify strong donor-specific effects. The metabolic activity results were based on three biological replicates (=three donors) and two technical replicates. A confidence level of 70% was selected.

With respect to the long-term SHIME® experiment, metabolic activity and microbial community composition results ($n = 3$) were analyzed by means of a two-tailed homoscedastic t-test. This type of statistical analysis was used to compare (i) the corresponding control and treatment periods for a specific colonic region and (ii) the PC and DC region during the control period. It should be stressed that statistical tests regarding the microbial community composition were performed on the $\log_{10}$-transformed data (to make them normally distributed). Differences were considered statistically significant if the p-value was less than 0.05. For the reciprocal Simpson diversity index, statistical analysis was performed via a fixed linear model analysis of variance (i.e., one-way ANOVA; $p < 0.05$) after verification of the normality of the log-transformed data by means of a Shapiro–Wilk test.

With respect to the targeted metabolomics performed on samples from the long-term SHIME® experiment ($n = 3$), outlier analysis, cleaning, imputation, and $\log_2$-transformation of the data was performed prior to statistical analysis. Outlier analysis was performed by means of the interquartile range (IQR) method developed by Tukey [47]. For this purpose, the data were divided into four groups of equal size, setting three thresholds, and the threshold values defining the groups were calculated. Then, the IQR was calculated as the range between the first and third threshold, and any data point that was more than 1.5 times the IQR above the upper or more than 1.5 times the IQR below the lower threshold was considered an outlying value. Samples with markedly above-average outlier numbers were excluded if a biological reason for the anomaly was unlikely. Data cleaning was performed in order to exclude metabolites of which concentration values were missing or below the limit of detection (LOD). As a consequence, analytes were only included for further statistical analyses if at least 80% of valid values above the LOD per analyte were available in at least one group of the samples. After cleaning, the dataset still contained 164 metabolites. Missing value imputation was used to logically replace missing values with non-zero values while maintaining the overall data structure, according to Kooperberg and Stone [48]. Then, the dataset was further processed by $\log_2$-transformation to make assumptions about statistical tests (e.g., symmetric distribution, correction of heteroscedasticity, and skewness of the data) and to improve interpretability and visualization. Moreover, the MetIDQ™ MetaboINDICATOR module was used to calculate sums and ratios of selected metabolites or metabolic classes. Next, the normality of the log-transformed data was checked by means of a Shapiro–Wilk test and statistical analysis was performed via a fixed linear model analysis of variance (i.e., one-way ANOVA). The significance level was set to 0.05, and p-values were calculated. In order to control the false discovery rate (FDR) in multiple comparisons, FDR-adjusted p-values (q-values) were calculated using the Benjamini and Hochberg method [49]. A q-value of <0.05 was considered statistically significant. This type of analysis was performed for the individual components as well as for the sums and ratios obtained while using the MetaboINDICATOR module. Moreover, for each colonic region, separate ANOVA tests were performed to elucidate potential differences between (i) corresponding control and treatment periods and (ii) the different treatments. Finally, separate heatmaps were prepared for each test product (i.e., BF, AG, and BF + AG) and each colonic region (i.e., PC and DC) to visualize

the metabolomic results. Each row represents a different metabolite, or sum/ratio of metabolites, while each column represents a different sample with a specific color coding being applied, ranging from red to blue. For some microbiome-related metabolites of interest, box plots were prepared as well. These box plots include the minimum, maximum, and median values for each test condition during the control and treatment period.

Finally, to investigate potential associations between the metabolites and the metagenomic taxa, the metabolomics, microbial metabolic activity, and microbial community datasets were integrated using non-square correlation matrices. Fold changes were calculated by comparing each treatment to their corresponding control, using the data collected during the final control and treatment week (n = 3). Values below the limit of detection (LOD) were replaced by $\frac{1}{2}$ of the LOD. As an additional pre-processing step, metabolites and taxa were excluded from analysis if the number of LODs exceeded half of the total observations per investigated group. Fold changes were subsequently normalized via $\log_{10}$-transformation. Pearson correlation coefficients and t-test-derived p-values were calculated in R (R version 4.3.1 (2023-06-16 ucrt)) [50], comparing each metabolite and microbial family or phylum for each treatment per vessel [51]. Then, non-square correlation matrices were generated using the corrplot package [52] to visualize the pairwise association between the metabolites and metagenomic families or phyla.

3. Results

3.1. Short-Term Fecal Batch Fermentation Experiment

The results of the short-term fecal batch fermentation experiment (Supplementary Figure S1) indicated that each of the donors could have been selected for the long-term SHIME® experiment. Nevertheless, it opted to select donor A since this specific donor resulted in (i) the strongest pH decrease, (ii) the highest levels of total SCFA, acetate, and propionate, and (iii) the lowest levels of bCFA and ammonium (individual results not shown).

3.2. Long-Term SHIME® Experiment

3.2.1. The SHIME® Model as a Tool for the In Vitro Simulation of the Gut Microbiota Present in the Different Colon Regions

The microbial community composition of the proximal colon (PC) and distal colon (DC) reactors during the long-term SHIME® experiment was initially examined at the end of the control period in order to verify whether the difference in experimental conditions (i.e., pH and retention time) clearly resulted in a differentiation between both colonic regions. Quantitative 16S-targeted Illumina sequencing was used to determine the average levels (log (cells/mL)) of the different bacterial families encountered in the PC and DC region (Table S2). It was observed that the main phyla in the microbial community of the donor prior to administration of BF and/or AG included Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. However, for the majority of the bacterial families, significant differences were clearly observed between the two distinct colonic regions. Several bacterial families such as *Christensenellaceae* and *Ruminococcaceae* from the Firmicutes phylum, *Barnesiellaceae*, and *Rikenellaceae* from the Bacteriodetes phylum, and *Bifidobacteriaceae* from the Actinobacteria phylum specifically colonized the DC region. Moreover, the low abundance of Verrucomicrobia phylum was specifically detected in this colonic region. Other bacterial families, such as *Bacillaceae* from the Firmicutes phylum and *Enterobacteriaceae* from the Proteobacteria phylum, were mainly present in the PC region. Nevertheless, the highest cell density was observed overall in the DC region.

3.2.2. Metabolic Activity

The metabolic activity of the gut microbiota during the long-term SHIME® experiment was assessed three times per week during the control and treatment period for each colonic vessel. First, upon assessment of the normalized kinetic SCFA profiles (Figure 1), a sudden increase in total SCFA concentrations was observed at the start of the treatment period

for all experimental test conditions, with the strongest increases upon supplementation of the individual test compounds. The total SCFA and acetate concentrations instantly increased, while propionate and butyrate levels enhanced more gradually. Moreover, the propionate concentration often declined at one point during the treatment period, while the butyrate concentration increased at the same time. For the BF treatment, similar final increases in total SCFA were observed in both colonic regions. For the AG treatment and the combined treatment (BF + AG), on the other hand, the increase in total SCFA was slightly enhanced in the DC region compared to the PC region. Furthermore, upon calculating the average levels of the measured metabolic parameters during each experimental period (Table 1), it was noticed that acetate, propionate, butyrate, and lactate concentrations were significantly higher during the treatment period compared to the control period. This was valid for each treatment condition and each colonic region with only one exception, i.e., for propionate in the DC region following BF supplementation, for which no significant differences were observed between the experimental periods. Finally, treatment with the different test products consistently reduced bCFA and ammonium levels in both colonic regions.

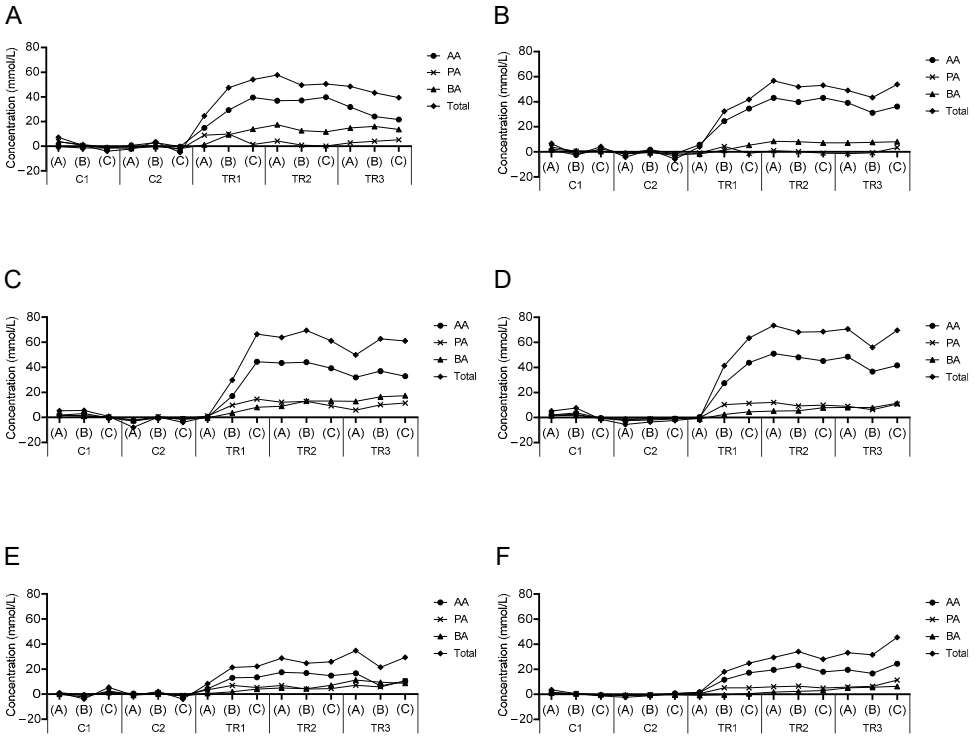

Figure 1. Normalized SCFA profiles during the long-term SHIME® experiment. Normalized kinetic values (mmol/L) of acetate (AA), propionate (PA), butyrate (BA), and total SCFA associated with treatment with baobab fiber (BF; (**A**,**B**)), Arabic gum (AG; (**C**,**D**)), and the combination of both (BF + AG; (**E**,**F**)) in the proximal (PC; (**A**,**C**,**E**)) and distal colon (DC; (**B**,**D**,**F**)) region. Samples were taken during two control (C1–C2) and three treatment (TR1–TR3) weeks, with each week three samples (A–C) being collected and presented as single data points. Average concentrations obtained during the control period (i.e., based on all six samples collected during the first (C1) and second (C2) week) were thereto subtracted from the corresponding concentration obtained at a specific time point.

Table 1. Microbial metabolic activity during the long-term SHIME® experiment. Absolute values (average ± stdev) of acetate (mM), propionate (mM), butyrate (mM), lactate (mM), bCFA (mM), and ammonium (mg/L) associated with the baobab fiber (BF), Arabic gum (AG), and combined (BF + AG) treatment in the proximal colon (PC) and distal colon (DC) vessels. Samples were taken during two control (C) and three treatment (TR) weeks (three samples/week). Statistically significant differences between the control and treatment periods are indicated in bold ($p < 0.05$).

	BF				**AG**				**BF + AG**			
	PC		**DC**		**PC**		**DC**		**PC**		**DC**	
	C	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**
Acetate (mM)	30.4 ± 1.6	**61.0 ± 8.8**	41.0 ± 2.1	**74.0 ± 11.8**	25.7 ± 2.1	**57.9 ± 14.8**	42.6 ± 2.4	**80.7 ± 15.8**	28.7 ± 1.0	**41.3 ± 4.7**	42.6 ± 0.9	**59.5 ± 6.8**
Propionate (mM)	28.1 ± 2.3	**32.3 ± 3.4**	29.0 ± 2.3	29.4 ± 2.1	23.3 ± 1.7	**33.0 ± 4.0**	24.9 ± 1.8	**33.7 ± 3.7**	26.0 ± 1.7	**32.0 ± 2.2**	25.7 ± 0.9	**31.4 ± 2.8**
Butyrate (mM)	14.6 ± 2.1	**26.8 ± 4.8**	15.3 ± 1.3	**21.0 ± 3.6**	15.1 ± 1.5	**25.4 ± 5.9**	15.5 ± 1.3	**21.3 ± 3.6**	13.4 ± 1.1	**19.3 ± 3.6**	13.8 ± 0.6	**16.4 ± 2.6**
Lactate (mM)	0.015 ± 0.005	**0.035 ± 0.004**	0.008 ± 0.004	**0.026 ± 0.007**	0.017 ± 0.002	**0.027 ± 0.004**	0.009 ± 0.001	**0.017 ± 0.003**	0.018 ± 0.003	**0.025 ± 0.006**	0.008 ± 0.004	**0.018 ± 0.007**
bCFA (mM)	**2.49 ± 0.14**	1.57 ± 0.18	**2.93 ± 0.13**	1.84 ± 0.32	**2.40 ± 0.15**	1.79 ± 0.19	**2.88 ± 0.11**	1.89 ± 0.30	**2.38 ± 0.16**	1.89 ± 0.18	**2.83 ± 0.09**	2.08 ± 0.26
Ammonium (mg/L)	**284 ± 25**	166 ± 39	**402 ± 21**	228 ± 63	**266 ± 28**	189 ± 41	**351 ± 58**	210 ± 57	**265 ± 31**	192 ± 28	**347 ± 49**	247 ± 46

3.2.3. Microbial Community Composition

The microbial community composition was determined both at the end of the control and treatment period for all treatment conditions and both colonic regions using 16S-targeted Illumina sequencing and flow cytometry. First, the reciprocal Simpson diversity index was calculated as a measure of both the diversity and evenness of the microbial community (Figure 2). Repeated administration of the different test products tended to increase the reciprocal Simpson diversity index and thus the population diversity (except in the PC upon BF supplementation), reaching significance in the DC region upon administration of the individual test products (i.e., BF and AG). However, the significance between the different test products was not reached.

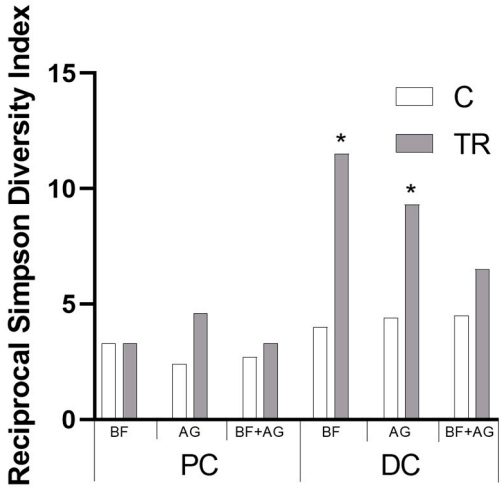

Figure 2. Microbial community diversity during the long-term SHIME® experiment. Average reciprocal Simpson diversity index of the proximal colon (PC) and distal colon (DC) upon treatment with baobab fiber (BF), AG (Arabic gum), and their combination (BF + AG) at different time points during the study, i.e., at the end of the control (C) and treatment period (TR) ($n = 3$). Significant differences between the corresponding control and treatment periods have been indicated by means of '*' ($p < 0.05$).

Next, treatment effects at different phylogenetic levels (i.e., phylum and family level) were investigated (Table 2). Results indicated that the enhanced Actinobacteria abundance

in the PC vessels upon treatment with BF and BF + AG was mainly attributed to increased levels of *Bifidobacteriaceae*. However, in the DC, increased Actinobacteria levels following BF supplementation were mainly attributed to an increased population of *Coriobacteriaceae*. No clear trend was observed for Actinobacteria levels in the DC region upon treatment with BF + AG. The decreased Bacteroidetes population observed in the PC region using BF and BF + AG was mainly the result of a decreased abundance of *Bacteroidaceae*. Nevertheless, the level of bacterial cells belonging to the *Prevotellaceae* family increased for all treatment conditions in the PC region despite the decreased or unchanged phylum levels. The increased Bacteroidetes level observed in the DC region for all treatment conditions was mainly linked to increased levels of *Bacteroidaceae*. For the AG treatment, in particular, increased levels of *Prevotellaceae* were also observed in the DC region. Although Firmicutes levels remained relatively stable in both colonic regions for all treatment conditions, some changes were observed at the family level. *Lachnospiraceae* were significantly stimulated upon treatment with AG in both colonic regions, whereas *Ruminococcaceae* specifically increased in the DC for all test conditions. In addition, BF and BF + AG treatment increased *Eubacteriaceae* levels, while BF + AG supplementation specifically stimulated the *Christensenellaceae* family. Finally, the increased abundance of the Verrucomicrobia phylum in the DC region following BF and BF + AG administration was mainly attributed to increased levels of *Akkermansiaceae*.

Table 2. Treatment effect on the microbial community composition at phylum and family level during the long-term SHIME® experiment. Relative levels (log (cells/mL)) of different families belonging to specific phyla observed in the proximal colon (PC) and distal colon (DC) vessels upon treatment with baobab fiber (BF), Arabic gum (AG), and the combination of both (BF + AG). Relative values were obtained by subtracting the average levels obtained at the end of the control period (n = 3) from the corresponding levels obtained at the end of the treatment period (n = 3). The intensity of the shading correlates with the relative abundance normalized for each of the different families (i.e., within each row), with the highest intensity being correlated with the highest value. Statistically significant differences between the absolute levels at the end of the control period and the absolute levels at the end of the treatment period were indicated by means of '*' ($p < 0.05$).

Phylum	Family	PC			DC		
		BF	AG	BF + AG	BF	AG	BF + AG
Actinobacteria	*Bifidobacteriaceae*	0.51 *	0.29	0.55 *	0.31	0.20 *	0.00
	Coriobacteriaceae	0.44	−0.59 *	−0.73 *	0.55 *	−0.56 *	−0.46 *
	Microbacteriaceae	0.87	−0.92	−0.76	0.49	−0.87	−0.73 *
	Micrococcaceae	−0.50	0.23	0.07	0.11	0.14	0.13
	Total	0.48 *	0.23	0.40 *	0.31 *	0.13	−0.07
Bacteroidetes	*Bacteroidaceae*	−0.80 *	−0.08	−0.41 *	0.22 *	0.25 *	0.28 *
	Marinifilaceae	<LOQ	<LOQ	<LOQ	0.67	0.39	0.29
	Prevotellaceae	1.40 *	2.96 *	1.34 *	0.68	1.59 *	0.75
	Rikenellaceae	−0.72 *	<LOQ	<LOQ	0.28	0.11	0.21
	Tannerellaceae	−0.98 *	0.44 *	0.10	−0.37 *	−0.23 *	−0.01
	Total	−0.47 *	0.13	−0.34 *	0.20 *	0.25 *	0.27 *
Firmicutes	*Acidaminococcaceae*	−0.88 *	−0.21	−0.45	−0.23	0.08	0.04
	Christensenellaceae	<LOQ	<LOQ	<LOQ	0.48	−0.56 *	1.14 *
	Clostridiaceae_1	<LOQ	−0.31	−1.03	<LOQ	<LOQ	<LOQ
	Enterococcaceae	<LOQ	−0.46	−0.25	<LOQ	−0.56 *	0.65
	Eubacteriaceae	<LOQ	<LOQ	<LOQ	0.65 *	0.31	0.45 *
	Family_XIII	<LOQ	<LOQ	<LOQ	0.10	−0.55 *	−0.14
	Lachnospiraceae	−0.09	0.46 *	0.00	0.12	0.23 *	0.04
	Ruminococcaceae	0.82	0.72	0.91	0.85 *	0.71 *	0.67 *
	Veillonellaceae	−0.09	−0.18	−0.22 *	−0.39	−0.16 *	−0.03
	Total	−0.08	−0.05	−0.19 *	−0.11	0.03	0.03
Lentisphaerae	*vadinBE97*	<LOQ	<LOQ	<LOQ	−0.16 *	−0.86 *	−0.65 *
	Victivallaceae	<LOQ	<LOQ	<LOQ	0.55	−0.99 *	0.10

Table 2. *Cont.*

Phylum	Family	PC			DC		
		BF	**AG**	**BF + AG**	**BF**	**AG**	**BF + AG**
	Total	<LOQ	<LOQ	<LOQ	0.41	−0.95 *	−0.32 *
Proteobacteria	*Burkholderiaceae*	−0.47 *	0.17	−0.48	−0.04	−0.02	−0.09
	Desulfovibrionaceae	−1.48	<LOQ	0.15	−0.17	−0.31	−0.08
	Enterobacteriaceae	0.09	−0.74	−0.58	0.28	−0.99	0.42
	Pseudomonadaceae	0.19	−0.30	−0.71	0.26	0.17	0.38 *
	uncultured	−1.89 *	<LOQ	<LOQ	−0.57	0.87	1.34
	Total	−0.22	−0.54	−0.59	−0.02	−0.18	0.21
Verrucomicrobia	*Akkermansiaceae*	<LOQ	<LOQ	<LOQ	0.95 *	−0.11	1.26 *
	Puniceicoccaceae	<LOQ	<LOQ	<LOQ	0.96	<LOQ	−0.11
	Total	<LOQ	<LOQ	<LOQ	0.95 *	−0.33	0.75 *

3.2.4. Metabolomics

Metabolomic analysis was performed on samples collected from the PC and DC reactors of the long-term SHIME® experiment, and these results have been presented by means of separate heatmaps (Figure S2A–F). Six microbiome-related metabolites of interest, detected either in the PC or DC region, were selected and further discussed (Figures 3 and 4). With respect to the PC region (Figure 3), significant changes between the control and treatment period occurred, amongst others, for three metabolites and/or classes/ratios of metabolites, i.e., ProBetaine, serotonin, and serotonin synthesis. For ProBetaine, treatment with BF, AG, and BF + AG resulted in significantly higher concentrations than the corresponding control periods. The highest and lowest values were obtained following treatment with BF and AG, respectively, while intermediate values were obtained for the combined treatment (BF + AG). Treatment with BF and BF + AG resulted in significantly higher serotonin levels as compared to the corresponding control periods, with the strongest effect being observed for the individual BF treatment. In addition, a similar trend was observed for the serotonin synthesis pathway. Treatment with AG alone had no significant effect on the serotonin levels in the PC region, though the serotonin synthesis pathway tended to be boosted. However, significance was only reached for BF and BF + AG. For the DC region (Figure 4), significant changes between the control and treatment period occurred, amongst others, for five metabolites, i.e., ProBetaine, spermidine, p-cresol-SO_4, serotonin, and sarcosine. For ProBetaine, all treatments increased ProBetaine levels as compared to the corresponding control periods, but significance was only reached upon supplementing BF alone. While the highest and lowest values were obtained for the BF and AG treatment, respectively, intermediate values were obtained for the combined treatment (BF + AG). For spermidine, all treatments significantly augmented concentrations than the corresponding control periods. No significant differences were observed between the treatments, although the combined treatment (BF + AG) proved to result in the highest values. For p-cresol-SO_4, treatment with BF, AG, and BF + AG significantly reduced the metabolite concentrations compared to the corresponding control periods. As for spermidine, no significant differences were observed between the different test products. Similar to the PC region, treatment with BF and BF + AG resulted in augmented serotonin levels as compared to the control periods, with a more pronounced effect again being observed for the individual BF treatment. Treatment with AG alone had no significant effect on the serotonin levels in the DC region. Finally, the sarcosine concentrations also decreased following treatment with BF, AG, and BF + AG, though only reaching significance for BF + AG. Furthermore, inter-treatment differences were observed, with the combined treatment (BF + AG) resulting in significantly lower sarcosine concentrations compared to AG treatment and intermediate values being obtained following BF supplementation.

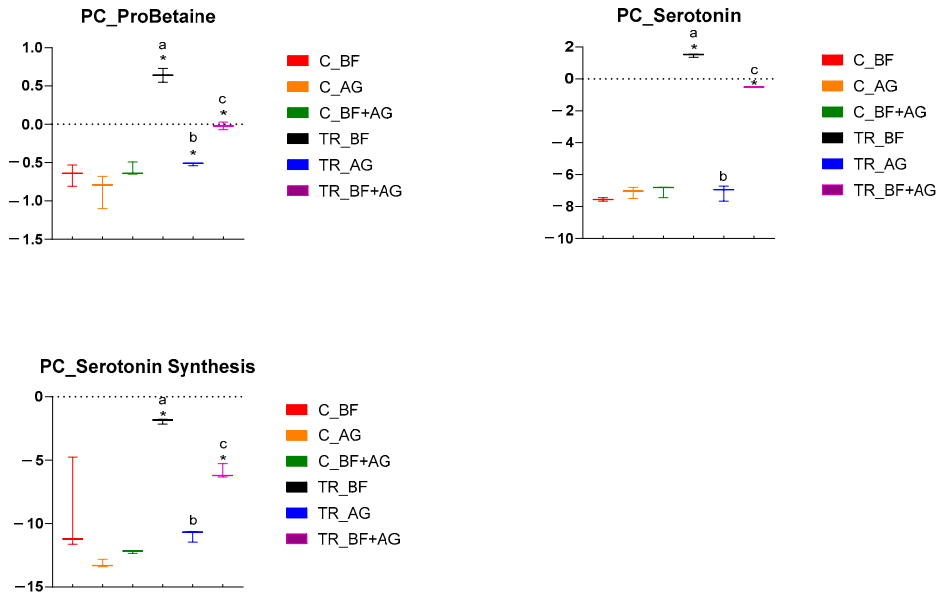

Figure 3. Specific metabolome-related changes observed in the proximal colon (PC) region following long-term product administration. Box plots for different metabolites and metaboINDICATORS (i.e., ProBetaine, serotonin, and serotonin synthesis) detected during the control (C) and treatment (TR) period upon repeated administration of baobab fiber (BF), Arabic gum (AG), and their combination (BF + AG). For each box plot, cleaned, imputed, and $\log_2$-transformed data from three independent replicates (n = 3) were used to determine the minimum, maximum, and median values ($\log_2(\mu M)$). Significant differences between corresponding control and treatment values have been indicated by means of '*', while significant differences between the treatments have been indicated by means of a different letter ($p < 0.05$ and $q < 0.05$).

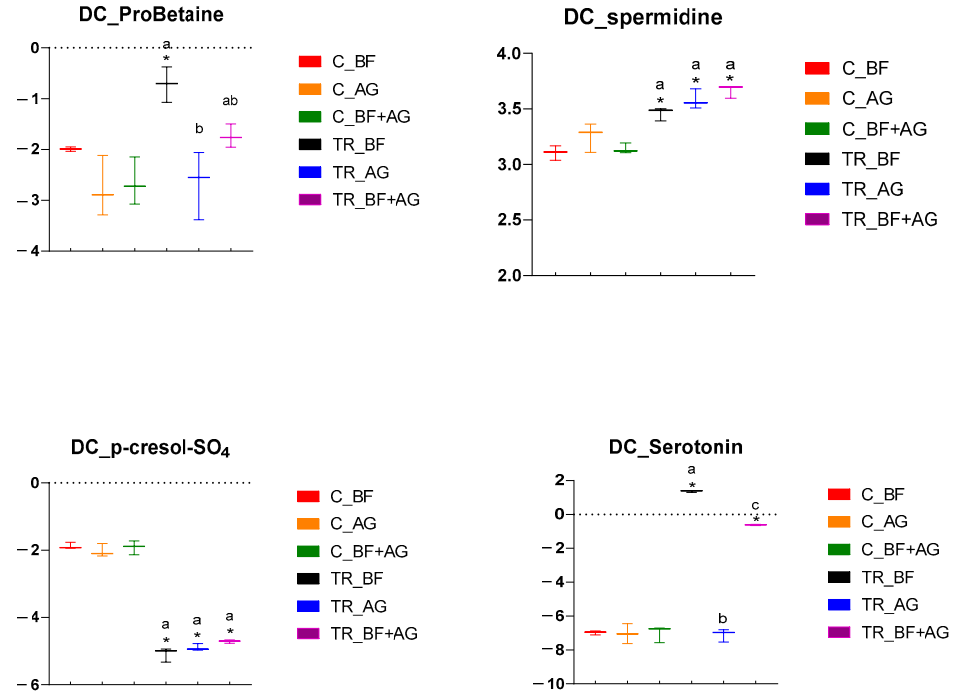

Figure 4. *Cont.*

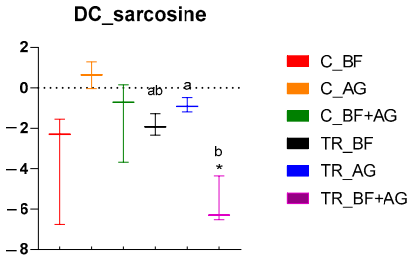

Figure 4. Specific metabolome-related changes observed in the distal colon (DC) region following long-term product administration. Box plots for different metabolites and metaboINDICATORS (i.e., ProBetaine, spermidine, p-cresol-SO_4, serotonin, and sarcosine) detected during the control (C) and treatment (TR) period upon repeated administration of baobab fiber (BF), Arabic gum (AG), and their combination (BF + AG). For each box plot, cleaned, imputed, and $\log_2$-transformed data from three independent replicates (n = 3) were used to determine the minimum, maximum, and median values ($\log_2(\mu M)$). Significant differences between corresponding control and treatment values have been indicated by means of '*', while significant differences between the treatments have been indicated by means of a different letter ($p < 0.05$ and $q < 0.05$).

3.2.5. Correlation Analysis between Metabolites and Metagenomic Families

Correlation analysis was performed in order to identify potential associations between the metabolites of interest and the identified metagenomic taxa (Figure 5). It was overall concluded that the correlations clearly depended on the colonic region (i.e., PC or DC) and the applied treatment (i.e., BF, AG, or BF + AG). In the PC region, a significant positive correlation was, for example, observed between acetate and the *Micrococcaceae*, *Rikenellaceae*, *Veillonellaceae*, and *Desulfovibrionaceae* families upon repeated BF administration. In the DC region, a significant negative correlation was, for example, observed between sarcosine and the *Rikenellaceae*, *Ruminococcaceae*, and *Victivallaceae* families upon repeated BF + AG administration.

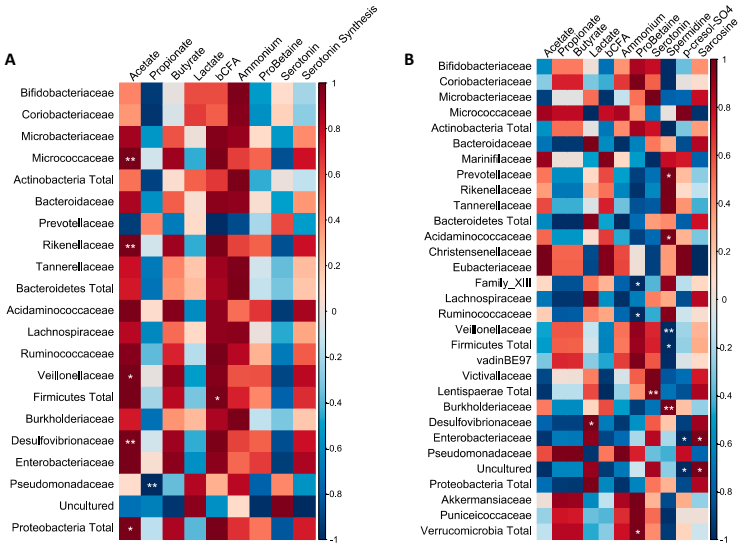

Figure 5. *Cont.*

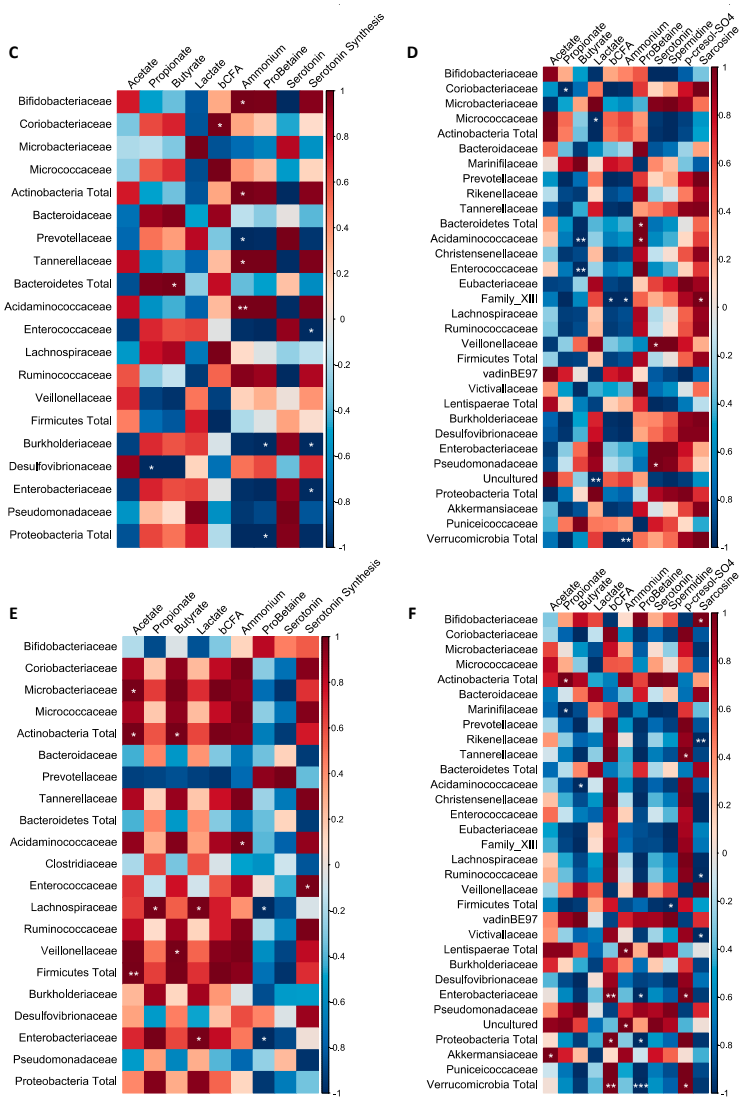

Figure 5. Non-square correlation matrices visualizing pairwise association between the metabolites and metagenomic families or phyla: (**A**) proximal colon–baobab fiber (PC–BF), (**B**) distal colon–baobab fiber (DC–BF), (**C**) proximal colon–Arabic gum (PC–AG), (**D**) distal colon–Arabic gum (DC–AG), (**E**) proximal colon–baobab fiber + Arabic gum (PC–BF + AG), (**F**) distal colon–baobab fiber + Arabic gum (DC–BF + AG). Statistically significant changes are indicated by means of * ($p \leq 0.05$), ** ($p \leq 0.01$), or *** ($p \leq 0.001$).

4. Discussion

4.1. The SHIME® Model as a Tool for the In Vitro Simulation of the Gut Microbiota Present in the Different Colon Regions

In the present study, the effect of repeated daily administration of AG, BF, and their combination on the human gut microbiome was investigated using the validated in vitro SHIME® model. While the microbial community of the selected donor was mainly dominated by members of the Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria phyla, which are indeed the phyla accounting for 93.5% of all identified species in the human gut microbiome [53], also low abundances of the Lentisphaera, Synergistetes, and Verrucomicrobia phyla were observed. Furthermore, the in vitro microbial community developed in a colon-region-specific way, confirming the literature findings [54]. For example, the *Ruminococcaceae* family (part of *Clostridium* cluster IV) was significantly enhanced in the DC region, as was observed in the study of Van den Abbeele et al. [54]. Furthermore, Van Herreweghen et al. [55] confirmed that the mucin-degrading *Akkermansia muciniphila*,

currently the only well-known representative of the Verrucomicrobia phylum in the human gut, specifically colonizes the DC, as was observed within the presented study. Thus, this region-specific colonization indicates that the SHIME® is a highly relevant in vitro model to simulate the effect of repeated administration of BF, AG, and their combination on the metabolic activity and community composition of the human gut microbiota.

4.2. Effect of Boabab Fiber and/or Arabic Gum on the Overall Gut Microbial Activity

Administration of BF, AG, and BF + AG enhanced levels of acetate and lactate, which are both key metabolites formed during primary substrate fermentation, indicating an effective breakdown of the test products. While acetate serves as an important energy source for the host [3], increased lactate levels can be considered favorable since it exerts strong antimicrobial effects against pathogens, especially at low pH values [56]. The highest final levels of acetate in the PC and DC region were obtained for BF and AG, respectively, indicating that BF was mainly fermented in the PC region, while AG was also (partially) fermented in the DC region. This region-specific fermentation was most likely the result of a difference in molecule structure/complexity, i.e., the structures of AG and BF were, respectively, reported as being complex [15,17,20] and relatively simple [31]. As previous studies indicated that most chronic colonic diseases mainly originate in the DC [14], prebiotic compounds should preferably exert their biological activity throughout the entire colon, suggesting the potential of co-supplementing BF and AG. Moreover, while the strongest increase in acetate production was obtained following administration of the individual compounds, significantly higher acetate levels were still obtained in both colonic regions upon combined BF + AG treatment, suggesting the potential to lower the test dose without losing the ability to exert prebiotic properties.

Next to increased levels of acetate and lactate, propionate and butyrate concentrations were also enhanced upon supplementation of the different test products, except for propionate in the DC region upon BF treatment. Butyrate has been linked with anti-inflammatory properties as it is responsible for maintaining intestinal homeostasis [57]. Moreover, it is able to reduce oxidative stress in the colon and plays a protective role against CRC and colitis [58].

Finally, ammonium and bCFA levels decreased for all treatment conditions, an effect that can be considered as beneficial as markers of proteolytic fermentation have been linked with direct and indirect health effects such as cancer development [59]. Although the combined treatment resulted in the highest final bSCFA and ammonium concentrations, probably due to the lower test dose, these levels were generally not significantly different from the individual treatment conditions, confirming that the lower combined treatment dosage was able to still entail potential health-promoting effects.

4.3. Effect of Boabab Fiber and/or Arabic Gum on the Gut Microbial Community Composition

The in vivo study of Calame et al. [21] previously confirmed that repeated administration of AG resulted in an increased abundance of *Bifidobacteriaceae* (Actinobacteria phylum), while the current long-term in vitro study is the first one to reveal a similar phenomenon for BF. Moreover, the current study identified potential complementary effects between both prebiotic fibers since BF and AG resulted in enhanced *Bifidobacteriaceae* levels in different colonic regions, i.e., in the PC and DC, respectively. Nevertheless, this complementarity was only confirmed in the PC region where the combined treatment, using a lower dosing strategy as compared to the individual treatments, still resulted in enhanced *Bifidobacteriaceae* levels. Interestingly, the community composition at the operational taxonomic unit (OTU) level (Table S3) indicated that administration of BF and AG resulted in an increased abundance of OTUs related to *Bifidobacterium adolescentis* and *Bifidobacterium longum*, respectively, which again suggests complementarity between both prebiotic compounds. The presence of *Bifidobacteriaceae* in the human gut microbiome can be considered beneficial since previous studies indicated that these bacteria have, amongst others, the potential to

(i) prevent and/or treat CRC, (ii) reduce symptoms of inflammatory bowel disease (IBD), and (iii) treat diarrhea [60].

At the level of the Bacteroidetes phylum, all treatments resulted in significantly increased *Bacteroidaceae* and *Prevotellaceae* levels in the DC and PC regions, respectively. For the DC region, in particular, a significantly enhanced *Prevotellaceae* abundance was only observed for AG. Thus, the current study is in line with the study of Kishimoto et al. [61], where a pig model indicated that *Prevotella ruminicola* was the predominant species responsible for AG fermentation and corresponding SCFA production.

In both colonic regions, but for the DC region in particular, increased levels of the *Ruminococcaceae* family (Firmicutes phylum) were observed for all treatments. Interestingly, the community composition results at the OTU level (Table S3) indicated that the administration of AG and BF resulted in an increased abundance of OTU 13 in the PC and DC regions, respectively. This specific OTU number is closely related to *Faecalibacterium prausnitzii*, which is able to exert a strong anti-inflammatory activity in the intestinal environment, mainly linked with the production of butyrate stimulation of regulatory T-cells [62]. Furthermore, increased *Faecalibacterium prausnitzii* levels have been associated with a reduction of endotoxemia in obese subjects [63]. A specific finding for the combinatory product (BF + AG) included the significant stimulation of the *Christensenellaceae* (Firmicutes phylum) abundance in the DC, which was not observed upon supplementation of the individual test compounds. *Christensenellaceae* include several butyrate-producing microorganisms and have been related to many health-promoting effects [64,65]. The study of Goodrich et al. [66], for example, indicated that the abundance of *Christensenellaceae* was significantly increased in individuals with a normal body mass index (BMI) compared to obese individuals, suggesting an anti-obesity effect of this bacterial family. In addition, long-term administration of *Christensenellaceae minuta* DSM33407 in a diet-induced obesity mouse model indicated that this specific strain had a positive effect on body weight gain, food metabolization, and fat accumulation [67].

At the level of the Verrucomicrobia phylum, an enhanced *Akkermansiaceae* abundance was observed for BF alone and the combined treatment (BF + AG). Interestingly, despite the lower test dose being applied, higher *Akkermansiaceae* levels were observed for the combined treatment as compared to BF treatment alone, indicating complementary effects between both fibers. *A. muciniphila*, the most well-known representative of the *Akkermansiaceae* family in the human gut, has anti-inflammatory properties and a positive effect on metabolic health [68]. In mice, *A. muciniphila* was linked with lowering body fat mass, improving glucose homeostasis, and decreasing adipose tissue inflammation [69–71]. In overweight and obese persons, on the other hand, a high abundance of *A. muciniphila* was correlated with lower fasting glucose levels, reduced waist-to-hip ratios, and lower subcutaneous adipocyte diameters. In addition, a high *A. muciniphila* abundance at baseline was linked with better glucose homeostasis, blood lipid profile, and body composition by the end of a 6-week calorie restriction period [72]. Thus, the increased *Akkermansiaceae* levels in the DC region indicate a strong prebiotic potential for the combination of BF and AG when supplemented at a concentration of 2.5 g/d.

4.4. Effect of Baobab Fiber and/or Arabic Gum on the Metabolomic Profile

For several of the metabolome-related metabolites of interest, similar trends were observed for the individual and combined treatment strategy, including an increase of ProBetaine in the PC, an increase of spermidine in the DC, and a reduction of p-cresol-SO_4 in the DC. ProBetaine is generally produced by microbial metabolization of betaine, the latter being available through dietary intake and/or synthesis via microbial oxidation of choline [73]. As no betaine was administered during the present in vitro study, the enhanced levels of ProBetaine probably originated from enhanced microbial betaine synthesis, with increased levels of betaine being correlated with antioxidative [73], osmoprotective [74], and anti-inflammatory effects [75]. Spermine and spermidine are essential for the proliferation of eukaryotic cells [76] and have been linked with health-promoting

properties, including protection against oxidative stress, maintenance of the intestinal mucosal barrier, and anti-inflammatory effects [77]. In addition, increased spermidine and spermine concentrations were previously linked with a decrease in body weight and an increased glucose tolerance in diet-induced obese mice (models) [78,79]. Thus, the increased microbial production in the DC region can be considered beneficial. P-cresol-SO_4, which is predominantly synthesized by the gut microbiota in the distal part of the colon, has been linked with detrimental health effects mainly due to oxidative stress [80,81]. Therefore, the reduction of these metabolites in the DC could be considered beneficial.

Also, some product-specific effects were observed at the metabolomic level. Serotonin levels increased in the PC and DC region following long-term administration of BF and BF + AG, while the individual treatment with AG had no direct effect on the serotonin levels. However, the serotonin synthesis pathway tended to be boosted in the PC region only upon administration of AG. Serotonin is a neurotransmitter that is critical for the development and functioning of the central nervous system. Most serotonin (i.e., 95%) is produced in the intestinal environment, where it is involved in hormonal, autocrine, paracrine, and endocrine actions [82]. As an example, serotonin has been found to modulate motility, inflammation, and epithelial development. It is involved in the regulation of peristaltic movement, but high serotonin levels are potentially correlated with increased pathogenesis in IBS patients. In addition, increased mucosal serotonin levels were previously linked with a proinflammatory effect [82]. Therefore, long-term intake of the combined treatment, entailing a more limited increase as compared to BF alone, would be recommended in order to find a balance between the critical functions of serotonin and its adverse side effects when too high levels are being reached. Finally, sarcosine levels decreased in the DC region following long-term administration of the combined treatment strategy, while the individual compounds resulted in unchanged levels. Sarcosine is an intermediate during glycine synthesis with neuroprotective properties [83]. Thus, the production of sarcosine in the colonic environment can be considered beneficial.

4.5. Correlation between the Different Metagenomic Taxa and the Metabolites of Interest

As indicated before, significantly enhanced acetate and lactate production was observed in both colonic regions for all treatments. In the literature, enhanced acetate and lactate levels were previously linked with enhanced *Bifidobacterium* spp. levels [84]. However, significant correlations between the *Bifidobacteriaceae* abundance and the production of acetate and lactate were not observed in the current study. Nevertheless, this does not necessarily mean that no acetate and lactate were formed by the members of the *Bifidobacteriaceae* family since acetate and lactate can be converted to secondary metabolites such as butyrate due to cross-feeding interactions within the gut microbial community.

The *Prevotellaceae* abundance was significantly enhanced in the PC region for all treatments. According to the literature, members of this family are able to produce acetate and succinate as a result of their metabolic activity [85]. This was not confirmed in the current study since negative (non-significant) correlations were observed between the *Prevotellaceae* abundance and the production of acetate. As for the *Bifidobacteriaceae* family, this does not necessarily mean that the members of the *Prevotellaceae* family did not contribute to the increased production of acetate, as acetate was likely converted to secondary metabolites such as propionate or butyrate.

Concerning the Firmicutes phylum, an enhanced *Ruminococcaceae* abundance was observed in the DC region for all treatments, while the *Christensenellaceae* abundance in the DC region was only enhanced for the combined treatment. Members of the *Ruminococcaceae* family are able to produce acetate, lactate, succinate, and butyrate, with *Faecalibacterium prausnitzii* being an important butyrate producer [86]. The *Christensenellaceae* members mainly produce acetate, but small amounts of butyrate can also be formed [64]. The *Ruminococcaceae* abundance was indeed positively correlated with acetate and/or lactate production, but significance was not reached. For the *Christensenellaceae* abundance, on the

other, a positive correlation with acetate production was indeed observed upon administration of BF + AG, but significance was again not reached.

Members of the *Akkermansiaceae* family were previously linked with an increased acetate and propionate production, with propionate being the most important endpoint [87]. Treatment with BF and BF + AG resulted in an enhanced *Akkermansiaceae* abundance in the DC region, which was indeed positively related with an enhanced acetate and propionate production. However, significance was only reached for the acetate production upon repeated administration of BF + AG.

The study of Gryp et al. [88] observed that the generation of p-cresol was correlated with bacterial species belonging to the *Bacteroidaceae*, *Bifidobacteriaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, *Ruminococcaceae*, and *Veillonellaceae*. While positive correlations between the p-cresol-SO_4 levels and the abundance of *Eubacteriaceae* (all treatments), *Lachnospiraceae* (AG and BF + AG), *Ruminococcaceae* (AG and BF + AG), and *Veillonellaceae* (AG) were indeed observed within currently presented studies, significance was not reached.

Finally, previous correlation studies have shown that high sarcosine levels were positively correlated with *Escherichia-Shigella* and negatively correlated with *Ruminococcaceae*, *Lachnospiraceae*, *Enterorhabdus*, and *Bacteroides* [89]. Therefore, the reduction in sarcosine concentration after co-supplementation of AG and BF could indicate a shift in microbiome composition away from *Escherichia-Shigella*, a group containing several pathogenic species, towards the other mentioned bacterial taxa, with the latter being confirmed in the current study (i.e., a negative correlation with the *Ruminococcaceae* and *Lachnospiraceae* family was observed, though only reaching significance for the *Ruminococcaceae* family).

5. Conclusions

This in vitro research is the first highlighting prebiotic activities of baobab fiber and indicated that repeated administration of baobab fiber, Arabic gum, and their combination had a significant effect on the metabolic activity and the microbial community composition of the gut microbiota present in the proximal and distal colon. Main prebiotic activities have been confirmed throughout lactate, short-chain fatty acid, diversity index, and *Bifidobacterium* enrichment. Despite the lower dosage strategy, co-supplementation of baobab fiber and Arabic gum resulted in some specific synergistic prebiotic effects, including the ability to exert their biological activity throughout the entire colon, specifically increased abundance of *Akkermansiaceae* and *Christensenellaceae* in the distal colon region, and increased levels of spermidine in the DC region.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu16111570/s1. Figure S1: Donor-specific metabolic activity effect during the short-term fecal batch fermentation experiments. Principle component analysis (PCA) of all metabolic activity results obtained from the short-term fecal batch fermentation experiments (i.e., pH, acetate, propionate, butyrate, bCFA, and total SCFA). Relative changes between 0 and 48 h of incubation were normalized for all donors (i.e., donor A, donor B, and donor C) and all test conditions (i.e., blank, 2.5 or 5 g/L baobab fiber (BF), 2.5 or 5 g/L Arabic gum (AG), and 2.5 or 5 g/L BF + AG). In addition, normalized values were calculated for an 'average donor' to identify donor-specific effects. A confidence level of 70% was selected, and the PCA was performed based on three biological replicates (= three donors) and two technical replicates. Figure S2: Metabolomic profile following long-term product administration. Heatmaps of the metabolites and metaboINDICATORS that were significantly different ($p < 0.05$ and $q < 0.05$) in the relevant group comparisons after data cleaning, imputation, and $\log_2$-transformation. Separate heatmaps have been included for each combination of the test product (i.e., baobab fiber (BF), Arabic gum (AG), and their combination (BF + AG)) and each colonic region (i.e., proximal colon (PC) and distal colon (DC)). (A) PC–BF, (B) PC–AG, (C) PC–BF + AG, (D) DC–BF, (E) DC–AG, and (F) DC–BF + AG. Each row and column of the heatmap represent a different metabolite and sample, respectively. For each metabolite, it was indicated as well as to which category of metabolites (see Table S1) it belongs. For both the control (C) and treatment (TR) periods, three replicates ($n = 3$) have been included, and the representative

log_2-transformed values have been indicated by means of a specific color range going from red to blue. Table S1: Classification of metabolome-related metabolites. Metabolite categories identified following targeted ultra-high-performance liquid chromatography coupled with high-resolution Orbitrap mass spectrometry (UHPLC-LC-MLS/MS). Table S2: Region-specific microbial colonization in the SHIME®. Average (± SD) levels (log (cells/mL)) of different bacterial families encountered in all proximal colon (PC) and distal colon (DC) vessels during the last week of the control period (n = 9). The p-value has been indicated in bold when significant differences ($p < 0.05$) were obtained between the different colon regions. In addition, the intensity of the shading correlates with the absolute abundance, normalized for each of the different families (i.e., within each row). Table S3: Treatment effect on the microbial community composition at OTU level during the long-term SHIME® experiment. Relative levels (log (cells/mL)) of the 25 most abundant OTUs, belonging to specific phyla and families, observed in the proximal colon (PC) and distal colon (DC) vessels upon treatment with baobab fiber (BF), Arabic gum (AG), and the combination of both (BF + AG). The closely related species, as identified by blasting of the corresponding sequence, were also included for each OTU. Relative values were obtained by subtracting the average levels obtained at the end of the control period (n = 3) from the corresponding levels obtained at the end of the treatment period (n = 3). The intensity of the shading correlates with the relative abundance, normalized for each of the different OTUs (i.e., within each row). Statistically significant differences between the absolute levels at the end of the control period and the absolute levels at the end of the treatment period were indicated by means of '*' ($p < 0.05$).

Author Contributions: Conceptualization, C.D., D.G. and M.M.; methodology, C.D.; software, C.D. and M.G.; validation, C.D.; formal analysis, C.D.; investigation, C.D.; resources, D.G. and M.M.; data curation, C.D. and M.G.; writing—original draft preparation, C.D. and M.G.; writing—review and editing, C.D., M.G. and M.M.; visualization, C.D. and M.G.; supervision, M.M.; project administration, M.M.; funding acquisition, D.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All fecal samples were collected according to the ethical approval of the University Hospital Ghent (reference number B670201836585, 3 August 2018).

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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Conflicts of Interest: Damien Guillemet is an employee of Nexira. Cindy Duysburgh, Marlies Govaert, and Massimo Marzorati are employees of ProDigest.

References

1. Ley, R.E.; Peterson, D.A.; Gordon, J.I. Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. *Cell* **2006**, *124*, 837–848. [CrossRef] [PubMed]
2. Steer, T.; Carpenter, H.; Tuohy, K.; Gibson, G.R. Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. *Nutr. Res. Rev.* **2000**, *13*, 229–254. [CrossRef] [PubMed]
3. den Besten, G.; van Eunen, K.; Groen, A.K.; Venema, K.; Reijngoud, D.-J.; Bakker, B.M. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340. [CrossRef] [PubMed]
4. Van der Beek, C.M.; Dejong CH, C.; Troost, F.J.; Masclee, A.A.M.; Lenaerts, K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. *Nutr. Rev.* **2017**, *75*, 286–305. [CrossRef] [PubMed]
5. Wang, P.X.; Deng, X.R.; Zhang, C.H.; Yuan, H.J. Gut microbiota and metabolic syndrome. *Chin. Med. J.* **2020**, *133*, 808–816. [CrossRef] [PubMed]
6. Cao, Y.; Shen, J.; Ran, Z.H. Association between *Faecalibacterium prausnitzii* Reduction and Inflammatory Bowel Disease: A Meta-Analysis and Systematic Review of the Literature. *Gastroenterol. Res. Pract.* **2014**, *2014*, 872725. [CrossRef] [PubMed]
7. Rebersek, M. Gut microbiome and its role in colorectal cancer. *BMC Cancer* **2021**, *21*, 1325. [CrossRef] [PubMed]

8. Gibson, G.R.; Hutkins, R.; Sanders, M.E.; Prescott, S.L.; Reimer, R.A.; Salminen, S.J.; Scott, K.; Stanton, C.; Swanson, K.S.; Cani, P.D. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 491–502. [CrossRef]
9. Davani-Davari, D.; Negahdaripour, M.; Karimzadeh, I.; Seifan, M.; Mohkam, M.; Masoumi, S.J.; Berenjian, A.; Ghasemi, Y. Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods* **2019**, *8*, 92. [CrossRef]
10. Markowiak, P.; Śliżewska, K. Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. *Nutrients* **2017**, *9*, 1021. [CrossRef]
11. Duncan, S.H.; Louis, P.; Thomson, J.M.; Flint, H.J. The role of pH in determining the species composition of the human colonic microbiota. *Environ. Microbiol.* **2009**, *11*, 2112–2122. [CrossRef]
12. Walker, A.W.; Duncan, S.H.; McWilliam Leitch, E.C.; Child, M.W.; Flint, H.J. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Appl. Environ. Microbiol.* **2005**, *71*, 3692–3700. [CrossRef]
13. Richardson, A.J.; McKain, N.; Wallace, R.J. Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. *BMC Microbiol.* **2013**, *13*, 6. [CrossRef] [PubMed]
14. Macfarlane, G.T.; Gibson, G.R.; Cummings, J.H. Comparison of fermentation reactions in different regions of the human colon. *J. Appl. Bacteriol.* **1992**, *72*, 57–64. [CrossRef]
15. Verbeken, D.; Dierckx, S.; Dewettinck, K. Exudate gums: Occurrence, production, and applications. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 10–21. [CrossRef]
16. Ahallil, H.; Abdullah, A.; Maskat, M.Y.; Sarbini, S.R. Fermentation of gum arabic by gut microbiota using in vitro colon model. *AIP Conf. Proc.* **2019**, *2111*, 050004. [CrossRef]
17. Rhazi, L.; Lakahal, L.; Andrieux, O.; Niamba, N.; Depeint, F.; Guillemet, D. Relationship between the molecular characteristics of Acacia gum and its functional properties. *Food Chem.* **2020**, *328*, 126860. [CrossRef]
18. Daguet, D.; Pinheiro, I.; Verhelst, A.; Possemiers, S.; Marzorati, M. Acacia gum improves the gut barrier functionality in vitro. *Agro Food Ind. Hi-Tech.* **2015**, *26*, 29–33.
19. Daguet, D.; Pinheiro, I.; Verhelst, A.; Possemiers, S.; Marzorati, M. Arabinogalactan and fructooligosaccharides improve the gut barrier function in distinct areas of the colon in the Simulator of the Human Intestinal Microbial Ecosystem. *J. Funct. Foods* **2016**, *20*, 369–379. [CrossRef]
20. Cherbut, C.; Michel, C.; Raison, V.; Kravtchenko, T.; Severine, M. Acacia Gum is a Bifidogenic Dietary Fibre with High Digestive Tolerance in Healthy Humans. *Microb. Ecol. Health Dis.* **2003**, *15*, 43–50. [CrossRef]
21. Calame, W.; Weseler, A.R.; Viebke, C.; Flynn, C.; Siemensma, A.D. Gum arabic establishes prebiotic functionality in healthy human volunteers in a dose-dependent manner. *Br. J. Nutr.* **2008**, *100*, 1269–1275. [CrossRef] [PubMed]
22. Duggan, C.; Gannon, J.; Walker, W.A. Protective nutrients and functional foods for the gastrointestinal tract. *Am. J. Clin. Nutr.* **2002**, *75*, 789–808. [CrossRef] [PubMed]
23. Salih, S.S.A.; Sabir, O.M.; Mshelbwala, M.; Gadour, M.O.E.H. Gum Arabic a superb anti-diarrheal agent. *Sudan J. Med. Sci.* **2012**, *7*, 83–88.
24. Elamin, S.; Alkhawaja, M.J.; Bukhamsin, A.Y.; Idris, M.A.S.; Abdelrahman, M.M.; Abutaleb, N.K.; Housawi, A.A. Gum Arabic Reduces C-Reactive Protein in Chronic Kidney Disease Patients without Affecting Urea or Indoxyl Sulfate Levels. *Int. J. Nephrol.* **2017**, *2017*, 9501470. [CrossRef] [PubMed]
25. Besco, E.; Braccioli, E.; Vertuani, S.; Ziosi, P.; Brazzo, F.; Bruni, R.; Sacchetti, G.; Manfredini, S. The use of photo chemiluminescence for the measurement of the integral antioxidant capacity of baobab products. *Food Chem.* **2007**, *102*, 1352–1356. [CrossRef]
26. Varudharaj, T.; Periyannan, M.; Narayanan, J.; Rajkishore, V.B. A Review on *Adansonia digitata*—Potential Herb. *Res. J. Pharmacogn. Phytochem.* **2015**, *7*, 57–60. [CrossRef]
27. Coe, S.A.; Clegg, M.; Armengol, M.; Ryan, L. The polyphenol-rich baobab fruit (*Adansonia digitata* L.) reduces starch digestion and glycemic response in humans. *Nutr. Res.* **2013**, *33*, 888–896. [CrossRef]
28. Gadour, M.O.; Khidir, H.B.; Adam, I.; Gasim, G.I. Effects of a powder of the fruit of *Adansonia digitata* (Tabaldia, Gongolase, or baobab tree) on serum lipids. *J. Herb. Med.* **2017**, *8*, 14–16. [CrossRef]
29. Garvey, R.; Clegg, M.; Coe, S. The acute effects of baobab fruit (*Adansonia digitata*) on satiety in healthy adults. *Nutr. Health* **2017**, *23*, 83–86. [CrossRef]
30. Foltz, M.; Zahradnik, A.C.; Van den Abbeele, P.; Ghyselinck, J.; Marzorati, M. A pectin-rich, baobab fruit pulp powder exerts prebiotic potential on the human gut microbiome in vitro. *Microorganisms* **2021**, *9*, 1981. [CrossRef]
31. Ibrahim, A.; Mahmoud, M.G.; Asker, M. Anti-inflammatory and Antioxidant Activities of Polysaccharide from *Adansonia digitata*: An in vitro Study. *Int. J. Pharm. Sci. Rev. Res.* **2014**, *25*, 174–182.
32. Van den Abbeele, P.; Taminiau, B.; Pinheiro, I.; Duysburgh, C.; Jacobs, H.; Pijls, L.; Marzorati, M. Arabinoxylo-Oligosaccharides and Inulin Impact Inter-Individual Variation on Microbial Metabolism and Composition, Which Immunomodulates Human Cells. *J. Agric. Food Chem.* **2018**, *66*, 1121–1130. [CrossRef] [PubMed]
33. Molly, K.; Vande Woestyne, M.; Verstraete, W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 254–258. [CrossRef] [PubMed]
34. Minekus, M.; Smeets-Peeters, M.; Bernalier, A.; Marol-Bonnin, S.; Havenaar, R.; Marteau, P.; Alric, M.; Fonty, G.; Huis'in't Veld, J.H. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Appl. Microbiol. Biotechnol.* **1999**, *53*, 108–114. [CrossRef] [PubMed]

35. Blaut, M.; Collins, M.D.; Welling, G.W.; Doré, J.; van Loo, J.; de Vos, W. Molecular biological methods for studying the gut microbiota: The EU human gut flora project. *Br. J. Nutr.* **2007**, *87*, S203–S211. [CrossRef] [PubMed]
36. Frank, D.N.; Amand, A.L.S.; Feldman, R.A.; Boedeker, E.C.; Harpaz, N.; Pace, N.R. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13780–13785. [CrossRef] [PubMed]
37. Vandeputte, D.; Kathagen, G.; D'hoe, K.; Vieira-Silva, S.; Valles-Colomer, M.; Sabino, J.; Wang, J.; Tito, R.Y.; De Commer, L.; Darzi, Y.; et al. Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **2017**, *551*, 507–511. [CrossRef] [PubMed]
38. Idris, O.H.M.; Williams, P.A.; Phillips, G.O. Characterisation of gum from *Acacolonizgal* trees of different age and location using multidetection gel permeation chromatography. *Food Hydrocoll.* **1998**, *12*, 379–388. [CrossRef]
39. Possemiers, S.; Verthé, K.; Uyttendaele, S.; Verstraete, W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. *FEMS Microbiol. Ecol.* **2004**, *49*, 495–507. [CrossRef]
40. De Weirdt, R.; Possemiers, S.; Vermeulen, G.; Moerdijk-Poortvliet, T.C.W.; Boschker, H.T.S.; Verstraete, W.; Van de Wiele, T. Human faecal microbiota display variable patterns of glycerol metabolism. *FEMS Microbiol. Ecol.* **2010**, *74*, 601–611. [CrossRef]
41. Duysburgh, C.; Van den Abbeele, P.; Krishnan, K.; Bayne, T.F.; Marzorati, M. A synbiotic concept containing spore-forming Bacillus strains and a prebiotic fiber blend consistently enhanced metabolic activity by modulation of the gut microbiome in vitro. *Int. J. Pharm X* **2019**, *1*, 100021. [CrossRef]
42. Props, R.; Kerckhof, F.-M.; Rubbens, P.; De Vrieze, J.; Hernandez Sanabria, E.; Waegeman, W.; Monsieurs, P.; Hammes, F.; Boon, N. Absolute quantification of microbial taxon abundances. *ISME J.* **2017**, *11*, 584–587. [CrossRef]
43. Schloss, P.D.; Westcott, S.L. Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl. Environ. Microbiol.* **2011**, *77*, 3219–3226. [CrossRef]
44. Kozich, J.J.; Westcott, S.L.; Baxter, N.T.; Highlander, S.K.; Schloss, P.D. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl. Environ. Microbiol.* **2013**, *79*, 5112–5120. [CrossRef]
45. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef] [PubMed]
46. Cole, J.R.; Wang, Q.; Cardenas, E.; Fish, J.; Chai, B.; Farris, R.J.; Kulam-Syed-Mohideen, A.S.; McGarrell, D.M.; Marsh, T.; Garrity, G.M.; et al. The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **2009**, *37*, D141–D145. [CrossRef]
47. Senthamarai Kannan, K.; Manoj, K.; Arumugam, S. Labeling methods for identifying outliers. *Int. J. Stat. Syst.* **2015**, *10*, 231–238.
48. Kooperberg, C.; Stone, C.J. Logspline Density Estimation for Censored Data. *J. Comput. Graph. Stat.* **1992**, *1*, 301–328. [CrossRef]
49. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Series B Methodol.* **1995**, *57*, 289–300. [CrossRef]
50. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2023; Available online: https://www.R-project.org/ (accessed on 14 May 2024).
51. Revelle, W. *Psych: Procedures for Psychological, Psychometric, and Personality Research*; Northwestern University: Evanston, IL, USA, 2024.
52. Wei, T.; Simko, V. R Package 'Corrplot': Visualization of a Correlation Matrix. (Version 0.92). 2021. Available online: https://github.com/taiyun/corrplot (accessed on 14 May 2024).
53. Laterza, L.; Rizzatti, G.; Gaetani, E.; Chiusolo, P.; Gasbarrini, A. The Gut Microbiota and Immune System Relationship in Human Graft-versus-Host Disease. *Mediterr. J. Hematol. Infect. Dis.* **2016**, *8*, e2016025. [CrossRef]
54. Van den Abbeele, P.; Grootaert, C.; Marzorati, M.; Possemiers, S.; Verstraete, W.; Gérard, P.; Rabot, S.; Bruneau, A.; El Aidy, S.; Derrien, M.; et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster IX. *Appl. Environ. Microbiol.* **2010**, *76*, 5237–5246. [CrossRef] [PubMed]
55. Van Herreweghen, F.; Van den Abbeele, P.; De Mulder, T.; De Weirdt, R.; Geirnaert, A.; Hernandez-Sanabria, E.; Vilchez-Vargas, R.; Jauregui, R.; Pieper, D.H.; Belzer, C.; et al. In vitolonizationion of the distal colon by *Akkermansia muciniphila* is largely mucin and pH dependent. *Benef. Microbes* **2017**, *8*, 81–96. [CrossRef] [PubMed]
56. Pessione, E. Lactic acid bacteria contribution to gut microbiota complexity: Lights and shadows. *Front. Cell Infect. Microbiol.* **2012**, *2*, 86. [CrossRef] [PubMed]
57. Donohoe, D.R.; Garge, N.; Zhang, X.; O'Connell, T.M.; Sun, W.; Bunger, M.K.; Bultman, S.J. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab.* **2011**, *13*, 517–526. [CrossRef] [PubMed]
58. Rivière, A.; Selak, M.; Lantin, D.; Leroy, F.; De Vuyst, L. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. *Front. Microbiol.* **2016**, *7*, 979. [CrossRef] [PubMed]
59. Wang, X.; Gibson, G.R.; Costabile, A.; Sailer, M.; Theis, S.; Rastall, R.A. Prebiotic Supplementation of In Vitro Fecal Fermentations Inhibits Proteolysis by Gut Bacteria, and Host Diet Shapes Gut Bacterial Metabolism and Response to Intervention. *Appl. Environ. Microbiol.* **2019**, *85*, e02749-18. [CrossRef] [PubMed]
60. O'Callaghan, A.; van Sinderen, D. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front. Microbiol.* **2016**, *7*, 925. [CrossRef] [PubMed]

61. Kishimoto, A.; Ushida, K.; Philips, G.O.; Ogasawara, T.; Sasaki, Y. Identification of Intestinal bacteria Responsible for Fermentation of Gum Arabic in Pig Model. *Curr. Microbiol.* **2006**, *53*, 173–177. [CrossRef] [PubMed]
62. Sokol, H.; Pigneur, B.; Watterlot, L.; Lakhdari, O.; Bermúdez-Humarán, L.G.; Gratadoux, J.-J.; Blugeon, S.; Bridonneau, C.; Furet, J.-P.; Corthier, G.; et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16731–16736. [CrossRef]
63. Gonzalez-Sarrias, A.; Romo-Vaquero, M.; Garcia-Villalba, R.; Cortes-Martin, A.; Selma, M.V.; Carlos Espin, J. The Endotoxemia Marker Lipopolysaccharide-Binding Protein is Reduced in Overweight-Obese Subjects Consuming Pomegranate Extract by Modulating the Gut Microbiota: A Randomized Clinical Trial. *Mol. Nutr. Food Res.* **2018**, *62*, e1800160. [CrossRef]
64. Morotomi, M.; Nagai, F.; Watanabe, Y. Description of *Christensenella minuta* gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order *Clostridiales*, and proposal of *Christensenellaceae* fam. nov. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 144–149. [CrossRef] [PubMed]
65. Waters, J.L.; Ley, R.E. The human gut bacteria *Christensenellaceae* are widespread, heritable, and associated with health. *BMC Biol.* **2019**, *17*, 83. [CrossRef] [PubMed]
66. Goodrich, J.K.; Waters, J.L.; Poole, A.C.; Sutter, J.L.; Koren, O.; Blekhman, R.; Beaumont, M.; Van Treuren, W.; Knight, R.; Bell, J.T.; et al. Human genetics shape the gut microbiome. *Cell* **2014**, *159*, 789–799. [CrossRef] [PubMed]
67. Mazier, W.; Le Corf, K.; Martinez, C.; Tudela, H.; Kissi, D.; Kropp, C.; Coubard, C.; Soto, M.; Elustondo, F.; Rawadi, G.; et al. A New Strain of *Christensenella minuta* as a Potential Biotherapy for Obesity and Associated Metabolic Diseases. *Cells* **2021**, *10*, 823. [CrossRef] [PubMed]
68. Cani, P.D.; de Vos, W.M. Next-Generation Beneficial Microbes: The Case of *Akkermansia muciniphila*. *Front. Microbiol.* **2017**, *8*, 1765. [CrossRef] [PubMed]
69. Everard, A.; Belzer, C.; Geurts, L.; Ouwerkerk, J.P.; Druart, C.; Bindels, L.B.; Guiot, Y.; Derrien, M.; Muccioli, G.G.; Delzenne, N.M.; et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9066–9071. [CrossRef] [PubMed]
70. Anhê, F.F.; Roy, D.; Pilon, G.; Dudonné, S.; Matamoros, S.; Varin, T.V.; Garofalo, C.; Moine, Q.; Desjardins, Y.; Levy, E.; et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. *Gut* **2015**, *64*, 872–883. [CrossRef] [PubMed]
71. Shin, N.-R.; Lee, J.-C.; Lee, H.-Y.; Kim, M.-S.; Whon, T.W.; Lee, M.-S.; Bae, J.-W. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* **2014**, *63*, 727–735. [CrossRef] [PubMed]
72. Dao, M.C.; Everard, A.; Aron-Wisnewsky, J.; Sokolovska, N.; Prifti, E.; Verger, E.O.; Kayser, B.D.; Levenez, F.; Chilloux, J.; Hoyles, L.; et al. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: Relationship with gut microbiome richness and ecology. *Gut* **2016**, *65*, 426–436. [CrossRef] [PubMed]
73. Koistinen, V.M.; Kärkkäinen, O.; Borewicz, K.; Zarei, I.; Jokkala, J.; Micard, V.; Rosa-Sibakov, N.; Auriola, S.; Aura, A.-M.; Smidt, H.; et al. Contribution of gut microbiota to metabolism of dietary glycine betaine in mice and in vitro colonic fermentation. *Microbiome* **2019**, *7*, 103. [CrossRef]
74. Ueland, P.M.; Holm, P.I.; Hustad, S. Betaine: A key modulator of one-carbon metabolism and homocysteine status. *Clin. Chem. Lab. Med.* **2005**, *43*, 1069–1075. [CrossRef] [PubMed]
75. Bingül, İ.; Başaran-Küçükgergin, C.; Fatih Aydın, A.; Çoban, J.; Doğan-Ekici, I.; Doğru-Abbasoğlu, S.; Uysal, M. Betaine treatment decreased oxidative stress, inflammation, and stellate cell activation in rats with alcoholic liver fibrosis. *Environ. Toxicol. Pharmacol.* **2016**, *45*, 170–178. [CrossRef]
76. Pegg, A.E.; Casero, R.A., Jr. Current status of the polyamine research field. *Methods Mol. Biol.* **2011**, *720*, 3–35. [CrossRef] [PubMed]
77. Rider, J.E.; Hacker, A.; Mackintosh, C.A.; Pegg, A.E.; Woster, P.M.; Casero, R.A., Jr. Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. *Amino Acids* **2007**, *33*, 231–240. [CrossRef]
78. Sadasivan, S.; Vasamsetti, B.; Singh, J.; Marikunte, V.V.; Oommen, A.M.; Jagannath, M.R.; Pralhada Rao, R. Exogenous administration of spermine improves glucose utilization and decreases bodyweight in mice. *Eur. J. Pharmacol.* **2014**, *729*, 94–99. [CrossRef]
79. Fernandez, A.; Bárcena, C.; Martínez-García, G.G.; Tamargo-Gómez, I.; Suárez, M.F.; Pietrocola, F.; Castoldi, F.; Esteban, L.; Sierra-Filardi, E.; Boya, P.; et al. Autophagy counteracts weight gain, lipotoxicity and pancreatic β-cell death upon hypercaloric pro-diabetic regimens. *Cell Death Dis.* **2017**, *8*, e2970. [CrossRef]
80. Smith, E.A.; Macfarlane, G.T. Enumeration of human colonic bacteria producing phenolic and indolic compounds: Effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J. Appl. Bacteriol.* **1996**, *81*, 288–302. [CrossRef]
81. Drazic, A.; Miura, H.; Peschek, J.; Le, Y.; Bach, N.C.; Kriehuber, T.; Winter, J. Methionine oxidation activates a transcription factor in response to oxidative stress. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9493–9498. [CrossRef] [PubMed]
82. Terry, N.; Margolis, K.G. Serotonergic Mechanisms Regulating the GI Tract: Experimental Evidence and Therapeutic Relevance. *Handb. Exp. Pharmacol.* **2017**, *239*, 319–342. [CrossRef]
83. Pinto, M.C.; Simão, F.; da Costa, F.L.P.; Rosa, D.V.; de Paiva, M.J.N.; Resende, R.R.; Romano-Silva, M.A.; Gomez, M.V.; Gomez, R.S. Sarcosine preconditioning induces ischemic tolerance against global cerebral ischemia. *Neuroscience* **2014**, *271*, 160–169. [CrossRef]

84. Bindels, L.B.; Delzenne, N.M.; Cani, P.D.; Walter, J. Towards a more comprehensive concept for prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2015**, *12*, 303–310. [CrossRef] [PubMed]
85. Morotomi, M.; Nagai, F.; Sakon, H.; Tanaka, R. *Paraprevotella clara* gen. nov., sp. nov. and *Paraprevotella xylaniphila* sp. nov., members of the family '*Prevotellaceae*' isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1895–1900. [CrossRef] [PubMed]
86. Moens, F.; De Vuyst, L. Inulin-type fructan degradation capacity of Clostridium cluster IV and XIVa butyrate-producing colon bacteria and their associated metabolic outcomes. *Benef. Microbes* **2017**, *8*, 473–490. [CrossRef] [PubMed]
87. Van der Ark, K.C.H.; Aalvink, S.; Suarez-Diez, M.; Schaap, P.J.; de Vos, W.M.; Belzer, C. Model-driven design of a minimal medium for *Akkermansia muciniphila* confirms mucus adaptation. *Microb. Biotechnol.* **2018**, *11*, 476–485. [CrossRef] [PubMed]
88. Gryp, T.; Vanholder, R.; Vaneechoutte, M.; Glorieux, G. p-Cresyl Sulfate. *Toxins* **2016**, *9*, 52. [CrossRef]
89. Choo, J.M.; Kanno, T.; Mohd Zain, N.M.; Leong, L.E.X.; Abell, G.C.J.; Keeble, J.E.; Bruce, K.D.; James Mason, A.; Rogers, G.B. Divergent Relationships between Fecal Microbiota and Metabolome following Distinct Antibiotic-Induced Disruptions. *mSphere* **2017**, *2*, e00005-17. [CrossRef]

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The Combination of Exercise and Konjac Glucomannan More Effectively Prevents Antibiotics-Induced Dysbiosis in Mice Compared with Singular Intervention

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Abstract: Our previous studies have demonstrated that konjac glucomannan (KGM) can prevent dysbiosis induced by antibiotics. While exercise may also impact the gut microbiome, there are limited studies reporting its protective effect on antibiotic-induced dysbiosis. Therefore, this study investigated the preventive and regulatory effects of a combination of 6-week exercise and KGM intervention on antibiotic-induced dysbiosis in C57BL/6J mice compared with a single intervention. The results showed that combined exercise and KGM intervention could restore the changes in the relative abundance of *Bacteroides* (3.73% with CTL versus 14.23% with ATBX versus 4.46% with EK) and *Prevotellaceae_Prevotella* (0.33% with CTL versus 0.00% with ATBX versus 0.30% with EK) induced by antibiotics ($p < 0.05$), and minimized the Bray–Curtis distance induced by antibiotics (0.55 with CTL versus 0.81 with ATBX versus 0.80 with EXC versus 0.83 with KGM versus 0.75 with EK). Compared with the combined intervention, exercise intervention also produced a certain level of recovery effects; the relative abundance of Rikenellaceae (1.96% with CTL versus 0.09% with ATBX versus 0.49% with EXC) was restored, while KGM supplementation showed the best preventive effect. In addition, the combination of exercise and KGM significantly enriched microbial purine metabolic pathways ($p < 0.05$). These findings indicate that combining exercise with KGM could be a promising approach to reducing the side effects of antibiotics on the gut microbiome.

Keywords: glucomannan dietary fiber; sports; gut microbiome; metagenomics; SCFA

1. Introduction

Antibiotics are highly effective in reducing bacterial infections and restoring good health and are widely utilized in clinical practices; in a population experiment, participants who received continuous antibiotic prophylaxis for 24 months were 14.4% less likely to develop a first urinary tract infection than untreated participants [1]. However, the inappropriate use of antibiotics can also lead to other health issues [2], such as triggering allergic reactions and toxic responses [3], as well as negatively impacting the gut microbiome, leading to gut microbiome dysbiosis [4] and causing gastrointestinal symptoms and other systemic diseases [5].

Microecological preparations mainly include probiotics, prebiotics, and synbiotics, which can regulate the intestinal microecological balance, improve the health level of the host, and promote the health state of the physiological live bacteria products and the metabolic products of these bacteria and promote the growth and reproduction of these physiological bacteria; they are considered an effective treatment for addressing dysbiosis resulting from antibiotic use [6–8]. According to our previous research, konjac glucomannan (KGM), a high molecular weight polysaccharide derived from konjac isolated

from the tuber of *Amorphophallus konjac C. Koch.*, has demonstrated its ability to prevent and regulate antibiotic-induced dysbiosis in the gut microbiome [9].

Exercise is an effective and personalized strategy to promote physical health and prevent disease. It can regulate metabolic function, boost the immune system, impact inflammation status, and ultimately improve overall quality of life [10]. Emerging evidence has demonstrated that exercise can influence the composition and distribution of the gut microbiome [11]. This includes regulating the richness and diversity of microorganisms, balancing intestinal microbiota through increased colonization of beneficial bacteria, and improving host immune function and metabolic capacity [12]. In animal models, the movement distance of the animals was negatively correlated with Bacteroidetes, and the proportion of harmful bacteria in the exercise mice was also significantly reduced [12]. Despite these findings, there is currently no research on whether exercise can prevent or regulate antibiotic-induced dysbiosis in the gut microbiota.

Therefore, this study aimed to investigate the impact of a singular aerobic exercise intervention and its combination with KGM on dysbiosis induced by antibiotics. The combination of nutritional and sports interventions is widely recognized as the most effective strategy for managing many chronic diseases. The combination of diet control and exercise is definitely more effective than a singular intervention and is well-documented in various chronic disease treatment, such as obesity [13], and based on the positive effects of exercise and KGM on gut microbiome [14,15], we hypothesize that the combination of exercise and KGM intervention is more effective in preventing dysbiosis and regulating the gut microbiome. This study holds significance in searching for potential solutions for better-managing dysbiosis.

2. Materials and Methods

2.1. Chemicals

Based on our previous study [9], the native KGM with the highest molecular weight was used in this study. A KGM with a purity of >95% was purchased from Johnson & Johnson (Ezhou, China). The KGM used in the study had a molecular weight of 1.82×10^7 Da, a specific viscosity of 9.48 dL/g, and a galactose/glucose ratio of 1.65:1. The six short-chain fatty acids standards, including acetic acid, propionic acid, *n*-butyric acid, *iso*-butyric acid, *n*-valeric acid, and *iso*-valeric acid, were purchased from Aradin (Shanghai, China). A DNA extraction kit was purchased from Tiangen Biotechnology Co., Ltd. (Beijing, China). All the chemicals used in this study are listed in Table S1, and the detailed information is from the NCBI PubChem Compound Database and the supplier sources.

2.2. Animal Experiment Design

Thirty male C57BL/6J mice (7-week-old) were purchased from Zhuhai BesTest Bio-Tech Co., Ltd. (Zhuhai, Guangdong, China) and randomly assigned to 5 groups. They were pre-adapted for 1 week prior to the experiment. Throughout the study, the mice were provided with standard feed (AIN-93G purified feed, detailed formula listed in Table S2) and distilled water. To eliminate potential confounding effects of female hormones on experimental data, only male mice were used as subjects in this study. The mice were housed in specific pathogen-free (SPF) facilities at Guangzhou Sport University Animal Center under standard conditions (22–24 °C, 50 ± 5% humidity), following a 12 h light/dark cycle. All experimental procedures adhered to the NRC Guidelines for the Care and Use of Laboratory Animals (2011). The animal experiment was reviewed and approved by the Animal Experimental Ethics Inspection of Guangzhou Sport University (Permit No. 2022DWLL-24, 19 August 2022).

In order to comply with the prevention time of KGM and ensure that the exercise duration meets the minimum standard for long-term exercise intervention, we implemented a 6-week experimental period [16]. Further details regarding the intervention time were discussed in the Discussion section. As depicted in Figure 1a, during the 6-week experi-

ment, mice in the control group (CTL), antibiotic group (ATBX), and exercise group (EXC) were provided with distilled water as their drinking water. The KGM group (KGM) and combined intervention group (EK) received KGM at a concentration of 2.5 g/L in their drinking water. During week 3, except for the CTL group, the other four groups received a combination of antibiotics (based on our previous study [9], the antibiotic formulation is ampicillin, streptomycin, and clindamycin at a ratio of 1 mg/mL each) added to their drinking water or KGM solution. The mice were free to access a drinking source and food. The water and food were replaced every two to three days, while the consumption of food and water was recorded and calculated weekly based. Based on the recorded data of provided and leftover weights of food and water, the total and daily average food intake and water intake of each mouse can be calculated throughout the experimental period. Mice underwent exercise training on the ZH-PT/5S treadmill (Zhenghua Bio-Instrument Facility, Huaibei, Anhui, China), with the EXC and EK groups undergoing a 6-week training regimen. The exercise protocol was designed based on a previously reported study, with slight modifications [17].

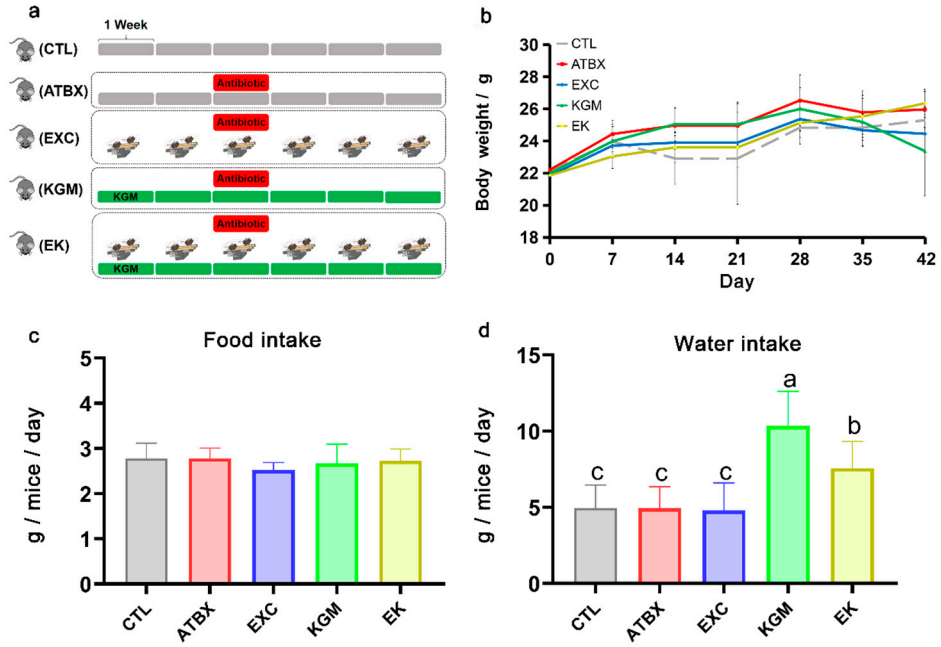

Figure 1. (**a**) The animal experiment diagram, (**b**) body weight, (**c**) food intake, (**d**) water intake in different groups for 42 days. Average of six samples. Error bars represent standard deviation at N = 6. Different letters indicate the significant difference among different groups for the same index, ANOVA with Bonferroni or Tamhane T2 post-hoc test, $p < 0.05$. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

As depicted in Table 1, the exercise program adopts progressive load exercise and determines the treadmill speed in the first week at 60% of the maximum running speed. Due to the reported impact of antibiotic intervention on the exercise ability of mice [18], the exercise load was kept unchanged in week 3 (13 m/min, 40 min), as it was in week 2. For the same reason, the speed increased in week 4 (14 m/min, 40 min) and remained at that level until week 6.

Table 1. The protocol of the exercise program.

Week	Velocity (m/min)	Duration (min)
1	13	30
2, 3	13	40
4, 5, 6	14	40

Fecal samples were collected from all groups on day 0, 14, 21, and 42 and stored at −80 °C in sterile tubes for subsequent analysis. We strictly adhered to the 3R principles in our experiments. In cases where the body condition of mice was poor, stool collection and exercise intervention were suspended until they returned to a normal state. Following the endurance test, the mice were given a day's rest and subsequently anesthetized with pentobarbital sodium. No incidents occurred at the end of the experiment. Blood samples were collected by cardiac puncture using a needle containing a solution of citric acid and glucose. The samples were immediately centrifuged at 3000 rpm at 4 °C for 15 min (Sorvall ST 8R small benchtop centrifuge, Thermo Fisher Scientific, Osterode am Harz, Germany), then plasma was collected and stored in a refrigerator at −80 °C for subsequent analysis.

2.3. Endurance Test

The mice were initially subjected to a speed of 10 m/min and underwent a 10 min running session, followed by incremental increases in treadmill speed every 2 min by 2 m/min until reaching exhaustion, enabling the assessment of their endurance performance [19]. According to our previous study [20], exhaustion was defined as the point at which the mice reached the electric grid at least five times within one minute or the inability of the animal to run on the treadmill for 10 s despite electrical prodding. Measures of mice exercise endurance included exhaustion time and running distance.

2.4. Histological Analysis

After rinsing the colon tissue with normal saline, it was subsequently fixed in a 4% paraformaldehyde solution at 4 °C for a duration of 24 h. Following fixation, the tissue was embedded in paraffin and subjected to hematoxylin and eosin (H and E) staining. A histological examination was performed using the Pannoramic 250 FLASH system (3DHISTECH Ltd., Budapest, Hungary), and an intestinal histological score was assigned (Table S3). The differences in intestinal pathological changes between different groups were analyzed utilizing CaseViewer Software 2.4.

2.5. Quantification of Short-Chain Fatty Acids in Plasma and Fecal Samples

Short chain fatty acids (SCFAs) in plasma and feces were determined using Shimadzu gas chromatograph (GC2010PLUS, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan) based on previous studies with minor modifications [21]. The six SCFA standards used for identification and quantification include acetic acid, propionic acid, *n*-butyric acid, *iso*-butyric acid, *n*-valeric acid, and *iso*-valeric acid (Aladdin®, Shanghai, China). In the final calculation, *n*- and *iso*-butyric acid and *n*-and *iso*-valeric acid were combined to be butyric acid and valeric acid, respectively.

A volume of 150 mL of Milli-Q water was added in the sterilized tubes containing feces, followed by vortexing to complete homogenization. Then, the supernatant was collected after 2 rounds of centrifugation at 12,000 rpm, 4 °C for 15 min using a Sorvall ST 8R small benchtop centrifuge (Thermo Fisher Scientific, Osterode am Harz, Germany). The pH of the supernatant was adjusted to 2–3 using 1M HCl. Crotonic acid was used as the internal standard. The sample was injected after filtration with a 0.45 μm membrane.

Agilent 7890B gas chromatography was used to determine SCFAs. Shimadzu SH-Rtx-Wax capillary column (DB-FFAP 1233232, 30 × 0.32 mm, Agilent Technologies Inc., Santa Clara, CA, USA) was used for separation. The carrier gas was high-purity nitrogen, and the flow rate was 0.6 mL/min. The initial column temperature (100 °C) was maintained for 2 min, and the temperature was raised to 150 °C at a rate of 5 °C/min for 2 min and then heated to 200 °C at a rate of 20 °C/min for 1 min. The FID temperature was 240 °C.

2.6. DNA Extraction and 16S rRNA Sequencing

Genomic DNA extraction was performed using the Tiangen Fecal DNA Extraction Kit (Tiangen, Beijing, China) and was carried out according to the manufacturer's instructions. The DNA concentration of microbial DNA was quantified using a NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA). Using Wekemo Technology Co., Ltd. (Shenzhen, China), 16S rRNA gene sequencing was performed. The data were demultiplexed and adapter-trimmed using a DADA2 plugin in a Qiime2 software, resulting in amplicon sequence variants (ASVs). Then, species annotation was obtained by referring to a Greengenes database 13_8.

2.7. Metagenomic Sequencing and Data Analysis of Fecal Samples

The fecal samples were sent to Wekemo Technology Co., Ltd. (Shenzhen, Guangdong, China) for metagenomic sequencing and data analysis of the gut. DNA was extracted according to the method in previous studies [22]; DNA purity and concentration were determined by agarose gel electrophoresis. Metagenomic sequencing using the Illumina NovaSeq platform (San Diego, CA, USA) was conducted, employing insert sizes of 350 bp and paired-end reads of 150 bp for each sample. Elimination of substandard and indeterminate bases was performed on the raw reads. The key step for quality control is to first remove the joint sequence then scan the rest of the sequence; if the average quality score is less than 20 (99% accuracy), then cut the subsequent sequence and remove the final length of the sequence less than 50 bp. (Trimmomatic parameters: illuminclip: adapter path: 2:30:10, sliding window: 4:20, minlen: 50). The effective sequences were obtained by eliminating the reads that were aligned to both the rat's genome reference and host DNA contamination, using Bowtie 2 with a parameter set as "very sensitive". A Kraken2 (2018) program was utilized to analyze the diversity and composition of species. The DNA sequence abundance of each metagenomic sample was calculated using the Bayesian resampling abundance as a statistical method based on the Kraken2 results. According to the results of Bracken, the percentage of sequences from the kingdom to the species was obtained for each sample in the total sequence. Principal component analysis (PCA) showed overall differences in species composition between groups. The similarity of species composition was studied by cluster analysis.

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify the characteristic species with high abundance in various group. The thresholds of LDA > 2 and LDA > 4 were used to distinguish high-abundance species. Gene function analysis was conducted by employing the HUMAnN 2.0 software, which involved comparing DNA sequences, post-quality-control, and removal of host sequences, with those present in the UniProt reference cluster 90 (UniRef90) database. The default comparison parameters in HUMAnN 2.0 were set to translated_query_corverage_threshold = 90.0, prescreen threshold_0.01, evalue_threshold = 10, and translated_subject_coverage_threshold = 50.0, and reads of inferior quality were eliminated correspondingly. Then, the protein expression levels in UNniRef90 were quantified as reads per kilobase per million and then compared with the clean reads of each sample to generate a functional database and Kyoto Encyclopedia of Genes and Genomes (KEGG) corresponding to their relative functional abundance. The LEfSe and a Dunn's test were used to identify the characteristic KEGG metabolic pathways, KEGG. pathways level 1 and KEGG. pathways level 2 in each group. The metabolic pathways were further verified in the MetaCyc database. The Circos diagrams were used to visualize the top 10 characteristic EC enzyme activity pathways in various samples.

2.8. Statistical Analysis

The differences in body weight, water intake, and food intake among the groups at each time point were analyzed using a Bonferroni or Tamhane T2 post hoc test or a one-way ANOVA with least-significant difference (Bonferroni) [23], intestinal morphological indicators, histological scores, running distance, concentration of SCFAs in feces, and SCFAs in plasma concentration. In line with the objective of this study, Bray–Curtis dissimilarity

was identified as the primary measure to assess the microbial shift caused by antibiotics and various interventions. The most stringent Bonferroni multiple-testing correction was employed for the primary outcome in order to compare the microbial shifts across different groups. The Bray–Curtis was analyzed using permutational multivariate analysis of variance (PERMANOVA). For the diversity and classification relative abundance of the gut microbiome, the metagenomic detection data were analyzed using one-way ANOVA and a Duncan post hoc test according to the literature report (Front Microbiol). For the data that do not meet the normal distribution, a Kruskal–Wallis test was used. $p < 0.05$ indicates statistically significant differences between groups. The statistical analysis was performed using an SPSS 26.0 software. The differences in microbial communities were analyzed using the Bray–Curtis dissimilarity principal component analysis (PCA), and the differences in fecal samples were evaluated using qiime2. Before using the above statistical methods to test the data, normality test is taken to ensure that the statistical method is correct. According to the data obtained from the statistical results, the differences in microbial diversity and different bacterial abundance between the groups can be compared, and the intervention effects of different intervention measures on the gut microbiome of mice can be judged by comparing with the CTL group. According to the PCA analysis, the difference of gut microbiome structure between the other four groups and the CTL group could be determined, and the most ideal intervention effect could be determined.

3. Results

3.1. Body Weight and Water and Food Intake

Figures 1 and S1 show the effect of different intervention methods on body weight (Figure 1b), food intake (Figure 1c), and water intake (Figures 1d and S1a) in mice. In general, the body weight of each group of mice increased gradually during the experiment, and although it showed some fluctuations, there was no significant difference between the groups at the end of the experiment. There was also no significant difference in total food intake among the groups.

As shown in Figure 1d, compared with the CTL group, the water intake of mice increased significantly when KGM was added, but exercise could offset this increased effect. The EK group significantly reduced the increase in water intake caused by the addition of KGM but still produced a significant difference with the CTL group, the ATBX group, and EXC group. At the end of the experiment, we conducted tests to determine the longest running distance achieved by mice in each group (Figure S1b). This was performed to assess the impact of antibiotics on their sport performance and evaluate the restorative effects of the three interventions. As shown in Figure S1b, even after administering antibiotics, there was a significant improvement in the longest running distance achieved by the exercise intervention group (the EXC and the EK groups) compared with the CTL group; the ATBX group was significantly lower than the CTL group, but there was no significant difference between the KGM group and the CTL group.

3.2. Intestinal Morphography

Figure 2a–e show the effects of different interventions on intestinal length and histology. Neither antibiotic intervention nor native KGM supplementation had an effect on colon length. However, compared with the CTL group, the EXC group significantly increased small intestine and whole intestine length. In addition, the H and E staining and histological scores showed no significant changes in colonic morphology among all groups.

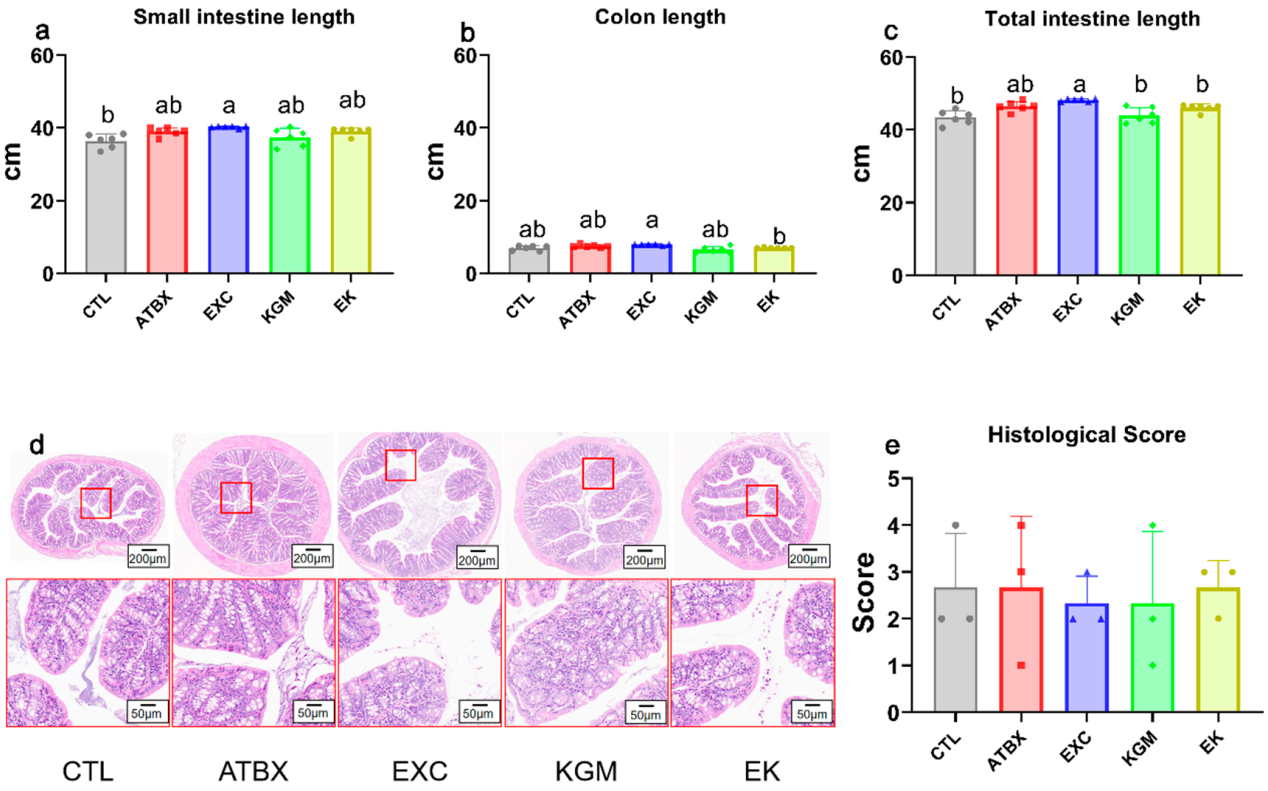

Figure 2. The effects of the different intervention methods on the intestinal tissue of mice. The length of the (**a**) small intestine, (**b**) colon, and (**c**) total intestine, and (**d**) representative pictures of H and E staining and (**e**) histological scores of mice colons. Average of six samples. Error bars represent standard deviation at N = 6 for (**a**–**c**), and N = 3 for (**e**). Different letters indicate the significant difference among different groups for the same index, ANOVA with Bonferroni or Tamhane T2 post hoc test, $p < 0.05$. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

3.3. Short-Chain Fatty Acids

Figure 3 shows the generation of SCFAs in feces (Figure 3a–e) and plasma (Figure 3f) in five groups on day 42. The main SCFAs in feces were acetic acid, propionic acid, and butyric acid, and the main SCFA in plasma was acetic acid. Compared with the CTL group, the total contents of SCFAs in the other four groups were significantly reduced, and the content of SCFAs in the EXC group was the lowest, which was significantly different from that in the ATBX group. Specifically, the acetic acid content could not recover to the CTL level after antibiotic perturbation and three intervention methods, and there was significant difference compared with the CTL group. There was no significant difference between the ATBX group and the CTL group in propionic acid, valeric acid, and butyric acid content, and the EXC, KGM, and EK groups did not recover to the CTL group level. In plasma, after the intervention of exercise combined with KGM (the EK group), the content of acetic acid was increased, and there was a significant difference compared with the other four groups. After supplementing KGM (the KGM group), the content of acetic acid in the plasma of the mice was lower than that in the CTL, ATBX, EXC, and EK groups and showed a significant difference.

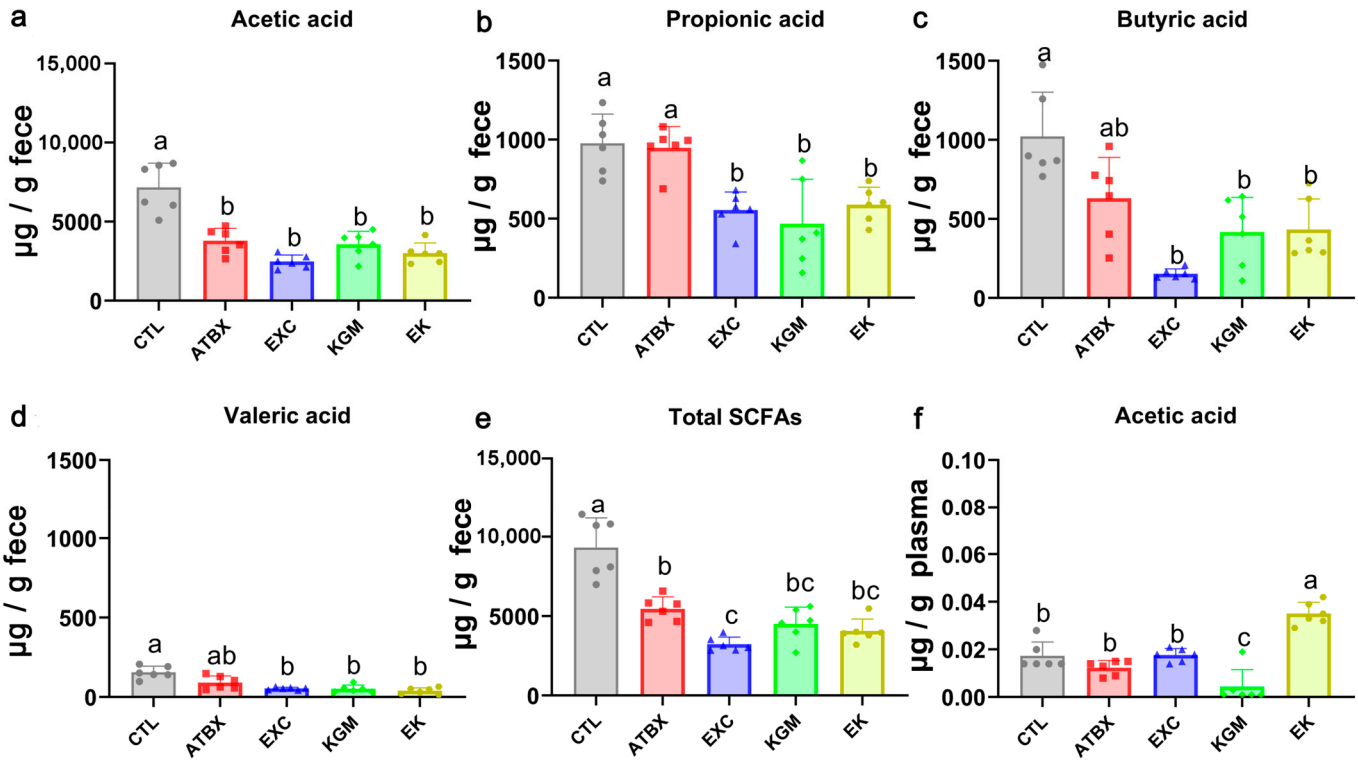

Figure 3. The effect of different intervention methods on SCFAs in mice feces and plasma on day 42. (**a**) Acetic acid, (**b**) propionic acid, (**c**) butyric acid, (**d**) valeric acid, (**e**) total SCFAs, (**f**) acetic acid in plasma. Different letters indicate the significant difference among different groups for the same index, ANOVA with Bonferroni or Tamhane T2 post hoc test, $p < 0.05$. Average of six samples. Error bars represent standard deviation at N = 6. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

3.4. Gut Microbiome (16S rRNA Sequencing\Bioinformatic Analysis)

In general, both exercise and KGM intervention led to significant changes in the gut microbiome. Table 2 shows the gut microbiome α diversity index on day 42. The diversity index is an analysis of species diversity in a sample, including the richness and evenness of species composition in the sample. Indices such as Observed OTUs and Shannon and Faith Phylogenetic Diversity (Faith_pd) are usually used to assess the species diversity of a sample, and the higher the index is, the more complex the diversity of the sample is. A chao1 index is an index used to reflect species richness (number of species). It extrapolates from the observed results a theoretical richness that is closer to the true richness. The observed OTUs index refers to the number of OTUs actually measured in the sample and the index that measures the richness of OTUs in the sample. The Shannon index, which is calculated taking into account the total number of OTUs in the sample and the proportion of each OTU. The Faith_pd is a diversity index calculated based on a phylogenetic tree, which uses the representative sequences of OTUs in individual samples to calculate the distance to build a phylogenetic tree, and adds the values of all the representative sequences in a sample to get the values. The Simpson index is used to estimate the similarity of the community, and it reflects the diversity based on accounting proportion of species in the community [24].

Table 2. The alpha-diversity indices of feces of mice on day 42. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

Groups.	Chao1	Faith_pd	Observed_otus	Shannon	Simpson
CTL	224 ± 43.67 ^{a}	20.16 ± 4.98 ^{a}	224 ± 43.67 ^{a}	4.62 ± 1.19	0.86 ± 0.17
ATBX	156.68 ± 28.12 ^{b}	12.81 ± 1.27 ^{bc}	156.68 ± 28.12 ^{b}	4.22 ± 0.57	0.88 ± 0.04
EXC	147.17 ± 35.05 ^{b}	13.47 ± 1.76 ^{bc}	147.17 ± 35.05 ^{b}	5.14 ± 0.40	0.95 ± 0.01
KGM	96.83 ± 27.78 ^{c}	10.55 ± 2.13 ^{c}	96.83 ± 27.78 ^{c}	4.19 ± 0.84	0.89 ± 0.09
EK	183 ± 32.54 ^{a}	15.75 ± 2.14 ^{b}	183 ± 32.54 ^{a}	4.85 ± 0.39	0.93 ± 0.03

Data are shown in average ± standard errors. Different letters indicate the significant difference among different groups for the same index, ANOVA, $p < 0.05$. N = 6.

The chao1 and observed_otus results were similar; only the EK group recovered to the CTL group level, while the ATBX group, EXC group, and KGM group were significantly lower than the CTL group, and the KGM group was significantly lower than the ATBX group and EXC group. The Faith_pd index of the ATBX group, EXC group, KGM group, and EK group decreased significantly compared with the CTL group, and the KGM group was significantly lower than the EK group. There was no significant difference between Shannon and Simpson among all groups.

3.5. Gut Microbiota

On day 0, as depicted in Figure 4a,b and Table S4, only minimal differences in gut microbial composition were observed within each group. The Table S3 presents the relative abundance of the top 20 taxa at different levels on day 0 and also showed no significant differences in the Bray–Curtis distance among all groups, indicating good comparability at the beginning of the experiment.

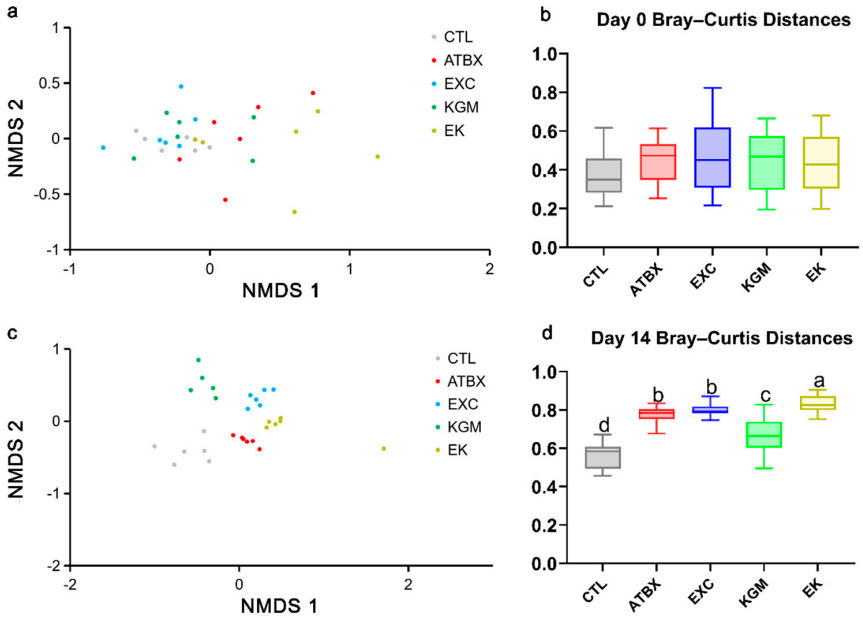

Figure 4. Effects of different intervention methods on fecal microbial composition of mice on day 0 and day 14: (**a**) the principal component analysis (PCoA) on day 0; (**b**) the Bray−Curtis distances from five groups to CTL group on day 0; (**c**) the principal component analysis (PCoA) on day 14; (**d**) the Bray−Curtis distances from five groups to CTL group on day 14. Not having the same letters indicates the significant difference among different groups for the same index. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

On day 14, as illustrated in Figure 4c,d and Table S5, there was a significant increase in Bacteroidetes at the phylum level for both the EXC group (77.64%) and the EK group (73.57%) compared with the CTL group (52.70%). In contrast, the relative abundance of Verrucomicrobia in the EXC group (0.31%) was significantly lower compared with the

CTL group (13.53%). Additionally, compared with the CTL group (3.40%), the relative abundance of Proteobacteria in the KGM group (0.33%) and the EK group (0.53%) was significantly reduced. At the family level, compared with the CTL group (13.53%), the relative abundance of Verrucomicroaceae in the EXC group (0.31%) and the EK group (2.01%) decreased significantly. The relative abundance of Paraprevotellaceae in the EK group (17.77%) was significantly higher than that in the CTL group (4.6%). However, the relative abundances of Bifidobacteriaceae (4.10% with the CTL versus 0.04% with KGM versus 0.02% with EK) decreased, while Alcaligenaceae (0.70% with the CTL versus 0.12% with KGM versus 0.45% with EK) and Prevotellaceae (0.69% with CTL versus 0.00% with KGM versus 0.71% with EK) showed a reduction due to KGM supplementation. At the genus level, exercise single intervention increased the relative abundances of unclassified taxa (43.83% with CTL versus 72.45% with EXC) but decreased *Akkermansia* (13.53% with CTL versus 16.67% with EXC) and *Parabacteroides* (4.14% with CTL versus 1.19% with EXC). Compared with the CTL group, the relative abundance of *Helicobacter* (2.48% with CTL versus 0.00% with KGM), *Sutterella* (0.69% with CTL versus 0.12% with KGM), and *Prevotellae_prevotella* (0.69% with CTL versus 0.00% with KGM) in the KGM group was significantly decreased. The relative abundance of *Akkermansia* (13.53% with CTL versus 2.06% with EK), *Helicobacter* (2.48% with CTL versus 0.17% with EK), and *Erysipelotrichacea_Clostridium* (0.74% with CTL versus 0.05% with EK) in the EK group also decreased significantly. In terms of the Bray–Curtis distance, the KGM, EXC, EK, and ATBX groups had significant differences compared with the CTL group.

On day 21, as illustrated in Figure 5a–e and Table S6, one-week perturbance of antibiotic resulted in more pronounced alterations in the gut microbiota of other four groups compared with the CTL group. At the phylum level, antibiotic intervention resulted in a decrease in the relative abundance of Bacteroidetes (72.83% with CTL versus 1.54% with ATBX), Firmicutes (17.31% with CTL versus 5.08% with ATBX), Verrucomicrobia (8.17% with CTL versus 0.05% with ATBX), Actinobacteria (0.99% with CTL versus 0.18% with ATBX), Tenericutes (0.13% with CTL versus 0.01% with ATBX), and *TM7* (0.01% with CTL versus 0.00% with ATBX) but an increase in Euryarchaeota (0.00% with CTL versus 0.07% with ATBX) compared with the CTL group. The KGM group partially mitigated these changes by preserving higher levels of Bacteroidetes (72.83% with CTL versus 1.54% with ATBX versus 15.85% with KGM), Firmicutes (17.31% with CTL versus 5.08% with ATBX versus 11.98% with KGM), and Verrucomicrobia (8.17% with CTL versus 0.05% with ATBX versus 0.87% with KGM), However, exercise intervention (EXC group and EK group) had no significant preventive effect on antibiotic-induced microbiome changes; the relative abundance of microbiome in the EXC group and the EK group had no significant difference compared with the ATBX group at the phylum level. At the family level (Figure 5b), compared with the CTL group, antibiotic intervention resulted in a decrease in the relative abundances of S24_7 (57.04% with CTL versus 0.41% with ATBX), Erysipelotrichaceae (8.64% with CTL versus 0.98% with ATBX), Verrucomicrobiaceae (8.17% with CTL versus 0.05% with ATBX), unclassified (5.12% with CTL versus 0.58% with ATBX), Paraprevotellaceae (4.78% with CTL versus 0.12% with ATBX), Porphyromonadaceae (3.78% with CTL versus 0.05% with ATBX), Lactobacillaceae (2.45% with CTL versus 0.35% with ATBX), Prevotellaceae (2.83% with CTL versus 0.16% with ATBX), and Rikenellaceae (1.56% with CTL versus 0.03% with ATBX). In contrast, there was an increase in the relative abundances of Pseudomonadaceae (0.00% with CTL versus 0.28% with ATBX). However, there was no significant difference between the KGM group and the CTL group. The Pseudomonadaceae in the EXC group (0.78%) also had no significant difference compared with the CTL group. At the genus level (Figure 5c), antibiotic intervention caused reductions in *Allobaculum* (7.27% with CTL versus 0.97% with ATBX), *Akkermansia* (8.17% with CTL versus 0.05% with ATBX), *Prevotella* (4.78% with CTL versus 0.09% with ATBX), *Parabacteroides* (3.78% with CTL versus 0.04% with ATBX), *Lactobacillus* (2.45% with CTL versus 0.35% with ATBX), *Prevotella_aceaeprevotella* (2.83% with CTL versus 0.16% with ATBX), *Serratia* (0.00% with CTL versus 0.54% with ATBX), and *Bifidobacterium* (0.64% with CTL versus 0.06% with

ATBX). On the other hand, antibiotics led to an increase in *Pseudomonas* (0.00% with CTL versus 0.28% with ATBX) and *Faecalibacterium* (0.00% with CTL versus 0.25% with ATBX). Notably, KGM intervention effectively attenuated most of these changes. As shown in Figure 5d, among the four groups with antibiotic perturbance, compared with the CTL group, the Bray–Curtis distances of the ATBX group, the EXC group, the KGM group, and the EK group were significantly different, but the Bray–Curtis distance of KGM group was closer to that of the CTL group and had a significant difference compared with the ATBX group, the EXC group, and the EK group.

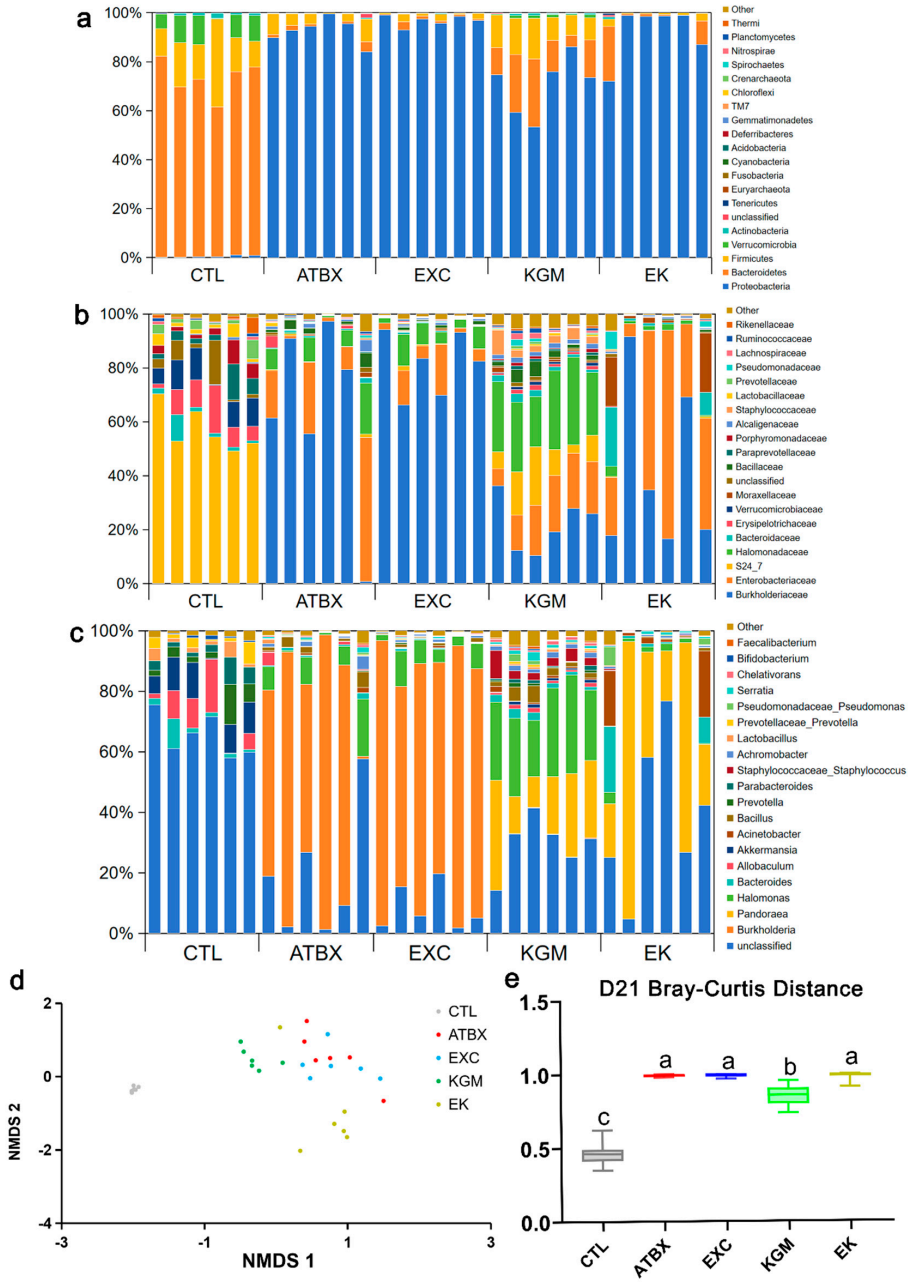

Figure 5. Effects of different intervention methods on fecal microbial composition of mice on day 21: (**a**) at phylum level, (**b**) at family level, and (**c**) at genus level. (**d**) The principal component analysis (PCoA), (**e**) Bray−Curtis distances from five groups to CTL group. Samples (N = 30) were colored by treatment. Not having the same letters indicates the significant difference among different groups for the same index. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

As illustrated in Figure 6a–e and Table S7, significant differences were observed in the relative abundance of Tenericutes (0.45% with CTL versus 0.00% with ATBX versus 0.05% with EK) at the phylum level on day 42 when comparing the ATBX group with the CTL group. However, the combination of exercise and the KGM restored these differences to a non-significant level, similar to that of the CTL group. There was no significant difference between the EXC group and the KGM group compared with ATBX group. At the family level (Figure 6b), compared with the CTL group, there is a significant difference in the relative abundance of Bacteroidaceae (3.73% with CTL versus 14.23% with ATBX), Rikenellaceae (1.96% with CTL versus 0.09% with ATBX), and Helicobacteraceae (0.11% with CTL versus 0.61% with ATBX) in the ATBX group, while there is no significant difference in the relative abundance of Bacteroidaceae (3.73% with CTL versus 14.23% with ATBX versus 7.30% with EXC versus 4.46% with EK) in the EK group and EXC group compared with the CTL group. The relative abundance of Rikenellaceae (1.96% with CTL versus 0.09% with ATBX versus 0.49% with EXC) in the EXC group was not significantly different from that in the CTL group. The addition of KGM (KGM group, EK group) showed no significant difference in relative abundance of Helicobacteraceae (0.11% with CTL versus 0.12% with KGM versus 0.00% with EK) compared with the CTL group. At the genus level (Figure 6c), compared with the CTL group, the relative abundance of unclassified (79.72% with CTL versus 45.27% with ATBX), *Bacteroides* (3.73% with CTL versus 14.23% with ATBX), *Prevotellaceae_Prevotella* (0.33% with CTL versus 0.00% with ATBX), *Lachnospiraceae_Clostridium* (0.01% with CTL versus 0.45% with ATBX), and *Pseudomonas* (0.77% with CTL versus 0.00% with ATBX) in the ATBX group had significant differences. However, the relative abundance of the unclassified (79.72% with CTL versus 65.07% with EK), *Bacteroides* (3.73% with CTL versus 4.46% with EK), *Prevotellaceae_Prevotella* (0.33% with CTL versus 0.30% with EK), and *Lachnospiraceae_Clostridium* (0.01% with CTL versus 0.14% with EK) in the EK group had no significant difference compared with the CTL group. FIG S1C represents the ratio of Firmicutes/Bacteroidetes (F/B) at each time point. The results obtained were similar to those described previously, with antibiotic intervention leading to large fluctuations in the gut microbiota on day 21, and KGM had the best protective effect compared with other interventions. On day 42, there was no significant difference in the F/B ratio among the groups. As shown in Figure 6d, compared with the CTL group, the Bray–Curtis distance of the other four groups was significantly different, and the Bray–Curtis distance between the KGM group and the CTL group was the farthest, which indicates that the species composition and structure were the most different. There was a significant difference between the EK group and the ATBX group ($p = 0.04$, $\eta^2 = 0.43$), indicating that the recovery effect of the EK group was better than the ATBX group.

As depicted in Figure 7a–c, on the KEGG level 1, "Human Diseases" were enriched in the ATBX group. On the KEGG level 2 (Figure 7b), antibiotic supplementation increased "Translation" and "Signal transduction", while exercise increased "Drug resistance_antineoplastic". The combined intervention of exercise and KGM (EK group) improved the LDA score of "Infectious disease_bacterial", "Nervous system", and "Transcription".

At the pathway level (Figure 7c), the intervention of ATBX significantly enriched multiple KEGG pathways, including Ribosome (map03010), Glycolysis/Gluconeogenesis (map00010: KO0850, K00001), HIF-1 signaling pathway (map04066), viral carcinogenesis (Map05203), and AMPK signaling pathway (map04152: K000850).

The EXC group mainly enriched biosynthesis and energy metabolism pathways, such as Monobactam biosynthesis (map00261: K01714); Antifolate resistance (map01523: K00560); Glycine, serine, and threonine metabolism (map00260: K00382); Tetracycline biosynthesis (map00253: K18221); and Adipocytokine signaling pathway (map04920: K01897).

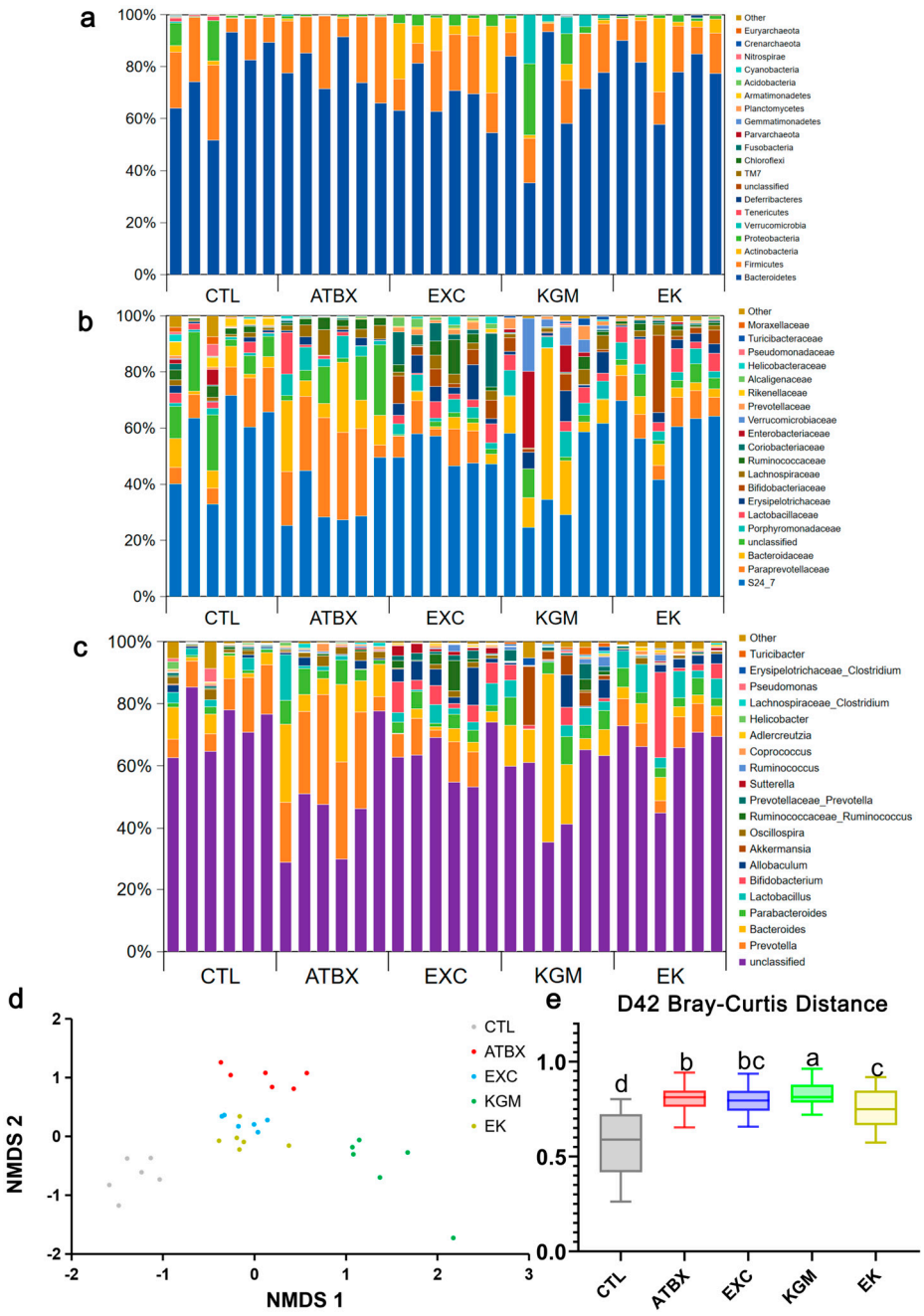

Figure 6. Effects of different intervention methods on fecal microbial composition of mice on day 42: (**a**) at phylum level, (**b**) at family level, and (**c**) at genus level. (**d**) The principal component analysis (PCoA), (**e**) Bray−Curtis distances from five groups to the CTL group. Not having the same letters indicates the significant difference among different groups for the same index. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM. Samples (N = 30) were colored by treatment.

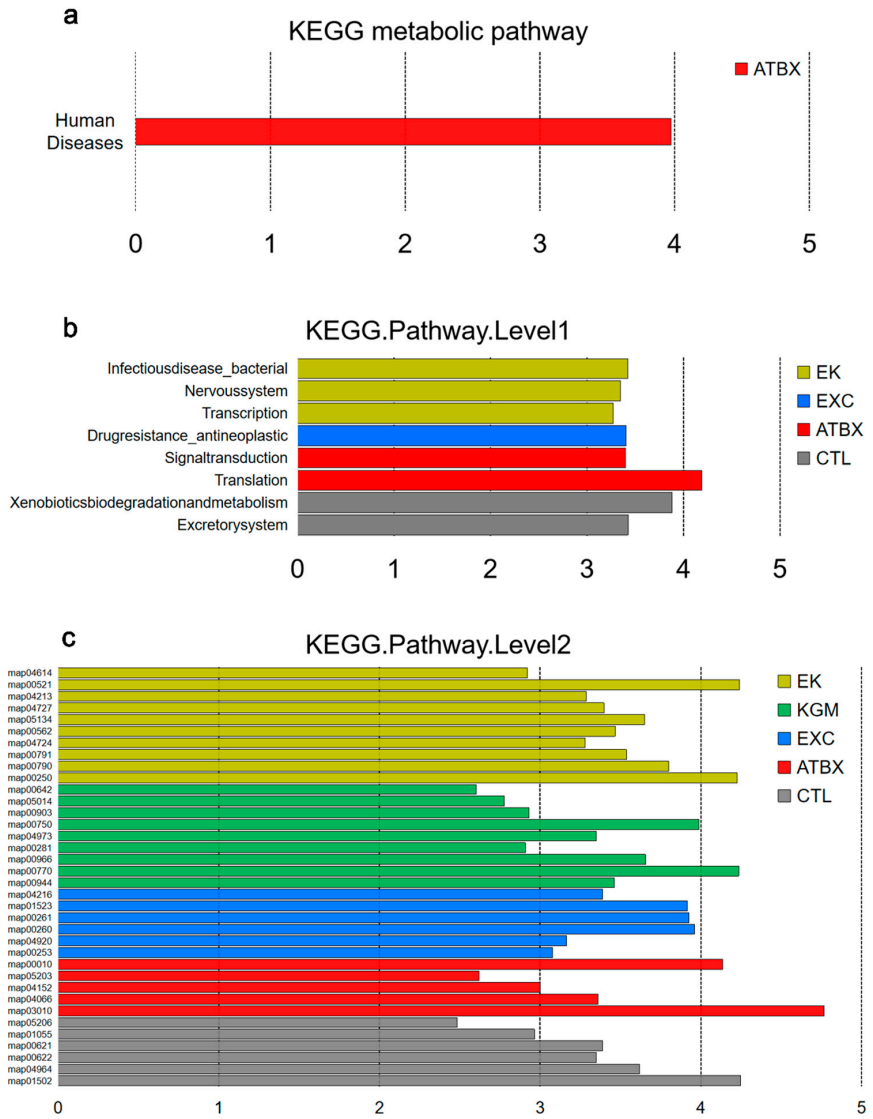

Figure 7. Effects of different intervention methods on KEGG metabolic pathway in mice at day 42: (**a**) KEGG metabolic pathways characteristics of CTL, ATBX, EXC, KGM, and EK groups at LDA2 level were analyzed via LEfSe, (**b**) KEGG. pathways level 1 characteristics of CTL, ATBX, EXC, KGM, and EK groups at LDA2 level were analyzed by LEfSe (**c**) KEGG. pathways level 2 characteristics of CTL, ATBX, EXC, KGM, and EK groups at LDA2 level were analyzed by LEfSe. Samples (N = 15) were colored by treatment. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

In the KGM group, there was enrichment in biosynthesis- and glucose-metabolism-related pathways like Pantothenate and CoA biosynthesis (map00770), Glucosinolate biosynthesis (map00966), Flavone and flavonol biosynthesis (map00944), and Vitamin B6 metabolism (map00750), along with Carbohydrate digestion and absorption (map04973). The combined intervention of exercise and KGM resulted in enrichment of Streptomycin biosynthesis (map00521: K01858), Alanine, aspartate and glutamate metabolism (map00250: K00265, K01915), Folate biosynthesis (map00790), Inositol phosphate metabolism (map00562), and Glutamatergic synapse (map04724: K01915) among other metabolic pathways.

In addition, the differential metabolic pathways in the MetaCyc database are displayed as heat maps (Figure 8a). For the ATBX group and the EK group, UMP biosynthesis III (PWY-7791), UMP biosynthesis I (PWY-5686), UMP biosynthesis II (PWY-7790), fo-

late transformations II (PWY-3841), and TRNA-CHARGING-PWY were not significantly different compared with the CTL group. The inosine-5′-phosphate biosynthesis I (PWY-6123), inosine-5′-phosphate biosynthesis II (PWY-6124), 5-aminoimidazole ribonucleotide biosynthesis II (PWY-6122), superpathway of 5-aminoimidazole ribonucleotide biosynthesis (PWY-6277), guanosine ribonucleotides de novo biosynthesis (PWY-7221), and other pathways related to purine metabolism were enriched in the EK group.

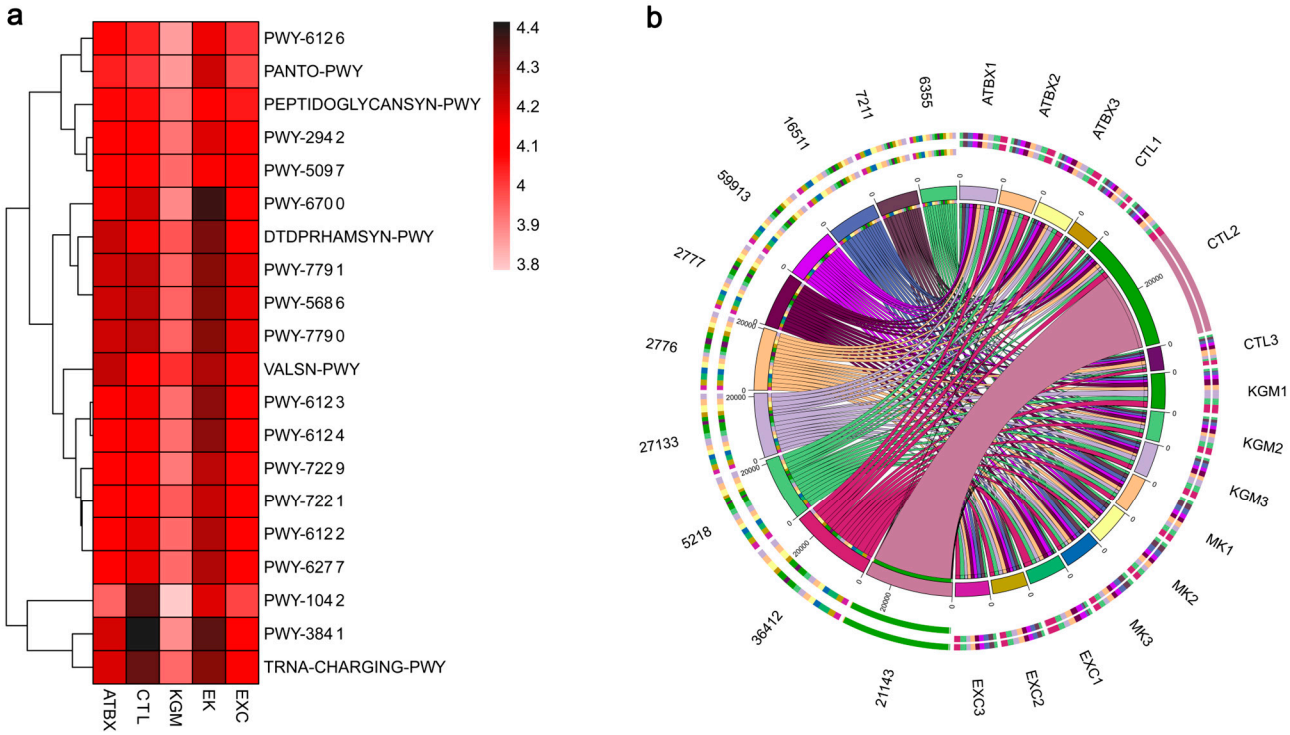

Figure 8. Effects of different intervention methods on MetaCyc metabolic pathway in mice at day 42. (**a**) MetaCyc metabolic pathway heat map after different intervention methods. (**b**) As shown in the Circos diagram, genes for enzyme activity were altered in five groups (Enzyme nomenclature database). CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM. Samples (N = 15) were colored by treatment.

The Circos diagram (Figure 8b) shows the proportion of each EC enzyme activity (top 10 abundance) in each sample, and the proportion of each EC enzyme activity in each sample. The ATBX group and the EK group DNA-directed DNA polymerase (EC 2.7.7.7.), DNA topoisomerase-ATP-hydrolyzing (EC 5.99.1.3.), and 6-phosphofructokinase (EC 2.7.1.11), restoring them to the CTL level, but the EXC group and the KGM group had significant differences compared with the CTL groups. In addition, except for the KGM group, beta-galactosidase (EC 3.2.1.23) in ATBX group, EXC group, and EK group had no significant difference compared with the CTL group.

3.6. Correlation Analysis

At the end of the experiment, the correlation between the relative abundance of different levels of gut microbiome and SCFAs was analyzed. The heat maps (Figure 9a) show that at the phylum level, Actinobacteria is significantly negatively correlated with acetic acid, propionic acid, butyric acid, and Valeric acid, while Tenericutes and TM7 are significantly positively correlated with acetic acid, propionic acid, and butyric acid. In addition, Actinobacteria, Deferribacteres, and Cyanobacteria are positively correlated with acetic acid levels in plasma. At the genus level (Figure 9c), *Prevotella*, *Parabacteroides*, *Bifidobacterium*, *Coprococcus*, and *Erysipelotrichaceae_Clostridium* are significantly negatively correlated with butyric acid. *Helicobacter* is significantly positively correlated with butyric

acid. *Paraprevotella*, *Sutterella*, and *Adlercreutzia* are significantly positively correlated with acetic acid level in plasma, and *Helicobacter* are significantly negatively correlated with acetic acid level in plasma. Meanwhile, an increase in *Adlercreutzia* and a decrease in *Helicobacter* were observed in the EK group, with a similar trend seen in the EXC group.

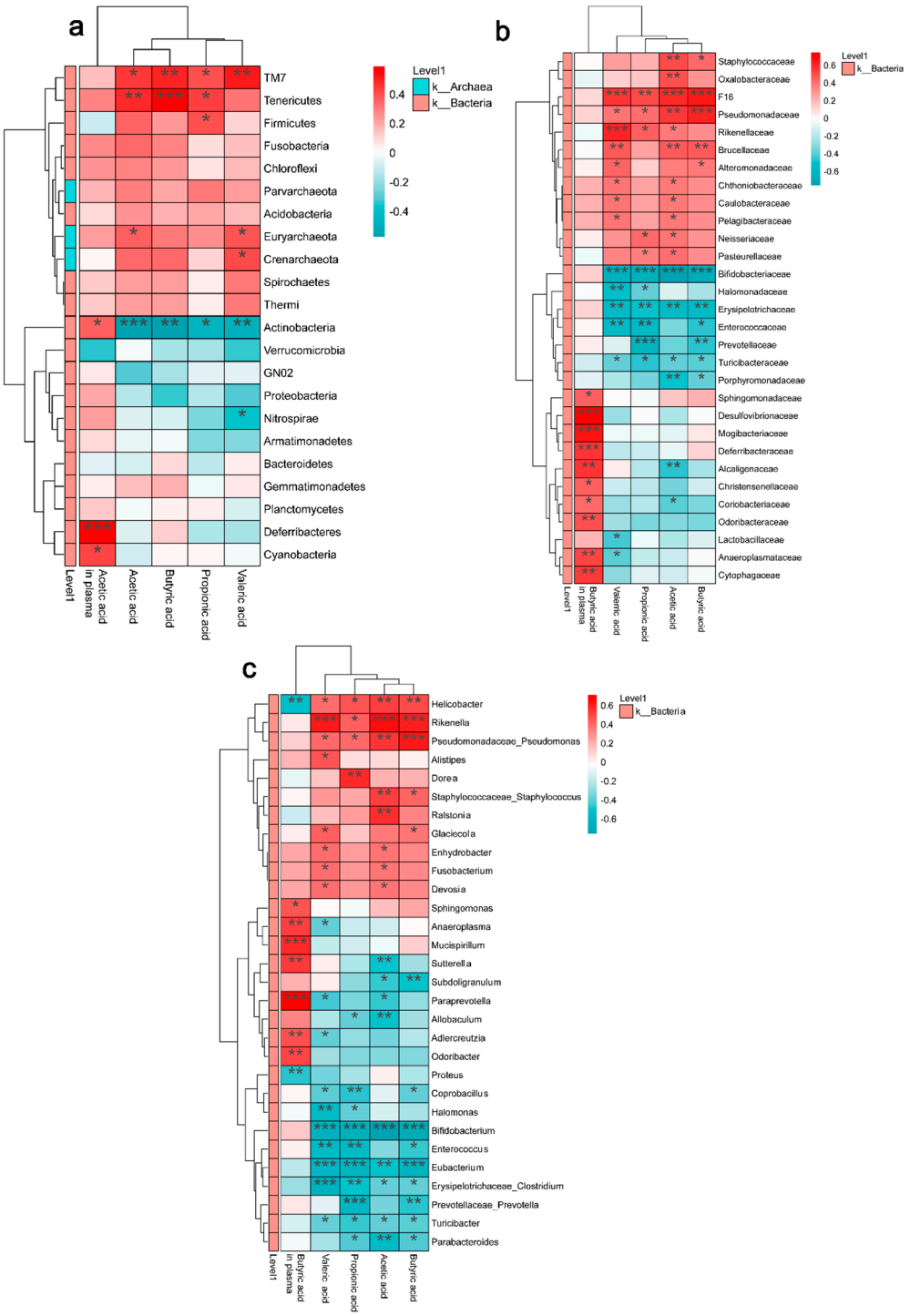

Figure 9. A correlation heatmap linking the top 20 significantly differentiated bacteria with the SCFAs: (**a**) phylum level; (**b**) family level; and (**c**) genus level. Samples (N = 15) were colored by treatment. * $0.01 \leq p < 0.05$, ** $0.001 \leq p < 0.01$, *** $p < 0.001$. CTL: control; ATBX: antibiotic; EXC: exercise; KGM: the native KGM; EK: combination of exercise and KGM.

4. Discussion

In our previous research, we found that compared with the lower molecular weight KGMs, the native KGM with higher molecular weight can more effectively prevent and counteract the perturbation of the gut microbiome caused by antibiotics [9,25]. Additionally, compared with both higher and lower concentrations, the moderate concentration of KGM (2.5 g/L in drinking water) was found to be more effective in alleviating the side effects of excessive exercise on the gut microbial composition and function [20]. Therefore, in this experiment, we employed a strategy of free access to water supplemented with 2.5 g/L of native KGM for mice. In this experiment, we found that the combination intervention using this representative concentration exhibited the best effect in counteracting dysbiosis. It is important to consider more concentrations in future studies.

In the present study, the average daily water intake in the KGM group (10.33 g/day/mice) was significantly higher than that in the CTL group (4.76 g/day/mice) (Figure 1d). Specifically, each mouse in the KGM group consumed approximately 1000 mg/kg BW of KGM per day, while mice in the EK (7.49 g/day/mice) group consumed around 750 mg/kg BW of KGM per day. The increase in water intake in the KGM group was consistent with our previous study [20], and we observed that exercise partially counteracted this trend, although there was still a significant difference compared with water intake in the CTL group. And a study has also reported that the amount of water that mice drink may fluctuate due to many factors [26]. This led to differences in dietary fiber intake between the KGM group and the EK group. Although this issue may affect the comparability between the two groups, we can still comprehensively consider the final outcome while taking into account the influence of KGM on water intake as a confounder factor. To improve this, to address this issue and improve the study, future research should consider isolating the effect of adding exercise while maintaining the same dietary fiber concentration.

Some studies have reported varied results regarding the recovery time following antibiotic perturbation, as a result of the influence of various factors (animal models, environment, antibiotic cocktail formulations, etc.) on this process [5,27,28]. A study indicated that it takes about four weeks for the gut microbiota to recover to some extent after antibiotic treatment [29]. However, another study showed that the recovery time of four weeks post-antibiotic perturbance was still far from reaching the fecal alpha diversity and bacterial richness of the control group [30]. In our previous study, we found that a two-week period of natural recovery following antibiotic perturbance resulted in a limited level of improvement, while the supplementation of singular KGM significantly accelerated this process [9]. Therefore, considering both of the test cost and effects, the present study employed a compromised three-week recovery period to investigate the extent of natural recovery in mice during this time and to determine if any interventions can accelerate the recovery process. The results showed (Table 2) that although there was a certain degree of recovery in alpha diversity, bacterial richness, and beta diversity in mice, the effect was more pronounced after exercise or exercise combined with KGM intervention. As far as we know, this is the first time that dysbiosis induced by antibiotics has been addressed with exercise in vivo.

Following a two-week intervention with exercise (prior to the administration of antibiotics), an increase in the relative abundance of Bacteroides in the feces of mice was observed in the EXC group and the EK group (Table S4). Bacteroides is the main phylum for digestion of dietary fiber [31], and it is able to convert carbohydrates into usable nutrients; it is widely recognized to be positively correlated with a healthier metabolic status [32]. Bacteroides were also inversely associated with type 2 diabetes in humans [33]. This is consistent with previous studies [33]; the changes in EXC and EK groups are mainly caused by exercise, and the main reason for the changes may be due to the positive effect of exercise on promoting intestinal health; the changes in individual metabolic characteristics caused by exercise also lead to this result. Additionally, Bacteroidetes have been reported to play a role in the processing of complex carbohydrates [30,32]. This may also explain the increase induced by exercise, as more energy is required under such conditions. Meanwhile, the two weeks of

KGM intervention resulted in a decrease in the Proteobacteria phylum, some Proteobacteria species may be associated with intestinal barrier dysfunction, chronic inflammation, and insulin resistance and also may be associated with the severity of some diseases [34].

However, after one week of antibiotic intervention (at the end of third week), the addition of KGM effectively mitigated the antibiotic-induced changes in microbiota better than the EXC group (Figure 5e). This finding further supports the protective effects of KGM, which is consistent with our previous finding [9]. After analysis and comparison, the F/B ratio of the four time points can also support this conclusion. In order to comprehensively observe the changes caused by antibiotics and to exclude other confounding factors that may affect the experimental results, histological scoring was conducted on colon tissues. The experimental results were consistent with our previous studies, and no significant differences were found between groups [9]. It is noteworthy that exercise alone or combined with KGM intervention did not exhibit significant preventive effects. Exercise leads to blood redistribution to the muscles, causing ischemia in the gastrointestinal tract, which can result in gastrointestinal disorders [35], and this may lead to a prolonged stay or an increased absorption of antibiotics in the gastrointestinal tract and an increased risk of harm to the body. Meanwhile, antibiotic intervention also reduces exercise capacity [18], and inappropriate exercise load can lead to decreased immunity, ultimately resulting in the occurrence of an "Open Window" phenomenon [36]. Considering this complex dynamic interaction, we have chosen a low–moderate intensity of exercise. Several studies have also reported a relationship between exercise and antibiotics. Doxorubicin is an effective chemotherapy antibiotic utilized in cancer treatment. Previous studies have demonstrated that a 4- to 12-week preconditioning regimen of treadmill exercise can mitigate its side effects (liver toxicity or cardiac injury) [37,38], while another study reported that 10-day exercise preconditioning has a limited effect on doxorubicin-induced tissue toxicity [39]. In our previous study, supplementing KGM for two weeks and intervening with antibiotics at the same time can achieve a good preventive effect. Therefore, in this experiment, the time of exercise preconditioning was also decided to be two weeks, and exercise training was still carried out within the weeks of antibiotic intervention, which is different from the exercise preconditioning method mentioned above. This may also explain why exercise is less effective in prevention. Therefore, the timing of exercise as a preventive measure holds significant importance; inappropriate timing may exacerbate the adverse effects caused by antibiotics. The limited preventive effect observed in the EXC and the EK groups may be a result of a combination of factors (low–moderate exercise intensity, increased of absorption antibiotics, and timing of exercise). According to some of the literature mentioned above, in the later experiments, stopping the exercise intervention in mice during antibiotic intervention, which might get different results. In addition, in future studies, the exercise intensity of the intervention mice will be gradually increased to determine the different effects of different exercise regimens.

At the end of the experiment (on day 42), although the composition of gut microbiome in the stool of the ATBX group was partially recovered, the gut microbial composition in the EK group was closer to that of the CTL group from the perspective of species structure (Figure 6e). Specifically, antibiotic intervention significantly decreased the relative abundance of Tenericutes phylum and unclassified taxa; however, only the EK group could restore these levels to the CTL level (Table S6). Previous studies have reported lower levels of Tenericutes in elderly patients with type 2 diabetes and obese individuals with metabolic dysfunction [40,41]. Furthermore, while there was no significant difference in the relative abundance of Verrucomicrobia between the KGM group and the CTL group after one-week of antibiotic intervention, showed a significantly higher abundance of Verrucomicrobia was observed in the KGM group compared with the CTL group after three-week recovery. This increase was also observed within specific taxa, such as Verrucomicrobiaceae and *Akkermansia*, which belong to the Verrucomicrobia phylum. *Akkermansia* has been identified as a promising probiotic candidate for improving indicators related to type 2 diabetes both in mice and humans [42], and it has been demonstrated to upregulate genes involved

in maintaining intestinal barrier function, thereby promoting intestinal homeostasis [43]. Additionally, a higher relative abundance of *Akkermansia* is often associated with a healthier metabolic status among adults, as it correlates negatively with body fat mass and glucose intolerance [44]. Consistent with previous findings from other studies [45], our results indicate that supplementation with KGM can effectively increase the relative abundance of *Akkermansia*.

In the current study, the KGM group demonstrated a superior preventive effect on day 21 compared with the two groups with exercise intervention. In contrast, the EK group exhibited the most effective restorative effect on day 42 among the three intervention groups. Our previous study reported that KGM with high viscosity could adsorb antibiotics, which partially contribute to the protective effect on gut bacteria [46]. However, whether the presence of KGM affects the therapeutic efficacy of antibiotics is still doubted. For patients, antibiotics are essential for treating inflammation and infections, but it is also important to consider the balance of gut microbiota. In other words, we aim to minimize harm to the gut microbiome while maximizing the effectiveness of antibiotics. If the use of KGM for prevention compromises the efficacy of antibiotics, then it is not primarily recommended. However, further experiments are required to confirm this. From this perspective, exercise may be a more effective preventive strategy and also help to avoid any potential impact on the efficacy of antibiotics. However, the increased energy requirement during exercise enhances the metabolism and utilization of KGM by gut microbiota [47], consequently reducing the concentration in the gut and decreasing the absorption of antibiotics. These factors may explain why the preventive effect in the EK group was inferior to that in the KGM group at day 21. On day 42, despite natural recovery over 3 weeks, the gut microbiome was still not fully restored (CTL vs. ATBX). However, exercise continuously regulated the gut microbiome through energy homeostasis and restored microbial composition, especially with sufficient usable carbohydrate substrates (for example, KGM). As a result, the EK group better reduced the impact induced by antibiotics compared with singular KGM intervention. Therefore, the recovery effect of the EK group was better than that of both the EXC and KGM groups on day 42.

We found that the concentration of acetic acid in plasma was significantly increased in the EK group (Figure 3f). Studies reported that the antibiotic treatment reduced the cross-sectional area of muscle fibers, and acetic acid supplementation could counteract this negative effect [48]. Acetic acid in plasma concentration was significantly increased in the EK group compared with other four groups, suggesting that the combination of exercise and KGM intervention may be able to better utilize acetic as an energy source [49], even at the same fecal acetic concentration. In addition, two weeks of exercise led to an increase in the relative abundance of many SCFA-producing bacteria (such as Bacteroides, Prevotella, and so on), but this effect was offset after perturbance of antibiotics, and failed to fully recover even after a three-week recovery period. The correlation analysis between SCFAs and the relative abundance of gut microbiome (Figure 9c) showed that *Adlercreutzia* levels were significantly positively correlated with the acetic acid level in plasma, and *Helicobacter* levels were significantly negatively correlated with the acetic acid level in plasma. Meanwhile, at the experiment end, the EK group was *Adlercreutzia*-increased and *Helicobacter*-decreased. A recent study identified *Adlercreutzia* as a protective bacterium for Alzheimer's disease [50], while *Helicobacte* was highly associated with chronic gastritis incidence [51]. This suggests that exercise combined with KGM intervention can not only increase the acetic content in plasma but also may have a positive effect on the body by altering the gut microbiome.

Additionally, the EK group was enriched with a variety of purine-metabolism-related pathways (Figure 8a). Some gut microbiomes can use purines as carbon and energy sources anaerobically, consequently affecting the host's purine homeostasis [52]. A study demonstrated that antibiotic intervention disrupted the gut microbiota of mice, resulting in elevated serum uric acid levels and disturbed purine metabolism [53]. Some animal studies reported that *Lactobacillus* can reduce the serum uric acid level of high-uric-acid animal

models [54,55]. In the present study, there was no significant difference in *Lactobacillus* levels between the KGM group and the CTL group on day 21; the relative abundance of *Lactobacillus* in the ATBX, EXC, and EK groups was significantly decreased compared with the CTL group. At the experiment end, the relative abundance of *Lactobacillus* in the EK group was significantly higher than that in the CTL group (Table S6). Based on these results and the Bray–Curtis distance (Figure 6e), exercise combined with KGM intervention can not only promote the recovery of gut microbiome but may also regulate uric acid levels by increasing the relative abundance of *Lactobacillus*. Moreover, these two effects may also contain an interaction [56]. Previous studies have demonstrated that nucleotide metabolism actively promotes antibiotic-induced bacterial death [57], which may also provide a possible explanation for the limited efficacy of the exercise–KGM combination in preventing antibiotic disruption. Furthermore, a study has reported a significant increase in the presence of bacteria containing purine-metabolizing proteins (*Bifidobacterium bifidum* and *Ruminococus gnavus*) in the feces of mice with high uric acid. Conversely, there was a notable decrease in the presence of bacteria-containing proteins associated with purine-recycling pathways (*Lactobacillus vaginalis* and *Allobaculum*) [53]. In the current study, there was a trend of higher relative abundance of *Allobaculum* in the EXC, KGM, and EK groups compared to the CTL group. However, this difference was not statistically significant, possibly due to the short duration of exercise. This suggests that single exercise and KGM supplement may also have a regulatory effect on the serum uric acid level induced by antibiotics. However, the measurement of uric acid excretion was not applicable in the present study, due to the lack of urine collection. Further studies are necessary to confirm the relevant characteristics and clarify the relationships.

5. Conclusions

In conclusion, the combination of exercise and KGM demonstrated a more effective reversal of the damage caused by antibiotics on gut microbiome compared with two singular interventions. It is worth noting that supplementing KGM showed the best preventive effect, while exercise exacerbated the dysbiosis immediately after antibiotic perturbation. Additionally, the combination of exercise and KGM exhibited a certain degree of regulatory effect on purine metabolism, but the causal relationship between restoring the species structure of the gut microbiome community and regulating purine metabolism still needs further research. In summary, the present study provides a promising solution and research direction for preventing and regulating dysbiosis caused by antibiotics or even other factors. Further studies are required to investigate the timing and broader impact of exercise on gut microbial metabolites.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16172942/s1, Figure S1: The daily water intake of the mice in the experiment (a) and maximum running distance (b) and Firmicutes/Bacteroidetes at different time points (c); Table S1: The chemical compounds used in this work with information from NCBI PubChem compound database and the supplier sources; Table S2: The formula of AIN93 purified diet used in the study; Table S3: Histological scoring system; Table S4: The relative abundance of top 20 taxa in feces at different levels on day 0; Table S5: The relative abundance of top 20 taxa in feces at different levels on day 14; Table S6: The relative abundance of top 20 taxa in feces at different levels on day 21; Table S7: The relative abundance of top 20 taxa in feces at different levels on day 42.

Author Contributions: M.W.: methodology, investigation, writing—original draft. Y.C.: methodology, investigation. A.-X.S.: methodology. X.W.: methodology, investigation. Y.M.: investigation. J.L.: methodology. Y.-H.M.: project administration, resources, writing—revision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are not openly available due to reasons of sensitivity but are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Guangzhou Sport University.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Morello, W.; Baskin, E.; Jankauskiene, A.; Yalcinkaya, F.; Zurowska, A.; Puccio, G.; Serafinelli, J.; La Manna, A.; Krzemień, G.; Pennesi, M.; et al. Antibiotic prophylaxis in infants with grade III, IV, or V vesicoureteral reflux. *N. Engl. J. Med.* **2023**, *389*, 987–997. [CrossRef] [PubMed]
2. Zhao, H.; Wei, L.; Li, H.; Zhang, M.; Cao, B.; Bian, J.; Zhan, S. Appropriateness of antibiotic prescriptions in ambulatory care in China: A nationwide descriptive database study. *Lancet Infect. Dis.* **2021**, *21*, 847–857. [CrossRef] [PubMed]
3. Zven, S.; Susi, A.; Nylund, C. Multiple Classes of Antibiotic Use in Infancy and Allergic Disease in Childhood-Reply. *JAMA Pediatr.* **2020**, *174*, 1003–1004. [CrossRef] [PubMed]
4. Lee, J.Y.; Tiffany, C.R.; Mahan, S.P.; Kellom, M.; Rogers, A.W.L.; Nguyen, H.; Stevens, E.T.; Masson, H.L.P.; Yamazaki, K.; Marco, M.L.; et al. High fat intake sustains sorbitol intolerance after antibiotic-mediated *Clostridia* depletion from the gut microbiota. *Cell* **2024**, *187*, 1191–1205.e15. [CrossRef]
5. Fishbein, S.R.S.; Mahmud, B.; Dantas, G. Antibiotic perturbations to the gut microbiome. *Nat. Rev. Microbiol.* **2023**, *21*, 772–788. [CrossRef]
6. Hempel, S.; Newberry, S.J.; Maher, A.R.; Wang, Z.; Miles, J.N.; Shanman, R.; Johnsen, B.; Shekelle, P.G. Probiotics for the prevention and treatment of antibiotic-associated diarrhea: A systematic review and meta-analysis. *JAMA* **2012**, *307*, 1959–1969. [CrossRef]
7. Pandey, K.R.; Naik, S.R.; Vakil, B.V. Probiotics, prebiotics and synbiotics—A review. *J. Food Sci. Technol.* **2015**, *52*, 7577–7587. [CrossRef]
8. Dahiya, D.; Nigam, P.S. Antibiotic-therapy-induced gut dysbiosis affecting gut microbiota-brain axis and cognition: Restoration by intake of probiotics and synbiotics. *Int. J. Mol. Sci.* **2023**, *24*, 3074. [CrossRef]
9. Mao, Y.H.; Xu, Y.; Song, F.; Wang, Z.M.; Li, Y.H.; Zhao, M.; He, F.; Tian, Z.; Yang, Y. Protective effects of konjac glucomannan on gut microbiome with antibiotic perturbation in mice. *Carbohydr. Polym.* **2022**, *290*, 119476. [CrossRef]
10. Conroy, G. Why is exercise good for you? Scientists are finding answers in our cells. *Nature* **2024**, *629*, 26–28. [CrossRef]
11. Kamal, F.D.; Dagar, M.; Reza, T.; Karim Mandokhail, A.; Bakht, D.; Shahzad, M.W.; Silloca-Cabana, E.O.; Mohsin, S.N.; Chilla, S.P.; Bokhari, S.F.H. Beyond Diet and Exercise: The impact of gut microbiota on control of obesity. *Cureus* **2023**, *15*, e49339. [CrossRef] [PubMed]
12. Wegierska, A.E.; Charitos, I.A.; Topi, S.; Potenza, M.A.; Montagnani, M.; Santacroce, L. The connection between physical exercise and gut microbiota: Implications for competitive sports athletes. *Sports Med.* **2022**, *52*, 2355–2369. [CrossRef]
13. Eglseer, D.; Traxler, M.; Embacher, S.; Reiter, L.; Schoufour, J.D.; Weijs, P.J.M.; Voortman, T.; Boirie, Y.; Cruz-Jentoft, A.; Bauer, S.; et al. Nutrition and Exercise Interventions to Improve Body Composition for Persons with Overweight or Obesity Near Retirement Age: A Systematic Review and Network Meta-Analysis of Randomized Controlled Trials. *Adv. Nutr.* **2023**, *14*, 516–538. [CrossRef] [PubMed]
14. de Geus, M.; Dam, M.; Visser, W.J.; Ipema, K.J.R.; de Mik-van Egmond, A.M.E.; Tieland, M.; Weijs, P.J.M.; Kruizenga, H.M. The impact of combined nutrition and exercise interventions in patients with chronic kidney disease. *Nutrients* **2024**, *16*, 406. [CrossRef] [PubMed]
15. Brauwers, B.; Machado, F.V.C.; Beijers, R.J.H.C.G.; Spruit, M.A.; Franssen, F.M.E. Combined exercise training and nutritional interventions or pharmacological treatments to improve exercise capacity and body composition in chronic obstructive pulmonary disease: A narrative review. *Nutrients* **2023**, *15*, 5136. [CrossRef]
16. Guo, S.; Huang, Y.; Zhang, Y.; Huang, H.; Hong, S.; Liu, T. Impacts of exercise interventions on different diseases and organ functions in mice. *J. Sport. Health Sci.* **2020**, *9*, 53–73. [CrossRef]
17. Xu, L.; Li, M.; Wei, A.; Yang, M.; Li, C.; Liu, R.; Zheng, Y.; Chen, Y.; Wang, Z.; Wang, K.; et al. Treadmill exercise promotes E3 ubiquitin ligase to remove amyloid β and P-tau and improve cognitive ability in APP/PS1 transgenic mice. *J. Neuroinflamm.* **2022**, *19*, 243. [CrossRef]

18. Okamoto, T.; Morino, K.; Ugi, S.; Nakagawa, F.; Lemecha, M.; Ida, S.; Ohashi, N.; Sato, D.; Fujita, Y.; Maegawa, H. Microbiome potentiates endurance exercise through intestinal acetate production. *Am. J. Physiol. Endocrinol. Metab.* **2019**, *316*, E956–E966. [CrossRef]
19. Bi, P.; Yue, F.; Sato, Y.; Wirbisky, S.; Liu, W.; Shan, T.; Wen, Y.; Zhou, D.; Freeman, J.; Kuang, S. Stage-specific effects of Notch activation during skeletal myogenesis. *Elife* **2016**, *5*, e17355. [CrossRef]
20. Mao, Y.H.; Wang, M.; Yuan, Y.; Yan, J.K.; Peng, Y.; Xu, G.; Weng, X. Konjac glucomannan counteracted the side effects of excessive exercise on gut microbiome, endurance, and strength in an overtraining mice model. *Nutrients* **2023**, *15*, 4206. [CrossRef]
21. Chen, Z.H.; Yuan, X.H.; Tu, T.T.; Wang, L.; Mao, Y.H.; Luo, Y.; Qiu, S.Y.; Song, A.X. Characterization and prebiotic potential of polysaccharides from Rosa roxburghii Tratt pomace by ultrasound-assisted extraction. *Int. J. Biol. Macromol.* **2024**, *268 Pt 2*, 131910. [CrossRef] [PubMed]
22. Zheng, Y.; Zhou, X.; Wang, C.; Zhang, J.; Chang, D.; Liu, W.; Zhu, M.; Zhuang, S.; Shi, H.; Wang, X.; et al. Effect of tanshinone IIA on gut microbiome in diabetes-induced cognitive impairment. *Front. Pharmacol.* **2022**, *13*, 890444. [CrossRef] [PubMed]
23. Wang, Y.; Cui, X.; Lin, Q.; Cai, J.; Tang, L.; Liang, Y. Active peptide KF-8 from rice bran attenuates oxidative stress in a mouse model of aging induced by d-Galactose. *J. Agric. Food Chem.* **2020**, *68*, 12271–12283. [CrossRef]
24. Simpson, A.M.R.; De Souza, M.J.; Damani, J.; Rogers, C.J.; Williams, N.I.; Weaver, C.M.; Ferruzzi, M.G.; Nakatsu, C.H. Gut microbes differ in postmenopausal women responding to prunes to maintain hip bone mineral density. *Front. Nutr.* **2024**, *11*, 1389638. [CrossRef] [PubMed]
25. Mao, Y.H.; Xu, Y.X.; Li, Y.H.; Cao, J.; Song, F.L.; Zhao, D.; Zhao, Y.; Wang, Z.M.; Yang, Y. Effects of konjac glucomannan with different molecular weights on gut microflora with antibiotic perturbance in in vitro fecal fermentation. *Carbohydr. Polym.* **2021**, *273*, 118546. [CrossRef] [PubMed]
26. Tordoff, M.G.; Bachmanov, A.A.; Reed, D.R. Forty mouse strain survey of water and sodium intake. *Physiol. Behav.* **2007**, *91*, 620–631. [CrossRef]
27. Ng, K.M.; Aranda-Díaz, A.; Tropini, C.; Frankel, M.R.; Van Treuren, W.; O'Loughlin, C.T.; Merrill, B.D.; Yu, F.B.; Pruss, K.M.; Oliveira, R.A.; et al. Recovery of the gut microbiota after antibiotics depends on host diet, community context, and environmental reservoirs. *Cell Host Microbe* **2019**, *26*, 650–665.e4. [CrossRef]
28. Duan, H.; Yu, L.; Tian, F.; Zhai, Q.; Fan, L.; Chen, W. Antibiotic-induced gut dysbiosis and barrier disruption and the potential protective strategies. *Crit. Rev. Food Sci. Nutr.* **2022**, *62*, 1427–1452. [CrossRef]
29. Dethlefsen, L.; Relman, D.A. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci. USA* **2011**, *108* (Suppl. S1), 4554–4561. [CrossRef]
30. Suez, J.; Zmora, N.; Zilberman-Schapira, G.; Mor, U.; Dori-Bachash, M.; Bashiardes, S.; Zur, M.; Regev-Lehavi, D.; Ben-Zeev Brik, R.; Federici, S.; et al. Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell* **2018**, *174*, 1406–1423.e16. [CrossRef]
31. McKee, L.S.; La Rosa, S.L.; Westereng, B.; Eijsink, V.G.; Pope, P.B.; Larsbrink, J. Polysaccharide degradation by the Bacteroidetes: Mechanisms and nomenclature. *Environ. Microbiol. Rep.* **2021**, *13*, 559–581. [CrossRef] [PubMed]
32. Shin, J.H.; Tillotson, G.; MacKenzie, T.N.; Warren, C.A.; Wexler, H.M.; Goldstein, E.J.C. Bacteroides and related species: The keystone taxa of the human gut microbiota. *Anaerobe* **2024**, *85*, 102819. [CrossRef] [PubMed]
33. Min, L.; Ablitip, A.; Wang, R.; Luciana, T.; Wei, M.; Ma, X. Effects of exercise on gut microbiota of adults: A systematic review and meta-analysis. *Nutrients* **2024**, *16*, 1070. [CrossRef] [PubMed]
34. Fan, Y.; Pedersen, O. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* **2021**, *19*, 55–71. [CrossRef]
35. ter Steege, R.W.; Kolkman, J.J. Review article: The pathophysiology and management of gastrointestinal symptoms during physical exercise, and the role of splanchnic blood flow. *Aliment. Pharmacol. Ther.* **2012**, *35*, 516–528. [CrossRef]
36. You, M. Role of physical activity in the prevention and treatment of influenza: A review. *Sports Med. Open* **2023**, *9*, 115. [CrossRef]
37. Pereira, T.C.R.; Fidale, T.M.; Guimarães, L.C.; Deconte, S.R.; Herrera, G.C.; Mundim, A.V.; de Sales Cabral, E.; Lopes, P.R.; de Souza, F.R.; de Ulhôa Rocha Júnior, L.D.; et al. Cardioprotective effects of the 4-week aerobic running exercises before treatment with doxorubicin in rats. *Cardiovasc. Toxicol.* **2023**, *23*, 265–277. [CrossRef]
38. Santos-Alves, E.; Rizo-Roca, D.; Marques-Aleixo, I.; Coxito, P.; Martins, S.; Guimarães, J.T.; Oliveira, P.J.; Torrella, J.R.; Magalhães, J.; Ascensão, A. Physical exercise positively modulates DOX-induced hepatic oxidative stress, mitochondrial dysfunction and quality control signaling. *Mitochondrion* **2019**, *47*, 103–113. [CrossRef]
39. Boeno, F.P.; Patel, J.; Montalvo, R.N.; Lapierre-Nguyen, S.S.; Schreiber, C.M.; Smuder, A.J. Effects of exercise preconditioning on doxorubicin-induced liver and kidney toxicity in male and female rats. *Int. J. Mol. Sci.* **2023**, *24*, 10222. [CrossRef]
40. Zhang, Y.; Lu, S.; Yang, Y.; Wang, Z.; Wang, B.; Zhang, B.; Yu, J.; Lu, W.; Pan, M.; Zhao, J.; et al. The diversity of gut microbiota in type 2 diabetes with or without cognitive impairment. *Aging Clin. Exp. Res.* **2021**, *33*, 589–601. [CrossRef]
41. Yuan, X.; Chen, R.; McCormick, K.L.; Zhang, Y.; Lin, X.; Yang, X. The role of the gut microbiota on the metabolic status of obese children. *Microb. Cell Fact.* **2021**, *20*, 53. [CrossRef] [PubMed]
42. Karamzin, A.M.; Ropot, A.V.; Sergeyev, O.V.; Khalturina, E.O. *Akkermansia muciniphila* and host interaction within the intestinal tract. *Anaerobe* **2021**, *72*, 102472. [CrossRef] [PubMed]

43. Martin-Gallausiaux, C.; Garcia-Weber, D.; Lashermes, A.; Larraufie, P.; Marinelli, L.; Teixeira, V.; Rolland, A.; Béguet-Crespel, F.; Brochard, V.; Quatremare, T.; et al. *Akkermansia muciniphila* upregulates genes involved in maintaining the intestinal barrier function via ADP-heptose-dependent activation of the ALPK1/TIFA pathway. *Gut Microbes* **2022**, *14*, 2110639. [CrossRef] [PubMed]
44. Dao, M.C.; Everard, A.; Aron-Wisnewsky, J.; Sokolovska, N.; Prifti, E.; Verger, E.O.; Kayser, B.D.; Levenez, F.; Chilloux, J.; Hoyles, L.; et al. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: Relationship with gut microbiome richness and ecology. *Gut* **2016**, *65*, 426–436. [CrossRef]
45. Zhai, X.; Lin, D.; Zhao, Y.; Li, W.; Yang, X. Effects of dietary fiber supplementation on fatty acid metabolism and intestinal microbiota diversity in C57BL/6J mice fed with a high-fat diet. *J. Agric. Food Chem.* **2018**, *66*, 12706–12718. [CrossRef]
46. Mao, Y.H.; Song, A.X.; Yao, Z.P.; Wu, J.Y. Protective effects of natural and partially degraded konjac glucomannan on Bifidobacteria against antibiotic damage. *Carbohydr. Polym.* **2018**, *181*, 368–375. [CrossRef]
47. Bi, L.; Triadafilopoulos, G. Exercise and gastrointestinal function and disease: An evidence-based review of risks and benefits. *Clin. Gastroenterol. Hepatol.* **2003**, *1*, 345–355. [CrossRef]
48. Kobayashi, S.; Morino, K.; Okamoto, T.; Tanaka, M.; Ida, S.; Ohashi, N.; Murata, K.; Yanagimachi, T.; Sakai, J.; Maegawa, H.; et al. Acetate derived from the intestinal tract has a critical role in maintaining skeletal muscle mass and strength in mice. *Physiol. Rep.* **2024**, *12*, e16047. [CrossRef]
49. Sakakibara, I.; Fujino, T.; Ishii, M.; Tanaka, T.; Shimosawa, T.; Miura, S.; Zhang, W.; Tokutake, Y.; Yamamoto, J.; Awano, M.; et al. Fasting-induced hypothermia and reduced energy production in mice lacking acetyl-CoA synthetase 2. *Cell Metab.* **2009**, *9*, 191–202. [CrossRef]
50. Cammann, D.; Lu, Y.; Cummings, M.J.; Zhang, M.L.; Cue, J.M.; Do, J.; Ebersole, J.; Chen, X.; Oh, E.C.; Cummings, J.L.; et al. Genetic correlations between Alzheimer's disease and gut microbiome genera. *Sci. Rep.* **2023**, *13*, 5258. [CrossRef]
51. Bordin, D.; Livzan, M. History of chronic gastritis: How our perceptions have changed. *World J. Gastroenterol.* **2024**, *30*, 1851–1858. [CrossRef]
52. Kasahara, K.; Kerby, R.L.; Zhang, Q.; Pradhan, M.; Mehrabian, M.; Lusis, A.J.; Bergström, G.; Bäckhed, F.; Rey, F.E. Gut bacterial metabolism contributes to host global purine homeostasis. *Cell Host Microbe* **2023**, *31*, 1038–1053.e10. [CrossRef]
53. Liu, X.; Ke, L.; Lei, K.; Yu, Q.; Zhang, W.; Li, C.; Tian, Z. Antibiotic-induced gut microbiota dysbiosis has a functional impact on purine metabolism. *BMC Microbiol.* **2023**, *23*, 187. [CrossRef] [PubMed]
54. Kuo, Y.W.; Hsieh, S.H.; Chen, J.F.; Liu, C.R.; Chen, C.W.; Huang, Y.F.; Ho, H.H. *Lactobacillus reuteri* TSR332 and *Lactobacillus fermentum* TSF331 stabilize serum uric acid levels and prevent hyperuricemia in rats. *PeerJ* **2021**, *9*, e11209. [CrossRef] [PubMed]
55. Zhou, Y.; Zeng, Y.; Wang, R.; Pang, J.; Wang, X.; Pan, Z.; Jin, Y.; Chen, Y.; Yang, Y.; Ling, W. Resveratrol improves hyperuricemia and ameliorates renal injury by modulating the gut microbiota. *Nutrients* **2024**, *16*, 1086. [CrossRef] [PubMed]
56. Moradell, A.; Casajús, J.A.; Moreno, L.A.; Vicente-Rodríguez, G.; Gómez-Cabello, A. Effects of diet-exercise interaction on human health across a lifespan. *Nutrients* **2023**, *15*, 2520. [CrossRef]
57. Lopatkin, A.J.; Yang, J.H. Digital insights into nucleotide metabolism and antibiotic treatment failure. *Front. Digit. Health* **2021**, *3*, 583468. [CrossRef]

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Lactobacillus plantarum and *Bifidobacterium longum* Alleviate High-Fat Diet-Induced Obesity and Depression/Cognitive Impairment-like Behavior in Mice by Upregulating AMPK Activation and Downregulating Adipogenesis and Gut Dysbiosis

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Abstract: Background/Objective: Long-term intake of a high-fat diet (HFD) leads to obesity and gut dysbiosis. AMP-activated protein kinase (AMPK) is a key regulator of energy metabolism. Herein, we investigated the impacts of *Lactobacillus (Lactiplantibacillus) plantarum* P111 and *Bifidobacterium longum* P121, which suppressed dexamethasone-induced adipogenesis in 3T3 L1 cells and increased lipopolysaccharide-suppressed AMPK activation in HepG2 cells, on HFD-induced obesity, liver steatosis, gut inflammation and dysbiosis, and depression/cognitive impairment (DCi)-like behavior in mice. **Methods:** Obesity is induced in mice by feeding with HFD. Biomarker levels were measured using immunoblotting, enzyme-linked immunosorbent assay, and immunofluorescence staining. **Results:** Orally administered P111, P121, or their mix LpBl decreased HFD-induced body weight gain, epididymal fat pad weight, and triglyceride (TG), total cholesterol (TC), and lipopolysaccharide levels in the blood. Additionally, they downregulated HFD-increased NF-κB activation and TNF-α expression in the liver and colon, while HFD-decreased AMPK activation was upregulated. They also suppressed HFD-induced DCi-like behavior and hippocampal NF-κB activation, NF-κB-positive cell population, and IL-1β and TNF-α levels, while increasing the hippocampal BDNF-positive cell population and BDNF level. The combination of P111 and P122 (LpBl) also improved body weight gain, liver steatosis, and DCi-like behavior. LpBl also mitigated HFD-induced gut dysbiosis: it decreased *Desulfovibrionaceae*, *Helicobacteriaceae*, *Coriobacteriaceae*, and *Streptococcaceae* populations and lipopolysaccharide production, which were positively correlated with TNF-α expression; and increased *Akkermansiaceae*, *Bifidobacteriaceae*, and *Prevotellaceae* populations, which were positively correlated with the BDNF expression. **Conclusions:** P111 and/or P121 downregulated adipogenesis, gut dysbiosis, and NF-κB activation and upregulatde AMPK activation, leading to the alleviation of obesity, liver steatosis, and DCi.

Keywords: obesity; liver steatosis; *Lactobacillus plantarum*; *Bifidobacterium longum*; psychiatric disorder; gut microbiota

1. Introduction

Obesity is the accumulation of abnormal or excessive amounts of fat in the body [1]. The primary risk factor of obesity is the excessive intake of high-calorie diets, such as a high-fat diet (HFD), and physical inactivity [1–3]. Long-term HFD feeding causes gut dysbiosis and excessive endotoxin production, which induce inflammation and adipogenesis and suppress the activation of AMP-activated protein kinase (AMPK) [4–6]. The AMPK activation

downregulates lipid metabolism in cells and organisms, including the oxidative decomposition and biosynthesis of fatty acids and triglycerides [6,7]. Interestingly, endotoxin-induced expression of proinflammatory cytokines suppresses AMPK activation, which inhibits the inflammatory response of adipose tissue macrophages, and systemic inflammation, including neuroinflammation [8,9]. Inflammation-inducing stressors such as pathogen infection and gut bacterial endotoxin induce depression and cognitive impairment (DCi) and systemic inflammation in mice [10,11]. Therefore, suppressing inflammation and adipogenesis and/or inducing AMPK activation may be useful for the therapy of obesity and psychiatric disorders, including DCi.

The gut microbiota is associated with both obesity and psychiatric disorders [5,12]. Gut dysbiosis-ameliorating probiotics may alleviate obesity, depression, and cognitive impairment in mice and volunteers by modulating gut microbiota [13–15]. *Lactobacillus delbrueckii* subsp. *bulgaricus strain* TCI904 alleviates HFD-induced weight gain and anxiety in mice [16]. *Lactiplantibacillus* (*Lactobacillus*) *plantarum* LC27 and *Bifidobacterium longum* LC67 alleviate weight gain by suppressing gut bacteria lipopolysaccharide (LPS) production [17]. *Lactobacillus acidophilus* alleviates HFD-induced weight gain, hyperlipidemia, and inflammation in mice [18]. Anti-inflammatory *Lactobacillus plantarum* NK33 and *B. adolescentis* mix (NVP1704) can also alleviate depression/anxiety and systemic inflammation in LPS-producing *Escherichia coli*-exposed mice by modulating the gut microbiota [19]. Nevertheless, studies on obesity-ameliorating action mechanism(s) of probiotics remain elusive.

Therefore, we selected *L. plantarum* P111 and *B. longum* P121, which suppressed dexamethasone-induced fat accumulation in 3T3L1 cells and increased LPS-suppressed AMPK activation in HepG2 cells, from the bacteria collection of healthy human feces and investigated their effects on HFD-induced obesity, liver steatosis, depression, and cognitive impairment in mice.

2. Materials and Methods

2.1. Materials

LPS (L2630) and DAPI (4,6-diamidine-2-phenylindole dihydrochloride, F6057)) were bought from Sigma (St. Louis, MO, USA). A limulus amoebocyte lysate (LAL) assay kit (113412) was bought from Cape Cod Inc. (E. Falmouth, MA, USA). Antibodies targeting *p*-p65 (#3033), p65 (#6956), AMPK (#25325), and *p*-AMPK (#25315), were bought from Cell Signaling Technology (Danvers, MA, USA). BDNF (PA5-85730) and β-actin (AB8227) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, UK), respectively. Low-fat diet (LFD, D12450B, Research Diets Inc.) and HFD (D12492) were bought from Research Diets Inc. (New Brunswick, NJ, USA).

2.2. Culture of Gut Microbiota-Derived Probiotics and Their Dosage Regimen

Gut bacteria, including P111 (KCCM13475P, from Korean Culture Center of Microorganisms, Seoul, Republic of Korea) and P121 (KCCM13476), were cultured in GAM (D5422, Nissuei Pharm Inc., Tokyo, Japan) or MRS (288130, BD, Franklin Lakes, NJ, USA) broth (0.5 L) and then centrifuged at 5000× *g* for 20 min, washed with saline and distilled water, and freeze-dried. The freeze-dried cells were resuspended in phosphate-buffered saline for in vitro cell experiment or 1% trehalose for in vivo animal experiment.

To determine the appropriate dose of probiotics for in vivo studies, mice were subjected to oral gavage with P111 (2×10^8 and 1×10^9 colony-forming unit (CFU)/mouse/day) for 4 weeks in conjunction with HFD. Compared to mice receiving only HFD treatment, P111 at doses of 2×10^8 CFU/mouse and 1×10^9 CFU/mouse reduced weight gain by 22% and 36%, respectively. Consequently, a dose of 1×10^9 CFU/mouse/day was selected for further in vivo experiments.

2.3. HepG2 and 3T3-L1 Cell Cultures

HepG2 cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultured in DMEM (00741, GIBCO, Grand Island, NY, USA) containing 1% antibiotic–antimycotic (AA, 15240-062, GIBCO) and 10% fetal bovine serum (FBS, 26140-079, GIBCO) at 37 °C in a 5% CO_2/air atmosphere [20]. The cells (1×10^6 cells/mL) were treated with or without probiotics (1×10^5 CFU/mL) in the presence of palmitic acid (0.25 mM) or LPS (100 ng/mL) for 24 h. 3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS and 1% AA at 37 °C and 5% CO_2/air and differentiated into the adipocyte, as previously reported [21].

Lipid amount and AMPK activation activity were assayed using Oil Red O staining and immunoblotting, respectively, as previously reported [21].

2.4. Animals

Animal experiments were conducted using male C57BL/6 mice (18–21 g, 6 weeks old) obtained from Koatech (Pyeongtaek, Republic of Korea). Mice were housed in plastic cages with 5 cm elevated wire flooring, under controlled conditions, for one week prior to the commencement of experiments and used in animal experiments, as previously reported [19]. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC approval nos. KHSASP-20-177, 14 July 2020; and KHSASP-21-098, 11 March 2021) and conducted according to the Ethical Policies and Guidelines of the University for Laboratory Animals Care and Use and Use of Laboratory Animals and ARRIVE guideline [22].

2.5. Preparation of Mice with Obesity and Anti-Obesity Activity Assay of Probiotics

To understand the anti-obesity activities of Lpl, Blo, and their (4:1) mix (LpBl), we examined two sequential experiments in mice with HFD-induced obesity, which were prepared as previously reported [17]. First, to investigate the effects of Lpl and Blo, mice were randomly separated into 4 groups (LF, HF, Lpl, and Blo). Second, to examine the effects of LpBl, mice were randomly separated into 3 groups (LF, HF, and LpBl). Each group consisted of 8 mice. The LF group was fed an LFD for 8 weeks, while the HF, Lpl, Blo, and LpBl groups were subjected to an HFD for the same duration. The LF and HF groups were administered 1% trehalose (vehicle) via oral gavage once a day (six day/week) after feeding on their assigned diet for 4 weeks. Lpl, Blo, and LpBl groups were administered P111, P121, and their (4:1) mix (1×10^9 CFU/mouse/day, suspended in 1% trehalose) via oral gavage once a day (six day/week) from next day after HFD feeding for 4 weeks, respectively.

DCi-like behaviors were measured 24 h after the final gavage of probiotics. Mice were euthanized via exposure to CO_2, followed by cervical dislocation. Blood, colon, liver, and brain tissues were collected and stored at −80 °C for biochemical marker analysis.

2.6. Behavioral Tasks

Depression-like behaviors were assessed using the elevated plus-maze test (EPMT) and tail suspension test (TST) performed in a plus-maze apparatus and at the edge of a table, respectively, as previously described [19]. Cognitive function-like behaviors were evaluated using the Y-maze task (YMT) conducted in a three-arm horizontal maze (40 cm long, 3 cm wide, and 12 cm high walls), as previously reported [23]. Detailed protocols are indicated in the Supplementary Materials' Methods section.

2.7. Immunoblotting and ELISA

Colon, liver, and brain tissue samples were homogenized and lysed in RIPA buffer and then centrifuged at 14,000× *g* for 20 min. Proteins in the supernatant (20 μg) were analyzed by immunoblotting for p65, *p*-p65, AMPK, *p*-AMPK, BDNF, and β-actin, as previously described [23]. Cytokines in the liver, colon, and blood supernatants were measured using ELISA kits [17].

2.8. Determination of LPS Concentration

LPS levels in blood, liver, and feces were measured using an LAL assay kit, as previously reported [17].

2.9. Determination of Total Cholesterol (TC), HDL-Cholesterol (HC), and Triglyceride (TG) Levels in the Liver and Blood

Liver tissue samples were homogenized, lysed in RIPA buffer (pc2002-050-00, Biosesang, Yongin-si, Republic of Korea), and then centrifuged at 14,000 g for 20 min. TC, HC, and TG levels in the liver homogenate supernatant and blood were measured using each commercial kit (Asan pharmaceutical Co., Seoul, Republic of Korea).

2.10. Immunofluorescence Staining

Immunofluorescence staining was performed, as described by Jang et al. [19]. Detailed protocols are provided in the Supplementary Materials (Methods).

2.11. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative PCR (qPCR) was performed for (SIRT)-1, sREBP-1c, PGC-1α, LPL, Fiaf, G6PD, FAS, and β-actin, following the method described by Jang et al. [19]. The primers are indicated in Supplementary Table S1.

2.12. Gut Microbiota Composition Analysis

Gut microbiota composition was determined using Illumina iSeq 100 [23]. Detailed protocols are provided in the Supplementary Materials (Methods). Sequenced reads were deposited in the NCBI's short-read archive under accession number PRJNA163520.

2.13. Whole-Genome Analysis

The whole-genome sequences of P111 and P121 were analyzed, as previously reported [23]. Detailed protocols are provided in the Supplementary Materials (Methods).

2.14. Statistical Analysis

Data were indicated as the mean ± standard deviation (SD) and analyzed by a GraphPad Prism 9. The significance was analyzed by a one-way ANOVA, followed by Duncan's multiple-range test ($p < 0.05$).

3. Results

3.1. Effects of Probiotics on Lipid Accumulation and AMPK Activation in 3T3-L1 and HepG2 Cells

First, we screened probiotics suppressing lipid accumulation in 3T3 L1 cells from healthy human fecal microbiota-derived lactic acid bacteria collection. Of them, P111 and P121 significantly suppressed dexamethasone-mediated adipogenesis (fat accumulation) in 3T3 L1 cells (Figure 1 and Supplementary Figure S1). They also suppressed palmitic acid-induced fat (lipid) accumulation in HepG2 cells. When they were (4:1), (1:1), or (1:4) mixed, their lipid accumulation-inhibitory effects were not different. However, they increased LPS-suppressed AMPK activation in HepG2 cells. Based on the analysis of Gram staining, whole genome and 16S rRNA gene, and API 20A and 50 CHL kits (bioMérieux, Marcy-l'Étoile, France), P111 and P121 were named *L. plantarum* and *B. longum*, respectively. Their whole-genome sequences exhibited the highest phylogenetic similarity to *L. plantarum* NCTC13644 (99.1%) and *B. longum* DSM20211 (96.3%), respectively, using OrthoANI (Supplementary Figure S2).

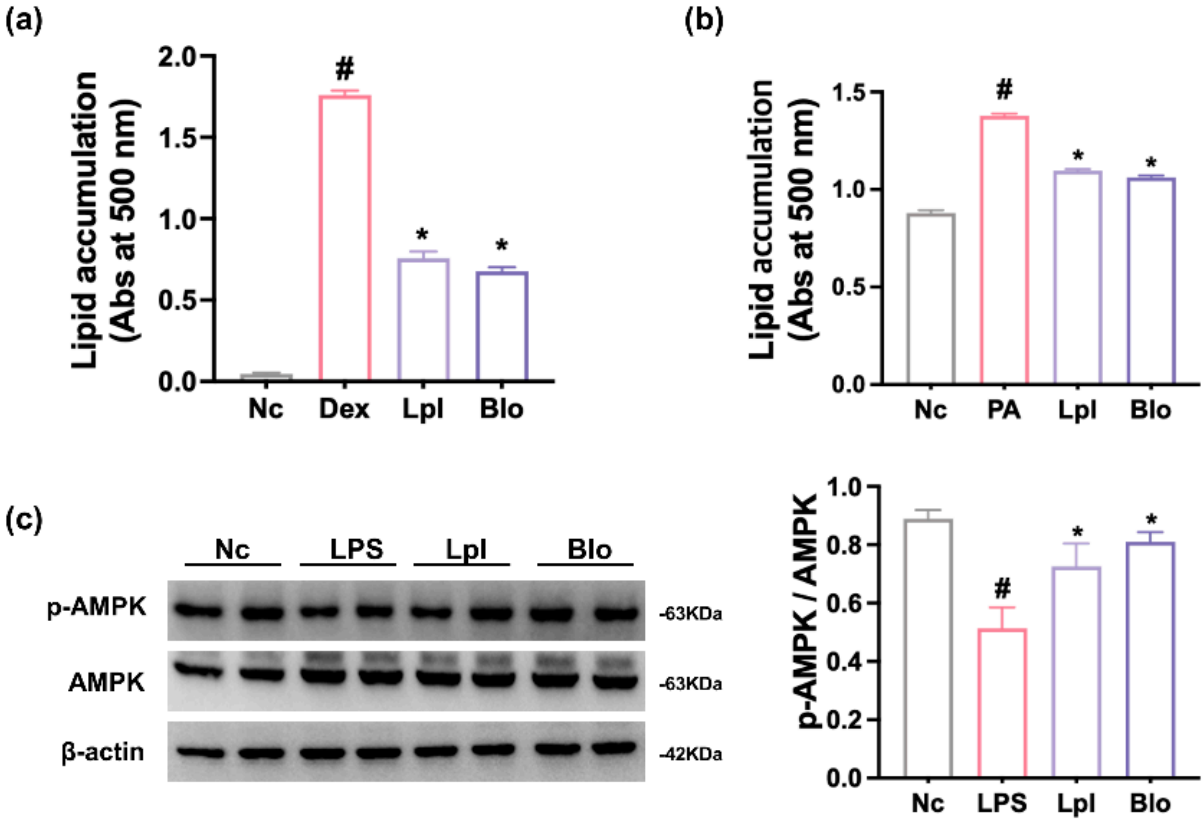

Figure 1. Effects of P111 and P121 on fat (lipid) deposition and AMPK activation in 3T3 L1 and HepG2 cells. (**a**) Effects on fat deposition in 3T3 L1 cells. Nc, vehicle alone; Dex, dexamethasone; Lpl, P111+dexamethasone; Blo, P121+dexamethasone. (**b**) Effects on fat deposition in HepG2 cells. Nc, vehicle alone; PA, palmitic acid; Lpl, P111+palmitic acid; Blo, P121+palmitic acid. (**c**) Effects on AMPK activation in HepG2 cells. Nc, vehicle alone; LPS, lipopolysaccharide (LPS); Lpl, P111+LPS; Blo, P121+LPS. Fat deposition and AMPK activation were assessed by Oil Red O staining and immunoblotting, respectively. 3T3 L1 cells were treated with probiotics (1×10^5 CFU/mL) and dexamethasone. HepG2 cells were treated with probiotics (1×10^5 CFU/mL) and palmitic acid. (n = 4). $^{\#}$ $p < 0.05$ vs. NC. * $p < 0.05$ vs. Dex or PA alone.

3.2. P111 and P121 Alleviated HFD-Induced Body Weight Gain, Liver Steatosis, and Depression in Mice

The effects of P111 and P121 on body weight changes were investigated in mice subjected to HFD. Long-term feeding of HFD significantly increased body weight gain compared to those of LFD feeding (Figure 2). Oral administration of P111 or P121 effectively mitigated HFD-induced weight gain. They also increased HFD-induced epididymal fat pad (EFP) weight and adipocyte size, as assessed by H&E staining.

HFD feeding also increased TG, TC, and HC levels in the blood. However, oral administration of P111 or P121 reduced HFD-increased TG and TC, while the HFD-decreased HC level increased. They also decreased HFD-induced corticosterone, IL-6, and LPS levels in the blood.

HFD feeding increased liver weight and lipid droplet number (Figure 3 and Supplementary Figure S3). Treatment with HFD also increased TG, TC, and HC levels in the liver. However, P111 and P121 significantly reduced HFD-induced liver weight; lipid droplet number; and TG, TC, and HC levels. Additionally, their treatments reduced TNF-α, IL-1β, IL-6, and LPS levels.

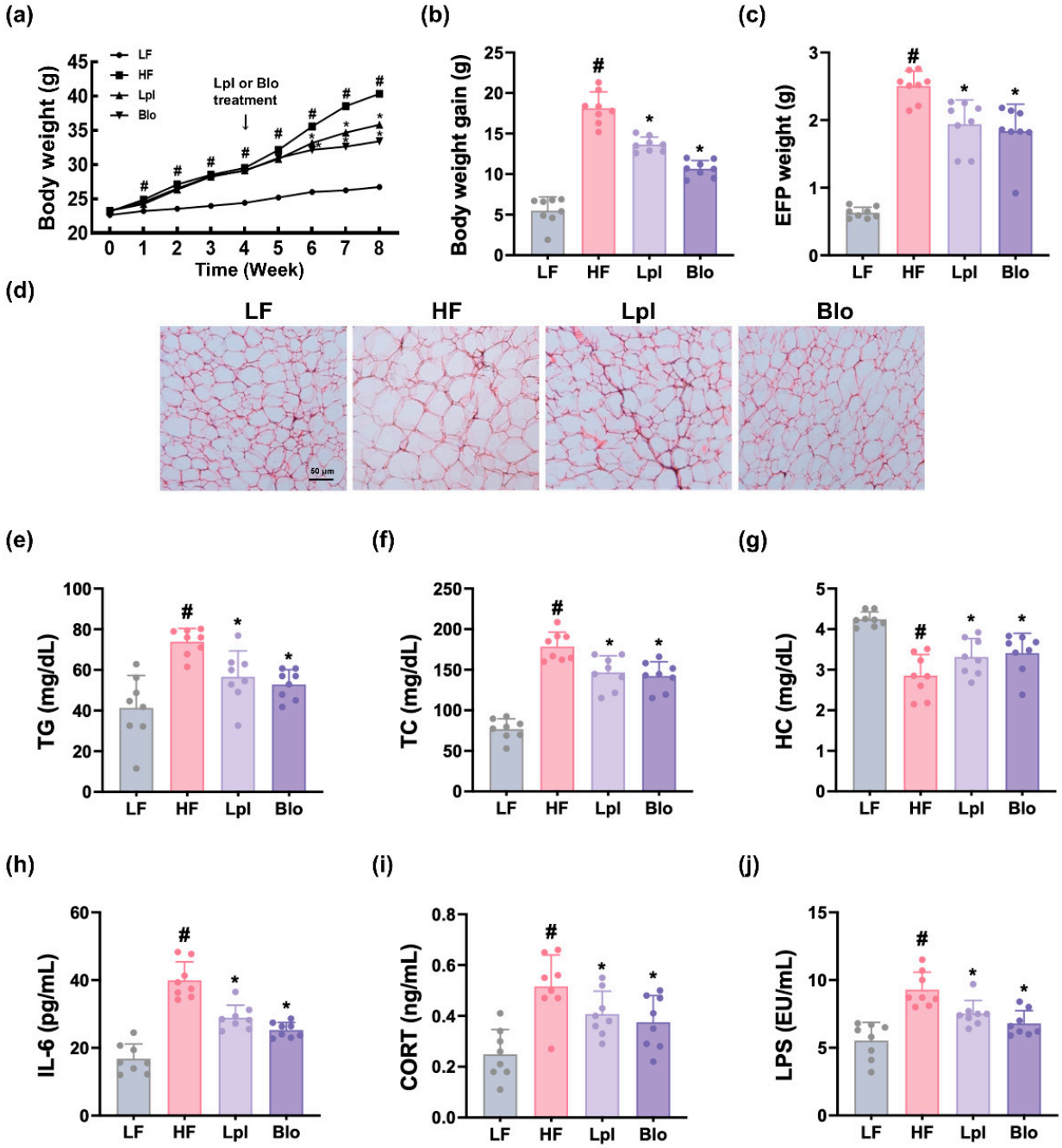

Figure 2. Effects of P111 and P121 on HFD-increased obesity in mice. Effects on body weight change (**a**), body weight gain (**b**), EFP weight (**c**), and EFP adipocyte size (**d**). Effects on TG (**e**), TC (**f**), and HC levels (**g**) in the blood. Effects on IL-6 (**h**), corticosterone (CORT, (**i**)), and LPS levels (**j**) in the blood. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; Lpl, P111 (4 weeks) with HFD (8 weeks); Blo, P121 (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

HFD feeding suppressed AMPK activation and induced NF-κB activation in the liver. Furthermore, HFD feeding decreased SIRT-1, PGC-1α, and Fiaf levels, while sREBP-1c, LPL, G6PD, and FAS levels increased. However, treatment with P111 or P121 induced HFD-suppressed AMPK activation and SIRT-1, PGC-1α, and Fiaf levels, while HFD-induced sREBP-1c, LPL, G6PD, and FAS levels and NF-κB activation decreased.

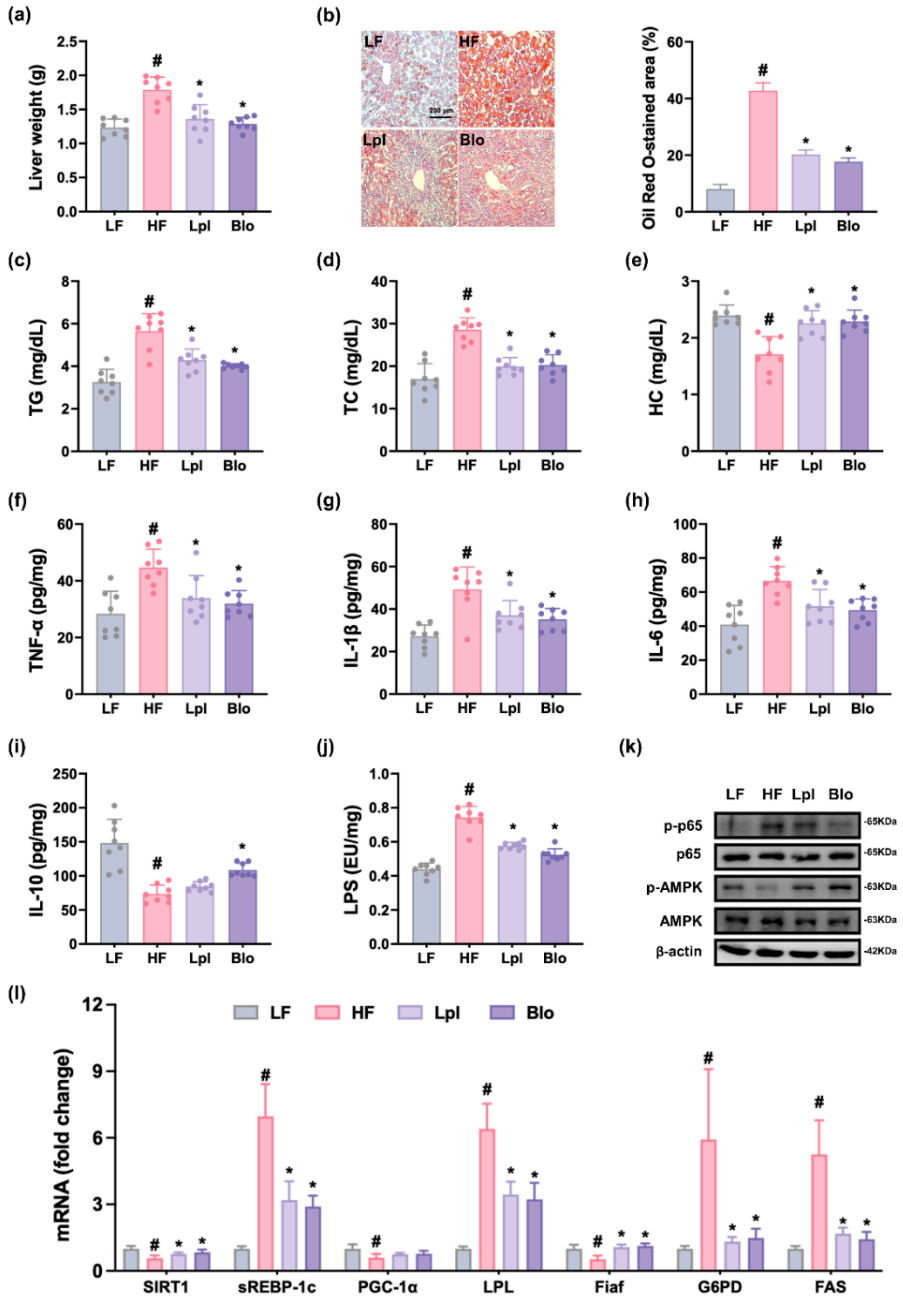

Figure 3. Effects of P111 and P121 on liver weight and steatohepatitis-related marker expression in the liver. Effects on liver weight (**a**) and lipid droplet number (**b**). Effects on TG (**c**), TC (**d**), and HC (**e**). Effects on TNF-α (**f**), IL-1β (**g**), IL-6 (**h**), IL-10 (**i**), and LPS (**j**) levels, as assessed by ELISA. (**k**) Effects on *p*-p65, p65, *p*-AMPK, AMPK, and β-actin expression, as assessed by immunoblotting. (**l**) Effects on SIRT1, sREBP-1c, PGC-1a, LPL, Fiaf, G6PD, and FAS levels, as assessed by qPCR. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; Lpl, P111 (4 weeks) with HFD (8 weeks); Blo, P121 (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

Long-term feeding of HFD increased depression-like behaviors, time spent in open arm (OT) and open arm entries (OE) in the EPMT, to 45.6% ($F_{3,28} = 11.12$, $p < 0.001$) and 50.0% ($F_{3,28} = 4.56$, $p < 0.01$) of LFD-fed mice, respectively, and immobility time (IT) in the TST to 151.3% ($F_{3,28} = 9.73$, $p < 0.001$) of LFD-fed mice, respectively (Figure 4 and Supplementary Figure S4). HFD also decreased spontaneous alternation (SA) in the YMT to 75.5% ($F_{3,28} = 5.71$, $p < 0.001$) of LFD-fed mice. Orally treated P111 and P121 alleviated HFD-induced depression-like behaviors OT to 67.2% and 67.9% of LFD-fed mice, respectively,

and IT to 122.4% and 124.8% of LFD-fed mice, respectively. They recovered HFD-decreased SA to 90.6% and 88.6% of LFD-fed mice, respectively.

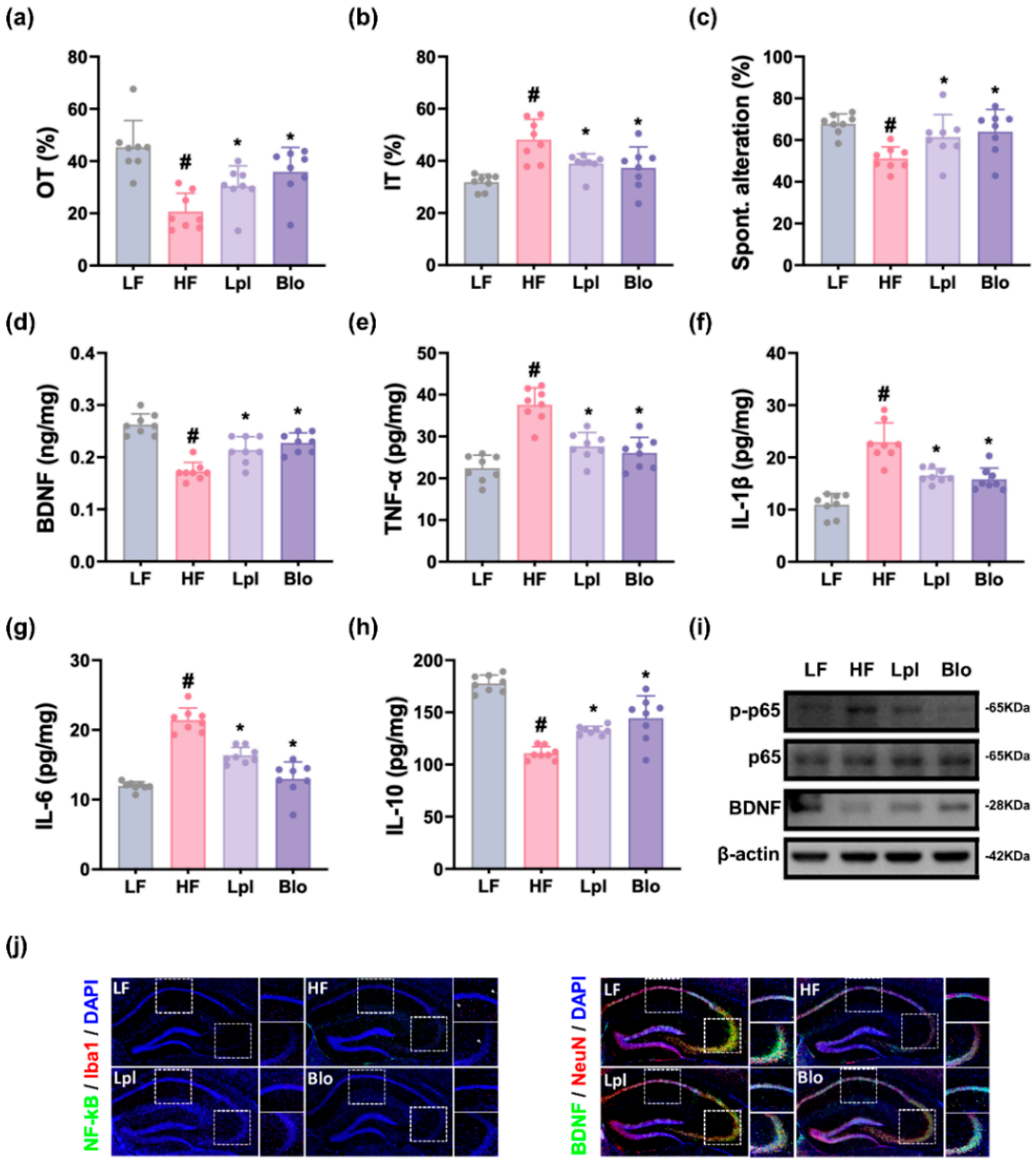

Figure 4. Effects of P111 and P121 on DCi-like symptoms in mice with HFD-induced obesity. Effects on OT in the EPMT (**a**), IT in the TST (**b**), and SA in the YMT (**c**). Effects on hippocampal BDNF (**d**), TNF-α (**e**), IL-1β (**f**), IL-6 (**g**), and IL-10 expression (**h**). (**i**) Effects on hippocampal *p*-p65, p65, BDNF, and β-actin expression. (**j**) Effects on hippocampal NF-κB$^+$Iba1$^+$ and BDNF$^+$NeuN$^+$ cell numbers. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; Lpl, P111 (4 weeks) with HFD (8 weeks); Blo, P121 (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

HFD treatment increased TNF-α, IL-1β, and IL-6 levels; NF-κB activation; and NF-κB$^+$Iba1$^+$ cell number in the hippocampus, while decreasing BDNF levels and the BDNF$^+$NeuN$^+$ cell number. However, oral treatment with P111 or P121 reduced HFD-induced TNF-α, IL-1β, and IL-6 levels; NF-κB activation; and NF-κB$^+$ cell number, while increasing the BDNF level and BDNF$^+$NeuN$^+$ cell number suppressed by HFD.

3.3. P11 and P121 Alleviated HFD-Induced Gut Inflammation and Dysbiosis in Mice

Long-term HFD feeding shortened the length of colon and enhanced the expression of myeloperoxidase, TNF-α, IL-1β, and IL-6, activation of NF-κB activation (*p*-p65/p65), and number of NF-κB^{+}CD11c^{+} cells in the colon, while decreasing IL-10 and SIRT1 expression and AMPK activation (*p*-AMPK/AMPK) (Figure 5 and Supplementary Figure S5). Orally administered P111 or P121 suppressed HFD-induced myeloperoxidase; IL-1β, IL-6, and TNF-α levels; NF-κB activation (*p*-p65/p65); and NF-κB-positive cell population and enhanced HFD-decreased IL-10 expression and AMPK activation (*p*-AMPK/AMPK).

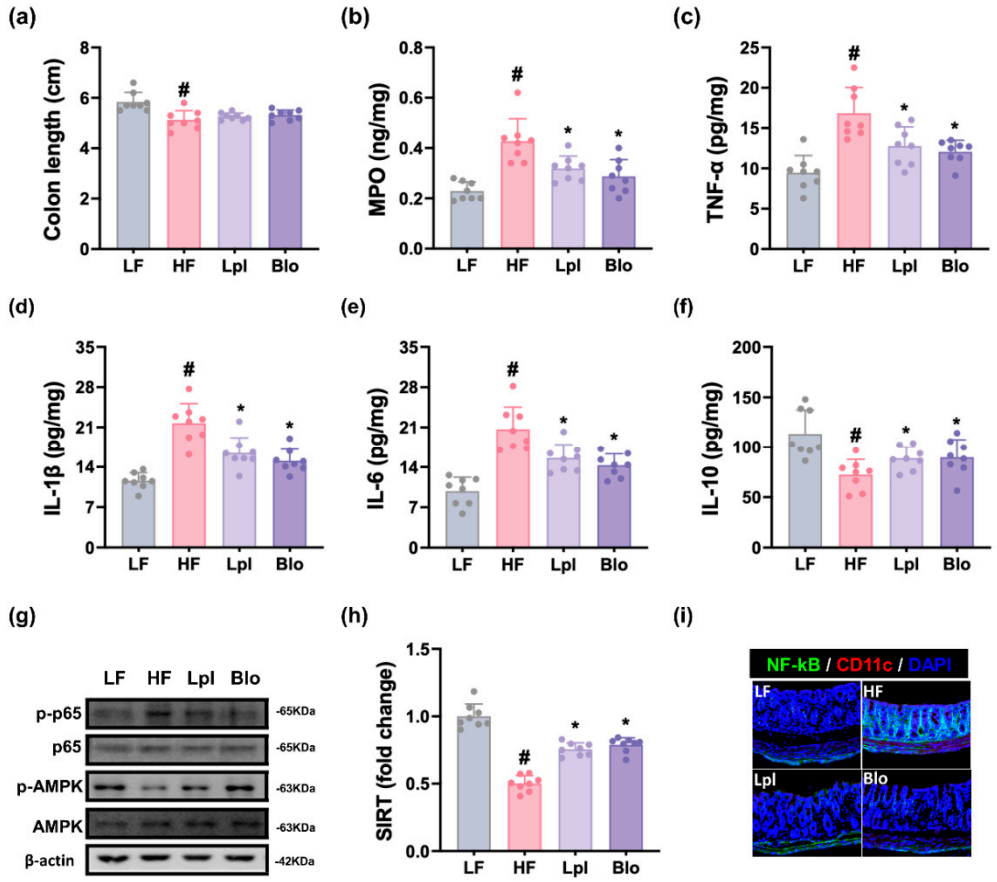

Figure 5. Effects of P111 and P121 on HFD-induced colitis in mice. (**a**) Effects on colon length. Effects on colonic myeloperoxidase (MPO, (**b**)), TNF-α (**c**), IL-1β (**d**), IL-6 (**e**), and IL-10 expression (**f**), as assessed by ELISA. (**g**) Effects on colonic *p*-p65, p65, *p*-AMPK, AMPK, and β-actin expression, as assessed by immunoblotting. (**h**) Effects on colonic SIRT1 expression, as assessed by qPCR. (**i**) Effects on colonic NF-κB^{+}CD11c^{+} cell populations. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; Lpl, P111 (4 weeks) with HFD (8 weeks); Blo, P121 (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

3.4. Effects of P111 and P121 Mix (LpBl) on HFD-Induced Body Weight, Liver Steatosis, and Their Related Biomarker Levels in Mice

When they were (4:1), (1:1), or (1:4) mixed, the lipid accumulation-inhibitory effects were not different in palmitic acid-treated HepG2 cells. Therefore, we examined the effect of LpBl (P111 and P121 (4:1) mix) on HFD-increased body weight gain in mice (Figure 6). HFD feeding significantly increased body weight gain compared to LFD feeding. However, oral administration of LpBl decreased HFD-induced body weight gain. Furthermore, they also decreased HFD-induced EFP weight and adipocyte size.

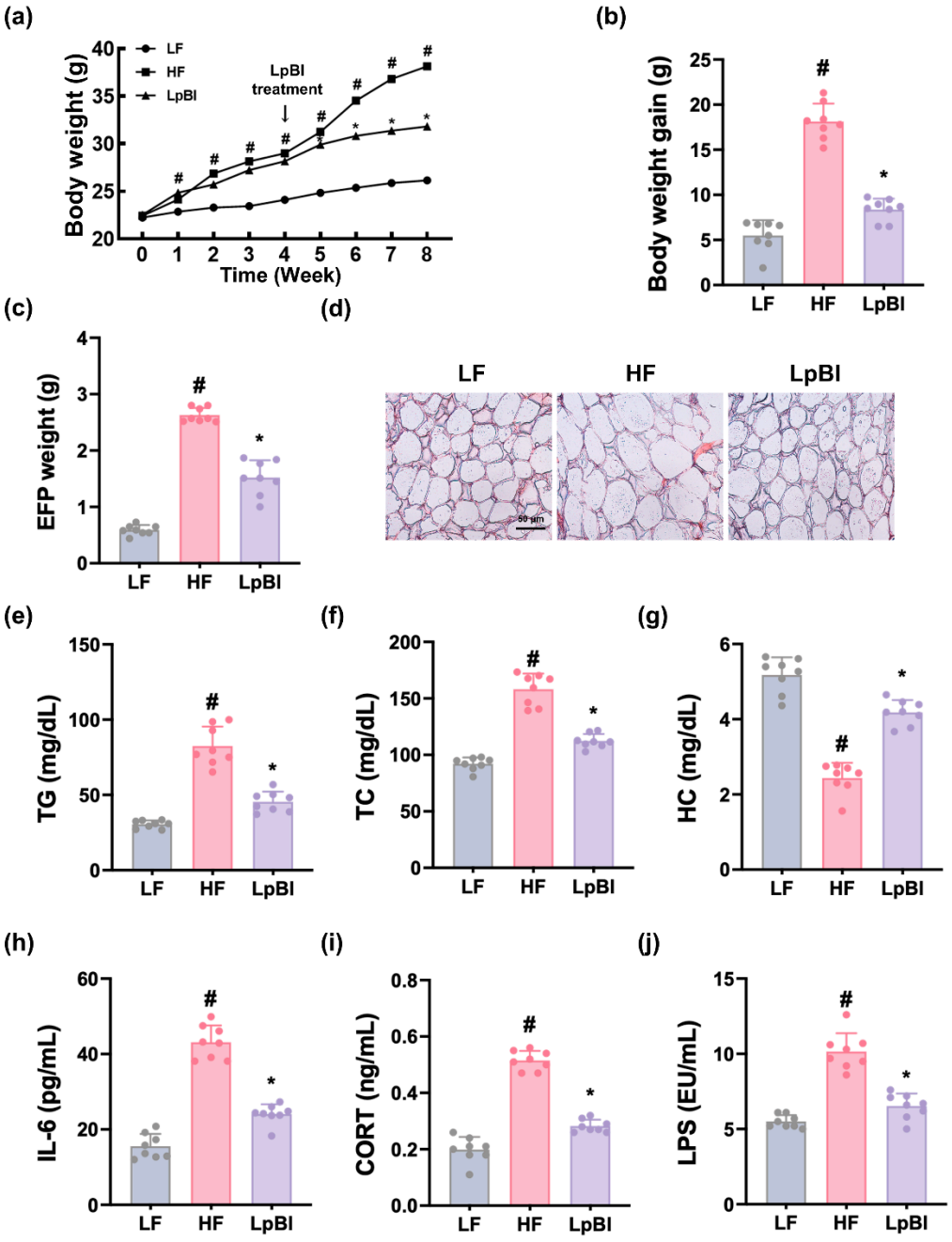

Figure 6. Effect of LpBl on HFD-induced body weight, liver steatosis, and their related biomarker levels in mice. Effect on body weight change (**a**), body weight gain (**b**), EFP weight (**c**), and EFP adipocyte size (**d**). Effects on blood TG (**e**), TC (**f**), and HC levels (**g**). Effect on blood IL-6 (**h**), corticosterone (CORT, (**i**)), and LPS levels (**j**), as assessed by ELISA. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; LpBl, P111 and P121 mix (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

HFD feeding enhanced blood TG, TC, and HC levels. On the contrary, oral treatment with LpBl decreased HFD-induced TG and TC, while the HFD-suppressed HC level increased. LpBl also reduced HFD-induced blood IL-6, corticosterone, and LPS levels.

HFD feeding also increased liver weight, lipid droplet number, and elevated levels of TG, TC, and HC in the liver (Figure 7 and Supplementary Figure S6). Conversely, oral treatment with LpBl significantly lowered HFD-increased liver weight; lipid droplet number; and TG, TC, and HC levels. Additionally, LpBl treatment attenuated HFD-induced elevations in TNF-α, IL-1β, IL-6, and LPS levels and NF-κB activation.

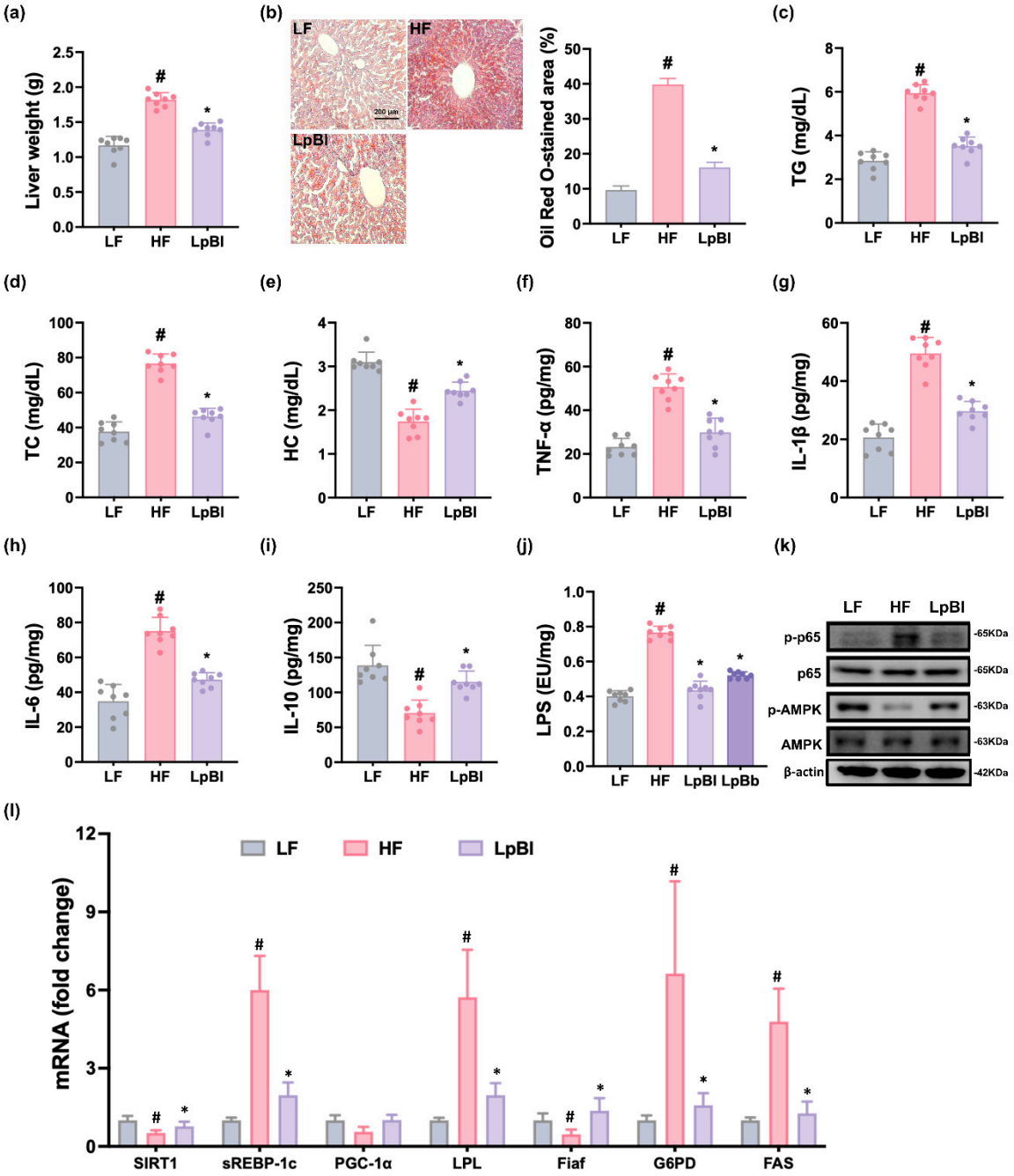

Figure 7. Effect of LpBl (P111 and P121 (4:1) mix) on liver steatosis-related marker levels. Effects on liver weight (**a**) and lipid droplet number (**b**). Effects on liver TG (**c**), TC (**d**), and HC (**e**). Effects on liver TNF-α (**f**), IL-1β (**g**), IL-6 (**h**), IL-10 (**i**), and LPS levels (**j**), as assessed by ELISA. (**k**) Effects on liver *p*-p65, p65. P-AMPK, AMPK, and β-actin levels, as assessed by immunoblotting. (**l**) Effects on liver SIRT1, sREBP-1c, PGC-1a, LPL, Fiaf, G6PD, and FAS levels, as assessed by qPCR. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; LpBl, P111 and P121 mix (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

Furthermore, LpBl upregulated HFD-decreased AMPK activation and PGC-1α, SIRT-1, and Fiaf levels, while HFD-induced sREBP-1c, LPL, G6PD, and FAS expression levels decreased.

HFD feeding decreased OT and OE in the EPMT to 44.7% ($F_{2,21} = 87.09$, $p < 0.001$) and 34.7% ($F_{2,21} = 104.2$, $p < 0.001$) of LFD-fed mice, respectively, and increased IT in the TST to 175.4% ($F_{2,21} = 71.99$, $p < 0.001$) of LFD-fed mice, respectively (Figure 8 and Supplementary

Figure S7). HFD treatment also decreased SA in the YMT to 71.5% ($F_{2,21}$ = 35.61, $p < 0.001$) of LFD-fed mice. Conversely, oral treatment with LpBl alleviated HFD-induced DCi-like behaviors: it recovered OT to 83.2% of LFD-fed mice, IT to 133.0% of LFD-fed mice, and SA to 93.4% of LFD-fed mice.

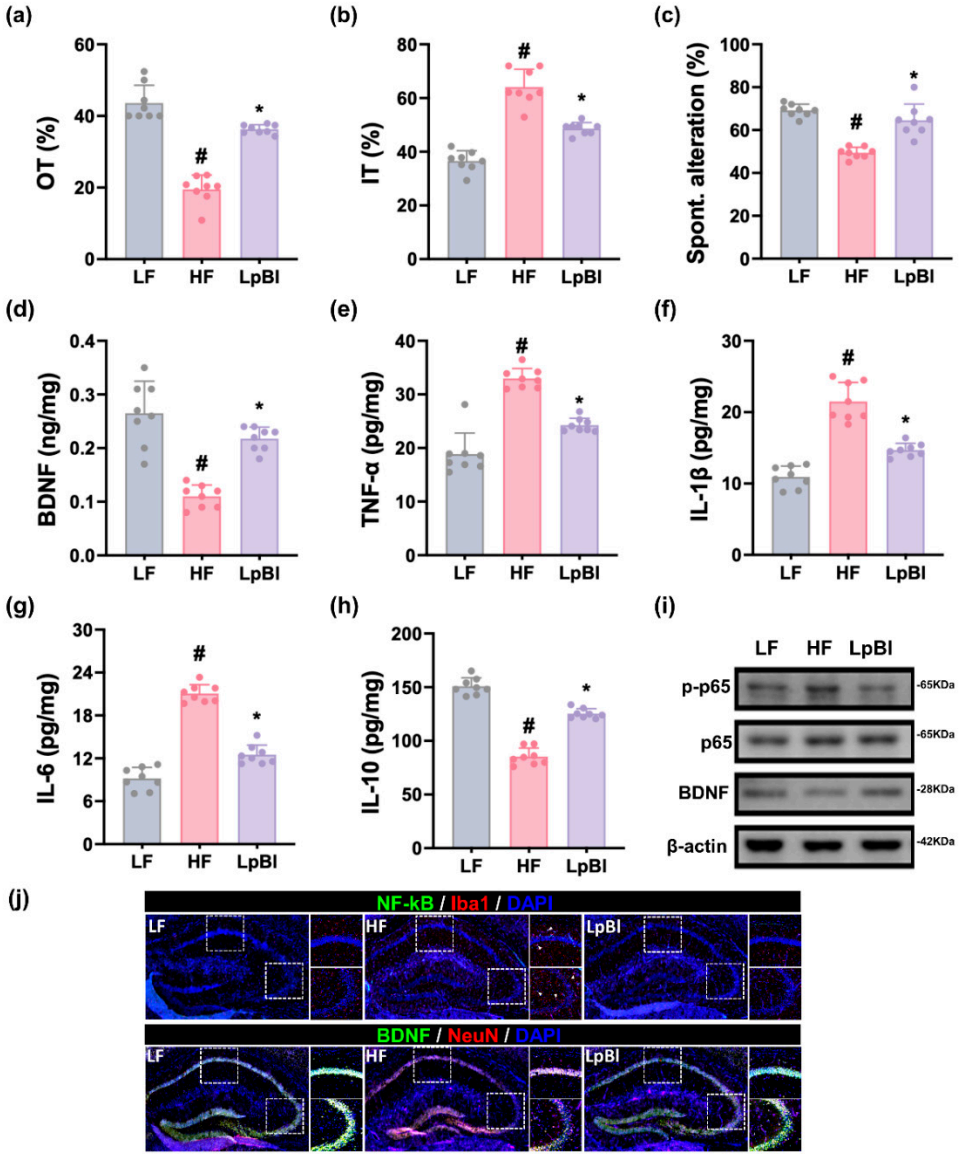

Figure 8. Effect of LpBl on HFD-induced DCi in mice. Effects on OT (**a**) in EPMT, IT in TST (**b**), and SA in YMT (**c**). Effect on hippocampal BDNF (**d**), TNF-α (**e**), IL-1β (**f**), IL-6 (**g**), and IL-10 levels (**h**), as assessed by ELISA. (**i**) Effect on hippocampal *p*-p65, p65, BDNF, and β-actin levels, as assessed by immunoblotting. (**j**) Effect on hippocampal NF-κB$^+$Iba1$^+$ and BDNF$^+$NeuN$^+$ cell number, as assessed by the confocal microscope. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; LpBl, P111 and P121 mix (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

Furthermore, LpBl treatment significantly lowered HFD-induced levels of TNF-α, IL-1β, and IL-6; activation of NF-κB; and number of NF-κB-positive cells in the hippocampus. Conversely, LpBl treatment enhanced HFD-suppressed levels of BDNF and the number of BDNF-positive cells.

3.5. Effect of LpBl on HFD-Induced Gut Inflammation and Dysbiosis in Mice

HFD feeding resulted in colitis: it elevated myeloperoxidase, TNF-α, IL-1β, and IL-6 levels, NF-κB activation (*p*-p65/p65), and NF-κB$^+$CD11c$^+$ cell number and decreased

IL-10 and SIRT1 levels and AMPK activation (*p*-AMPK/AMPK) in the colon (Figure 9 and Supplementary Figure S8). However, LpBl treatment lowered HFD-induced myeloperoxidase; IL-1β, IL-6, and TNF-α levels; NF-κB activation (*p*-p65/p65); and NF-κB-positive cell population and enhanced HFD-suppressed IL-10 level and AMPK activation (*p*-AMPK/AMPK).

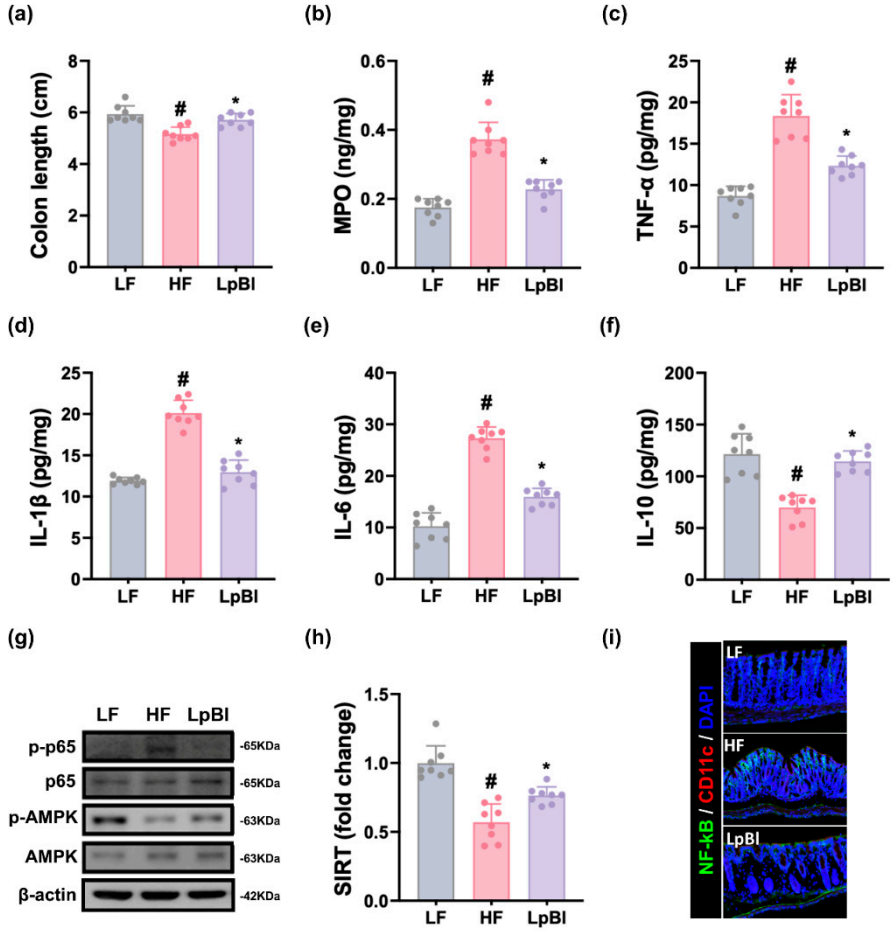

Figure 9. Effect of LpBl on HFD-induced gut inflammation in mice. (**a**) Effect on colon length. Effect on colonic myeloperoxidase (MPO, (**b**)), TNF-α (**c**), IL-1β (**d**), IL-6 (**e**), and IL-10 levels (**f**), as assessed by ELISA. (**g**) Effect on colonic *p*-p65, p65, *p*-AMPK, AMPK, and β-actin levels, as assessed by immunoblotting. (**h**) Effect on colonic SIRT1 level, as assessed by qPCR. (**i**) Effect on colonic NF-κB^{+}CD11c^{+} cell number. LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; LpBl, P111, and P121 mix (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

We examined the impact of LpBl on HFD-induced gut dysbiosis in mice. HFD feeding also fluctuated the composition of gut microbiota: it decreased α-diversity (OUT richness) and shifted β-diversity compared to that of LFD-fed mice (Figure 10 and Supplementary Tables S2 and S3). However, oral administration of LpBl increased HFD-suppressed α-diversity and partially shifted HFD-changed β-diversity to that of LFD-fed mice. In particular, HFD feeding increased the populations of *Firmicutes* and *Proteobacteria*, including *Streptococcaceae*, *Ruminococcaceae*, *Desulfovibrionaceae*, *Helicobacteriaceae*, *Coriobacteriaceae*, *AC160630_f*, and *Peptococcaceae*, while the populations of *Bacteroidetes* and *Verrucomicrobia*, including *Muribaculaceae*, *Akkermansiaceae*, *Prevotellaceae*, *Porphyromonadaceae*, *FR888536_f*, and *Bifidobacteriaceae*, decreased. However, oral administration of LpBl partially shifted the HFD-changed gut microbiota composition to that of LFD-fed mice. In particular, LpBl decreased *Streptococcaceae*, *Helicobacteriacae*, *Peptococcaceae*, and *AC160630_f* in mice with HFD-induced obesity, while

Akkermansiaceae, *Prevotellaceae*, *Lactobacillaceae*, and *Bifidobacteriaceae* populations increased. LpBl also decreased HFD-induced fecal LPS levels.

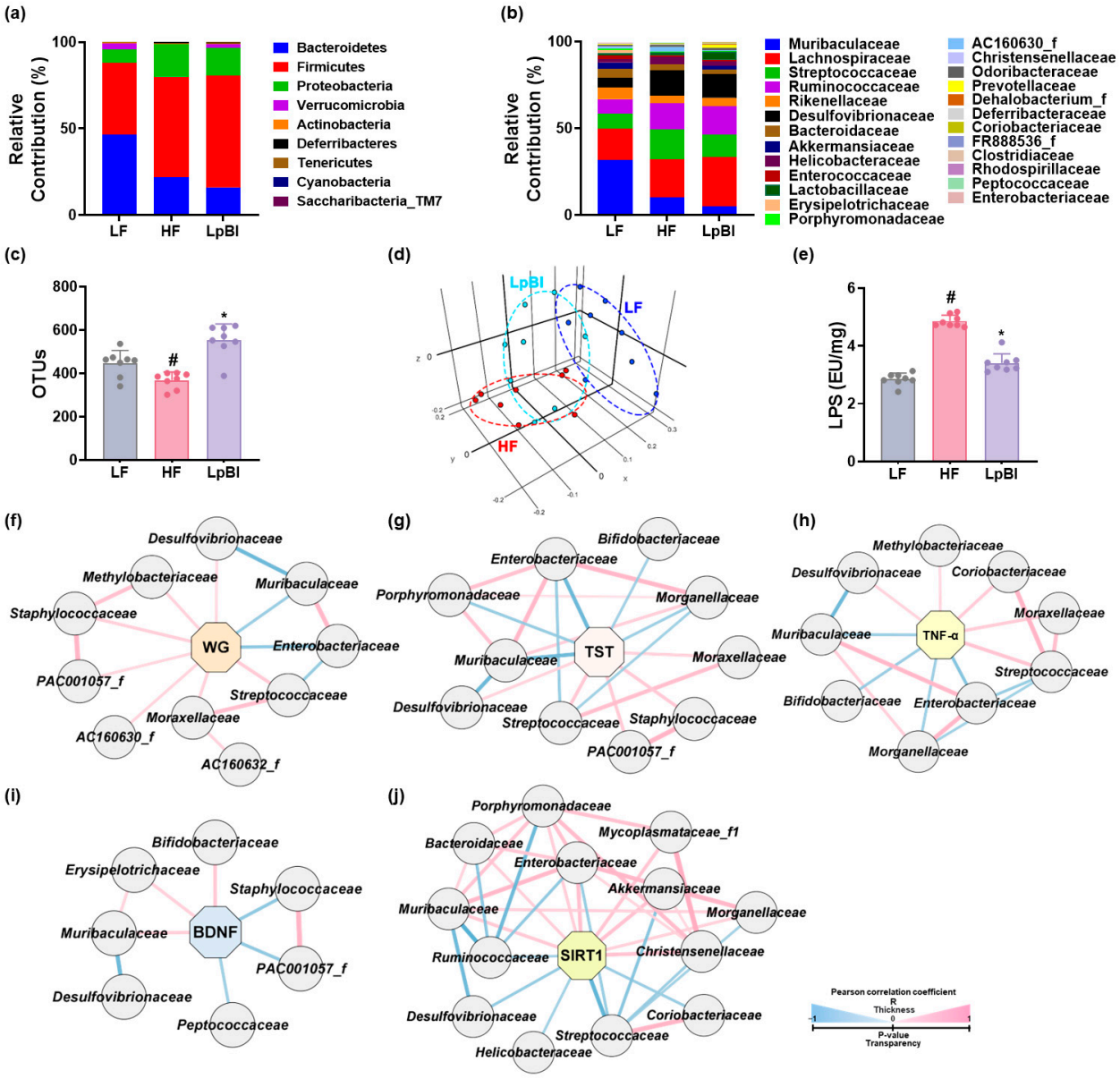

Figure 10. Effect of LpBl on HFD-induced gut dysbiosis in mice. Effect on the composition of fecal microbiota: (**a**) phylum and (**b**) family levels; (**c**) OTU (α-diversity); and (**d**) β-diversity (PCoA plot). (**e**) Effects on fecal microbiota LPS level. Network analysis between gut microbiota and body weight gain (WG, (**f**)), IT in the TST (**g**), TNF-α (**h**), BDNF (**i**), or SIRT1 level (**j**). LF, LFD (8 weeks) alone; HF, HFD (8 weeks) alone; LpBl, P111, and P121 mix (4 weeks) with HFD (8 weeks). n = 8. $^{\#}$ $p < 0.05$ vs. LF group. * $p < 0.05$ vs. HF group.

At a family level, *Streptococcaceae*, *Desulfovibrionaceae*, and *Moraxellaceae* populations had a positive correlation with body weight gain, while *Muribacullaceae* and *Enterobacteriaceae* populations were negatively correlated. *Staphylococcaceae*, *Moracellaceae*, *Streptococcaceae*, and *Desulfovibrionaceae* populations had a positive correlation with depression-like behaviors (IT in TST), while *Muribaculaceae*, *Enterobacteriaceae*, *Bifidobacteriaceae*, *Bifidobacteriaceae*, and *Morganellaceae* populations were negatively correlated. Liver TNF-α expression level had a positive correlation with *Moraxellaceae*, *Streptococcaceae*, *Corobacteriaceae*, and *Desulfovibrionaceae* populations, while *Enterobacteriacae*, *Bifidobacteriaceae*, *Muribacu-*

laceae, and *Morganellaceae* populations were negatively correlated. Hippocampal BDNF expression levels had a positive correlation with *Bifidobacteriaceae*, *Muribaculaceae*, and *Erysipelotrichaceae* populations, while *Peptococcaceae*, *Staphylococcaceae*, and *PAC001057_f* populations were negatively correlated. Liver SIRT1 expression level had a positive correlation with *Akkermansiaceae*, *Porphyromonadaceae*, *Enterobacteriaceae*, *Muribaculaceae*, and *Bacteroidaceae* populations, while *Streptococcaceae*, *Desulfovibrionaceae*, *Helicobacteriaceae*, and *Corobacteriaceae* populations were negatively correlated.

4. Discussion

Excessive, chronic feeding of an HFD induces obesity, which is the representative risk factor for heart disease, diabetes mellitus, and hepatic steatosis [24,25]. Furthermore, HFD induces gut dysbiosis and microbiota LPS production in humans and mice, inducing gut inflammation and membrane permeability [6,26]. Excessively exposed LPS also suppresses AMPK activation and induces adipogenesis in the gut and liver and causes neuroinflammation [7,27]. We also found that chronic feeding of an HFD induced body weight gain; inflammation in the colon, liver, and hippocampus; and DCi-like behavior in mice.

In the present study, oral administration of P111, P121, or LpBL decreased HFD-induced body weight gain and EFP weight. Furthermore, they enhanced HFD-suppressed AMPK activation and SIRT expression in both the colon and liver. They also increased HFD-suppressed PGC-1α and Fiaf expression and decreased HFD-induced sREBP-1c, LPL, G6PD, and FAS expression. The feeding of an HFD decreases AMPK activation, which regulates the expression of lipogenesis/lipolysis-involved metabolism-regulatory factors PGC1α, sREBP-1c, Fiaf, LDL, G6PD, and SIRT-1 [28–31]. An HFD suppresses SIRT1 expression, which increases PGC-1α expression and AMPK activation [28,32]. P111, P121, and LpBL suppressed lipid accumulation and induced AMPK activation in palmitic acid-stimulated HepG2 cells and suppressed adipogenesis in dexamethasone-stimulated 3T3 L1 cells. These findings imply that P111, P121, or LpBL are able to suppress lipogenesis and induce lipolysis in the liver and intestine by inducing AMPK activation and SIRT1 expression, leading to the alleviation of liver steatosis. They also decreased HFD-induced liver and EFP weights; liver lipid droplet number; and TC, TG, and HC levels in the liver and blood. These observations suggest that these probiotics can have an effect on liver steatosis.

Oral administration of P111, P121, or LpBL decreased HFD-induced proinflammatory cytokine expression, NF-κB activation (*p*-p65/065), and NF-κB-positive cell number in the colon, liver, and brain. Furthermore, they lowered HFD-induced LPS levels in the blood and feces. They also alleviated HFD-induced DCi-like behavior. LPS induces NF-κB-mediated proinflammatory cytokine expression, which triggers adipogenesis and hinders AMPK activation [4–6]. The AMPK activation inhibits systemic inflammation, including neuroinflammation [8,9]. Inflammation-inducing stressors such as pathogens and bacterial endotoxin induce DCi through systemic inflammation [10,11]. The combination of P111 with P121 (LpBl) additively alleviated HF-induced body weight gain, liver and EFP weights, liver steatosis, colitis, neuroinflammation, and DCi-like behavior. The efficacy of LpBl was more potent than those of P111 and P121, but not significantly. These findings suggest that P111 and/or P121 may suppress proinflammatory cytokine expression in the gut, liver, and brain by suppressing LPS-linked NF-κB signal, leading to the alleviation of colitis, hepatitis, neuroinflammation, and psychiatric disorders.

HFD feeding increased *Proteobacteria*, including *Helicobacteriaceae*, *Firmicutes*, *Streptococcaceae*, and *Staphylococcaceae* populations, and bacterial LPS production. However, LpBl, which most potently reduced HFD-induced body weight gain, suppressed HFD-induced populations of *Firmicutes* and *Proteobacteria*, in particular, *Streptococcaceae*, *Desulfovibrionaceae*, *Coriobacteriaceae*, and *Helicobacteriacae* populations, which had a positive correlation with body weight gain and a negative correlation with SIRT1 and BDNF expression levels in the liver. LpBl reduced the HFD-induced LPS level in the feces, blood, and liver that was positively correlated with TNF-α expression. The HFD-induced *Streptococcaceae* and *Helicobacteriaceae* number had a positive correlation with IT in the TST, while SA in the

YMT were negatively correlated. However, LpBl treatment increased HFD-suppressed *Bacteroidetes* and *Verrucomicrobiota*, including *Akkermansiaceae*, *Prevotellaceae*, *Bifidobacteriaceae*, and *Enterobacteriaceae* populations, which had a negative correlation with DCi-like behavior and a positive correlation with an increased hippocampal BDNF expression level. Furthermore, LpBl suppressed HFD-induced corticosterone and IL-6 levels in the blood and IL-6 levels in the colon, blood, liver, and hippocampus. HFD feeding suppresses the *Akkermansiaceae* population in mice, in turn suppressing HFD-induced obesity and the blood glucose level [33–35]. The probiotic *L. plantarum* KY1032 increases *Akkermansiaceae* and *Bifidobacteriaceae* populations in volunteers with overweight [36]. Dietary fibers increase gut *Prevotellaceae* and *Bifidobacteriaceae* populations and reduce body weight gain. LPS increases DCi-like behavior and TNF-α and corticosterone expression [19]. *Lactobacillus reuteri* NK33 alleviates depressive symptoms in mice by inhibiting gut dysbiosis and bacterial LPS production. These results suggest that HFD can cause gut dysbiosis, which may be closely associated with body weight gain, liver steatosis, gut inflammation, neuroinflammation, and DCi-like behavior; and probiotics, in particular, LpBl, may mitigate obesity, liver steatosis, colitis, and DCi by alleviating gut dysbiosis and bacterial LPS production.

Moreover, we discovered that chronic feeding of an HFD could cause gut dysbiosis, along with body weight gain, which leads to DCi, and P111 and/or P121 could alleviate body weight gain and DCi by regulating gut dysbiosis and AMPK activation. However, to understand the action mechanism of P111 and/or P121, future research is needed to identify their substances and to clarify the pathogenesis of overgrown fecal bacteria stemming from an HFD.

5. Conclusions

P111, P121, and their mix LpBl can alleviate obesity, liver steatosis, DCi, and systemic inflammation, including colitis, liver inflammation, and neuroinflammation, in vivo by inducing AMPK activation and suppressing gut dysbiosis and LPS production.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu16223810/s1. Figure S1. Effects of P111 (Lpl) and P121 (Bio) on lipid accumulation and AMPK activation in 3T3 L1 and HepG2 cells. Figure S2. Characterization of the whole genomes of *L. plantarum* P111 and *Bifidobacterium longum* P21. Figure S3. Effects of P111 (Lpl) and P121 (Blo) on steatohepatitis-related marker expression in the liver. Figure S4. Effects of P111 (Lpl) and P121 (Bio) on neuroinflammation in the hippocampus of mice with HF-induced obesity. Figure S5. Effects of P111 and P121 on gut inflammation in the colon of mice with HF-induced obesity. Figure S6. Effect of LpBl on steatohepatitis-related marker expression in the liver. Figure S7. Effect of LpBl on neuroinflammation in the hippocampus of mice with HF-induced obesity. Figure S8. Effect of LpBl on gut inflammation in the colon of mice with HF-induced obesity. Table S1. Primers used in the present study. Table S2. Effect of LpBl on the fecal microbiota composition of HF-treated mice at the phylum level. Table S3. Effect of LpBl on the fecal microbiota composition of HF-treated mice at the family level. Methods—Behavioral tasks and determination of LPS in the blood, liver, and feces. Quantitative real-time polymerase chain reaction (qPCR). Gut microbiota composition analysis. Immunofluorescence staining. References [37–40] are cited in the Supplementary Materials.

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References

1. Blüher, M. Obesity: Global epidemiology and pathogenesis. *Nat. Rev. Endocrinol.* **2019**, *15*, 288–298. [CrossRef] [PubMed]
2. Castro-Barquero, S.; Ruiz-León, A.M.; Sierra-Pérez, M.; Estruch, R.; Casas, R. Dietary Strategies for Metabolic Syndrome: A Comprehensive Review. *Nutrients* **2020**, *12*, 2983. [CrossRef] [PubMed]
3. Magkos, F.; Hjorth, M.F.; Astrup, A. Diet and exercise in the prevention and treatment of type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* **2020**, *16*, 545–555. [CrossRef] [PubMed]
4. Zou, J.; Liu, C.; Jiang, S.; Qian, D.; Duan, J. Cross Talk between Gut Microbiota and Intestinal Mucosal Immunity in the Development of Ulcerative Colitis. *Infect. Immun.* **2021**, *89*, e0001421. [CrossRef]
5. Wu, D.; Wang, H.; Xie, L.; Hu, F. Cross-Talk Between Gut Microbiota and Adipose Tissues in Obesity and Related Metabolic Diseases. *Front. Endocrinol.* **2022**, *13*, 908868. [CrossRef] [PubMed]
6. Kang, G.G.; Trevaskis, N.L.; Murphy, A.J.; Febbraio, M.A. Diet-induced gut dysbiosis and inflammation: Key drivers of obesity-driven NASH. *iScience* **2023**, *26*, 105905. [CrossRef]
7. Foretz, M.; Even, P.C.; Viollet, B. AMPK Activation Reduces Hepatic Lipid Content by Increasing Fat Oxidation In Vivo. *Int. J. Mol. Sci.* **2018**, *19*, 2826. [CrossRef]
8. Liu, X.; Yin, S.; Chen, Y.; Wu, Y.; Zheng, W.; Dong, H.; Bai, Y.; Qin, Y.; Li, J.; Feng, S.; et al. LPS-induced proinflammatory cytokine expression in human airway epithelial cells and macrophages via NF-κB, STAT3 or AP-1 activation. *Mol. Med. Rep.* **2018**, *17*, 5484–5491. [CrossRef]
9. Salminen, A.; Hyttinen, J.M.; Kaarniranta, K. AMP-activated protein kinase inhibits NF-κB signaling and inflammation: Impact on healthspan and lifespan. *J. Mol. Med.* **2011**, *89*, 667–676. [CrossRef]
10. Hassamal, S. Chronic stress, neuroinflammation, and depression: An overview of pathophysiological mechanisms and emerging anti-inflammatories. *Front. Psychiatry* **2023**, *14*, 1130989. [CrossRef]
11. Schedlowski, M.; Engler, H.; Grigoleit, J.S. Endotoxin-induced experimental systemic inflammation in humans: A model to disentangle immune-to-brain communication. *Brain Behav. Immun.* **2014**, *35*, 1–8. [CrossRef] [PubMed]
12. Rogers, G.B.; Keating, D.J.; Young, R.L.; Wong, M.L.; Licinio, J.; Wesselingh, S. From gut dysbiosis to altered brain function and mental illness: Mechanisms and pathways. *Mol. Psychiatry* **2016**, *21*, 738–748. [CrossRef]
13. Carpi, R.Z.; Barbalho, S.M.; Sloan, K.P.; Laurindo, L.F.; Gonzaga, H.F.; Grippa, P.C.; Zutin, T.L.M.; Girio, R.J.S.; Repetti, C.S.F.; Detregiachi, C.R.P.; et al. The Effects of Probiotics, Prebiotics and Synbiotics in Non-Alcoholic Fat Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH): A Systematic Review. *Int. J. Mol. Sci.* **2022**, *23*, 8805. [CrossRef]
14. Westfall, S.; Lomis, N.; Kahouli, I.; Dia, S.Y.; Singh, S.P.; Prakash, S. Microbiome, probiotics and neurodegenerative diseases: Deciphering the gut brain axis. *Cell. Mol. Life Sci.* **2017**, *74*, 3769–3787. [CrossRef]
15. Cheng, L.H.; Chou, P.Y.; Hou, A.T.; Huang, C.L.; Shiu, W.L.; Wang, S. Lactobacillus paracasei PS23 improves cognitive deficits via modulating the hippocampal gene expression and the gut microbiota in D-galactose-induced aging mice. *Food Funct.* **2022**, *13*, 5240–5251. [CrossRef]
16. Akbari, E.; Asemi, Z.; Daneshvar Kakhaki, R.; Bahmani, F.; Kouchaki, E.; Tamtaji, O.R.; Hamidi, G.A.; Salami, M. Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. *Front. Aging Neurosci.* **2016**, *8*, 256. [CrossRef]
17. In Kim, H.; Kim, J.K.; Kim, J.Y.; Jang, S.E.; Han, M.J.; Kim, D.H. Lactobacillus plantarum LC27 and Bifidobacterium longum LC67 simultaneously alleviate high-fat diet-induced colitis, endotoxemia, liver steatosis, and obesity in mice. *Nutr. Res.* **2019**, *67*, 78–89. [CrossRef]
18. Kang, Y.; Kang, X.; Yang, H.; Liu, H.; Yang, X.; Liu, Q.; Tian, H.; Xue, Y.; Ren, P.; Kuang, X.; et al. Lactobacillus acidophilus ameliorates obesity in mice through modulation of gut microbiota dysbiosis and intestinal permeability. *Pharmacol. Res.* **2022**, *175*, 106020. [CrossRef]
19. Jang, H.M.; Lee, K.E.; Kim, D.H. The Preventive and Curative Effects of Lactobacillus reuteri NK33 and Bifidobacterium adolescentis NK98 on Immobilization Stress-Induced Anxiety/Depression and Colitis in Mice. *Nutrients* **2019**, *11*, 819. [CrossRef]
20. Li, D.; Cui, Y.; Wang, X.; Liu, F.; Li, X. Apple polyphenol extract alleviates lipid accumulation in free-fatty-acid-exposed HepG2 cells via activating autophagy mediated by SIRT1/AMPK signaling. *Phytother. Res.* **2021**, *35*, 1416–1431. [CrossRef] [PubMed]
21. Gu, W.; Kim, K.A.; Kim, D.H. Ginsenoside Rh1 ameliorates high fat diet-induced obesity in mice by inhibiting adipocyte differentiation. *Biol. Pharm. Bull.* **2013**, *36*, 102–107. [CrossRef] [PubMed]
22. Percie du Sert, N.; Hurst, V.; Ahluwalia, A.; Alam, S.; Avey, M.T.; Baker, M.; Browne, W.J.; Clark, A.; Cuthill, I.C.; Dirnagl, U.; et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biol.* **2020**, *18*, e3000410. [CrossRef]

23. Lee, D.Y.; Shin, Y.J.; Kim, J.K.; Jang, H.M.; Joo, M.K.; Kim, D.H. Alleviation of cognitive impairment by gut microbiota lipopolysaccharide production-suppressing Lactobacillus plantarum and Bifidobacterium longum in mice. *Food Funct.* **2021**, *12*, 10750–10763. [CrossRef] [PubMed]
24. Mitrovic, B.; Gluvic, Z.M.; Obradovic, M.; Radunovic, M.; Rizzo, M.; Banach, M.; Isenovic, E.R. Non-alcoholic fatty liver disease, metabolic syndrome, and type 2 diabetes mellitus: Where do we stand today? *Arch. Med. Sci.* **2023**, *19*, 884–894. [CrossRef]
25. Dietrich, P.; Hellerbrand, C. Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best. Pract. Res. Clin. Gastroenterol.* **2014**, *28*, 637–653. [CrossRef] [PubMed]
26. Kociszewska, D.; Chan, J.; Thorne, P.R.; Vlajkovic, S.M. The Link between Gut Dysbiosis Caused by a High-Fat Diet and Hearing Loss. *Int. J. Mol. Sci.* **2021**, *22*, 13177. [CrossRef]
27. Chang, C.C.; Sia, K.C.; Chang, J.F.; Lin, C.M.; Yang, C.M.; Huang, K.Y.; Lin, W.N. Lipopolysaccharide promoted proliferation and adipogenesis of preadipocytes through JAK/STAT and AMPK-regulated cPLA2 expression. *Int. J. Med. Sci.* **2019**, *16*, 167–179. [CrossRef]
28. Cantó, C.; Auwerx, J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr. Opin. Lipidol.* **2009**, *20*, 98–105. [CrossRef] [PubMed]
29. Kohan, A.B.; Talukdar, I.; Walsh, C.M.; Salati, L.M. A role for AMPK in the inhibition of glucose-6-phosphate dehydrogenase by polyunsaturated fatty acids. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 117–121. [CrossRef]
30. Kim, H.K.; Youn, B.S.; Shin, M.S.; Namkoong, C.; Park, K.H.; Baik, J.H.; Kim, J.B.; Park, J.Y.; Lee, K.U.; Kim, Y.B.; et al. Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. *Diabetes* **2010**, *59*, 2772–2780. [CrossRef] [PubMed]
31. Kim, S.J.; Nian, C.; McIntosh, C.H. Activation of lipoprotein lipase by glucose-dependent insulinotropic polypeptide in adipocytes. A role for a protein kinase B, LKB1, and AMP-activated protein kinase cascade. *J. Biol. Chem.* **2007**, *282*, 8557–8567. [CrossRef] [PubMed]
32. Price, N.L.; Gomes, A.P.; Ling, A.J.; Duarte, F.V.; Martin-Montalvo, A.; North, B.J.; Agarwal, B.; Ye, L.; Ramadori, G.; Teodoro, J.S.; et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab.* **2012**, *15*, 675–690. [CrossRef] [PubMed]
33. Lin, X.Q.; Chen, W.; Ma, K.; Liu, Z.Z.; Gao, Y.; Zhang, J.G.; Wang, T.; Yang, Y.J. Akkermansia muciniphila Suppresses High-Fat Diet-Induced Obesity and Related Metabolic Disorders in Beagles. *Molecules* **2022**, *27*, 6074. [CrossRef] [PubMed]
34. Everard, A.; Belzer, C.; Geurts, L.; Ouwerkerk, J.P.; Druart, C.; Bindels, L.B.; Guiot, Y.; Derrien, M.; Muccioli, G.G.; Delzenne, N.M.; et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9066–9071. [CrossRef] [PubMed]
35. Shin, N.R.; Lee, J.C.; Lee, H.Y.; Kim, M.S.; Whon, T.W.; Lee, M.S.; Bae, J.W. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* **2014**, *63*, 727–735. [CrossRef]
36. Mo, S.J.; Lee, K.; Hong, H.J.; Hong, D.K.; Jung, S.H.; Park, S.D.; Shim, J.J.; Lee, J.L. Effects of Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032 on Overweight and the Gut Microbiota in Humans: Randomized, Double-Blinded, Placebo-Controlled Clinical Trial. *Nutrients* **2022**, *14*, 2484. [CrossRef]
37. Lee, H.J.; Lee, K.E.; Kim, J.K.; Kim, D.H. Suppression of gut dysbiosis by Bifidobacterium longum alleviates cognitive decline in 5XFAD transgenic and aged mice. *Sci. Rep.* **2019**, *9*, 11814. [CrossRef]
38. Kim, K.A.; Gu, W.; Lee, I.A.; Joh, E.H.; Kim, D.H. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* **2012**, *7*, e47713. [CrossRef] [PubMed]
39. Jang, H.M.; Han, S.K.; Kim, J.K.; Oh, S.J.; Jang, H.B.; Kim, D.H. Lactobacillus sakei Alleviates High-Fat-Diet-Induced Obesity and Anxiety in Mice by Inducing AMPK Activation and SIRT1 Expression and Inhibiting Gut Microbiota-Mediated NF-κB Activation. *Mol. Nutr. Food Res.* **2019**, *63*, e1800978. [CrossRef] [PubMed]
40. Kim, J.K.; Lee, K.E.; Lee, S.A.; Jang, H.M.; Kim, D.H. Interplay Between Human Gut Bacteria Escherichia coli and Lactobacillus mucosae in the Occurrence of Neuropsychiatric Disorders in Mice. *Front. Immunol.* **2020**, *11*, 273. [CrossRef] [PubMed]

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The Gut Microbiota and Its Metabolites and Their Association with the Risk of Autoimmune Thyroid Disease: A Mendelian Randomization Study

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Abstract: Objectives: Observational research shows associations of the gut microbiota and its metabolites with autoimmune thyroid disease (AITD), but the causality is undetermined. **Methods**: Two-sample Mendelian randomization (MR) was employed to analyze the association of the gut microbiota and its metabolites with AITD. A total of 119 gut microbiotas and nine fecal/circulating metabolites were the exposures. AITD, Graves' disease (GD), and Hashimoto's thyroiditis (HT) were the outcomes. Inverse-variance weighting (IVW) was primarily used to assess causality; Cochran's Q was used to assess heterogeneity. Sensitivity analyses (weighted median, MRPRESSO regression, MRPRESSO intercept, MRPRESSO global, Steiger filtering, leave-one-out) were conducted to assess causal estimate robustness. Multivariable MR (MVMR) was used to estimate the effects of body mass index (BMI) and alcohol consumption frequency on causality. **Results**: The outcomes were potentially causally associated with 22 gut microbiotas and three metabolites. After multiple-test correction, 3-indoleglyoxylic acid retained significant causality with AITD (IVW: odds ratio [OR] = 1.09, 95% confidence interval [CI] = 1.05–1.14, $p = 2.43 \times 10^{-5}$, FDR = 0.009). The sensitivity analyses were confirmatory (weighted median: OR = 1.06, 95% CI = 1.01–1.12, $p = 0.025$; MRPRESSO: OR = 1.09, 95% CI = 1.15–1.14, $p = 0.001$). MVMR revealed no confounding effects on this association (BMI: OR = 1.21, 95% CI =1.08–1.35, $p = 0.001$; drinks/week: OR = 1.22, 95% CI = 1.04–1.43, $p = 0.014$). **Conclusions**: MR revealed no significant causal effects of the gut microbiota on the outcomes. However, MR revealed the causal effects of 3-indoleglyoxylic acid on the risk of AITD.

Keywords: gut microbiota; metabolites; autoimmune thyroid disease; 3-indoleglyoxylic acid; mendelian randomization study

1. Introduction

Autoimmune thyroid disease (AITD) is the most prevalent autoimmune disease and the most common thyroid disorder [1]. AITD can result in thyroid dysfunction, cardiovascular disease [2], autoimmune encephalitis [3], and adverse pregnancy outcomes [4]. The two extremes of AITD are Hashimoto's thyroiditis (HT) and Graves' disease (GD) [5]. In addition to genetic factors, environmental factors (radiation, viral infections, medications, selenium, and iodine) can influence the incidence of AITD [5–7]. Recent studies have identified changes in the gut microbial composition as another potential environmental risk factor for AITD [8].

The gut microbiota plays a crucial role in the immune response. Certain microbiota species continuously present antigens that dictate the host's immune response [9], fostering the differentiation and maturation of regulatory T cells [10] and modulating the immune reactivity of B cells, dendritic cells, and macrophages [9]. Gut microbes produce metabolites that may affect the immune system via the bloodstream [11], including short-chain fatty acids (SCFAs) [12], trimethylamine *N*-oxide (TMAO) [13], tryptophan derivatives, and

bile acids (BAs) [14]. Additionally, lipopolysaccharides (LPSs), which are endotoxins produced by pathogenic bacteria, can potentially induce intestinal inflammation, compromise intestinal barrier integrity, and influence immune function [15].

Numerous studies have reported a link between the gut microbiota and AITD. A meta-analysis concluded that, compared with those in the control group, the abundances of *Bifidobacterium* and *Lactobacillus* in AITD were significantly lower, while the abundance of *Bacteroides fragilis* was significantly greater [16]. Two studies reported a decreased abundance of *Prevotella* in HT patients [17,18]. For GD patients, the abundances of *Lactobacillus* were correlated with thyroid-stimulating hormone receptor antibodies (TRAb), thyroid peroxidase antibodies (TPOAb), and thyroglobulin antibodies (TgAb) [19,20]; *Synergistetes* showed a negative association with thyroid-stimulating antibody (TSAb); and *Ruminococcus*, *Bifidobacterium*, and *Veillonella* were positively correlated with TRAb [21,22]. Other bacterial groups such as *Prevotella 9*, *Actinomyces odontolyticus*, and *Negativicutes* are positively associated with TPOAb [23]. Furthermore, the gut microbiota significantly impacts the intestinal absorption of iodine and selenium [24–26]. For example, *Bacteroides* can convert intracellular selenite into selenocysteine and selenomethionine, potentially enhancing organic selenium absorption [27] and contributing to a reduction in thyroid autoantibodies.

Metabolites also play a critical role in AITD development. SCFAs, through their interaction with G-protein-coupled receptors (GPCRs) on leukocytes and intestinal epithelial cells [28], can inhibit AITD. SCFA supplementation has been shown to upregulate IL-22 and IL-10, suppress AITDs and the NF-κB inflammatory pathway, reduce LPS stimulation, and regulate TNF-α expression [29]. In individuals with primary hypothyroidism, primarily due to AITD, a decrease in SCFA-producing bacteria leads to decreased SCFA levels, exacerbating damage to the intestinal barrier and increasing serum LPS levels [30]. Additionally, SCFAs, in conjunction with the sodium/iodide symporter, may enhance iodide uptake by thyroid follicular cells [31]. Gut microbiota metabolites also hold diagnostic potential in other thyroid disorders. In a mouse model of Graves' ophthalmopathy, fecal 16S rRNA functional predictions revealed significant changes in BA metabolism [32]; TMAO has shown potential in distinguishing between benign and malignant thyroid nodules [33]; and indole-3-carboxaldehyde in blood is a biomarker for papillary thyroid cancer [34]. While numerous studies have been conducted, their findings are often inconsistent, likely due to differences in detection methods, diet, and lifestyle, which contribute to substantial interindividual heterogeneity. Additionally, most studies have small sample sizes, limiting their ability to establish a strong relationship between the gut microbiota and AITD. Finally, as case–control studies, they are limited in assessing causality between the gut microbiota and AITD, adding to the challenge of fully understanding this relationship.

Randomized controlled studies focused on the gut microbiota may contribute to establishing causal relationships; however, high-quality evidence is lacking in the field of the gut–thyroid axis. In such scenarios, Mendelian randomization (MR) can serve as an alternative to randomized controlled trials, facilitating the exploration of causality [35]. The key idea of MR is to utilize the random allocation of genetic variation in nature, treating genetic variation as an instrumental variable (IV), effectively minimizing potential confounding factors like environmental and socioeconomic influences to enable more reliable causal assessment [35]. MR studies often leverage large genome-wide association study (GWAS) datasets, increasing their power to detect small effect sizes and enhancing their statistical robustness. By using genetic variation as an IV, MR also ensures that exposure (e.g., gut microbiota traits) precedes the outcome (AITD), effectively mitigating reverse causation bias. Therefore, MR is well suited for causal inference in studies examining the relationship between the gut microbiota and disease.

In this study, we used statistics from several GWASs of gut microbes and fecal/circulating metabolites to assess potential causal associations of gut microbes and metabolites with AITD using two-sample MR, and multivariate MR (MVMR) was used to adjust for possible confounders (body mass index [BMI] and alcohol consumption). Based on these analyses, we investigated the role of the gut microbiome in the pathogenesis of AITD, aiming to

provide evidence for the emergence of innovative therapeutic strategies, including dietary interventions, probiotic supplementation, and fecal microbiota transplantation.

2. Materials and Methods

2.1. Study Design and Data Sources

This study's results are reported in accordance with the STROBE-MR guidelines (Supplementary Table S1) [36]. Furthermore, such an MR study must satisfy three assumptions for the instrumental variables (IVs) [37]: (A) it predicts the exposure of interest; (B) it is independent of potential confounders; and (C) it influences the outcome only through risk factors. Figure 1 depicts the research design and assumptions for the MR analysis. Initially, we conducted a two-sample MR analysis of 119 different gut microbiotas and AITD. Then, based on reports in the literature (Supplementary Table S2), we identified nine gut microbiota metabolites in five categories that may be related to AITD. These SCFAs include three SCFAs (fecal butyrate, circulating acetate, and propionate) [38,39], circulating TMAO [40], two BAs (circulating cholic acid and taurocholic acid) [32], two indoles and their derivatives (circulating indole-3-propionate and 3-indoleglyoxylic acid) [41,42], and LPS (proxied by 3-hydroxymyristate) [43,44].

Supplementary Table S3 provides detailed information on the data sources. The first part of the exposure data comprises gut microbiota GWAS data from the MiBioGen consortium (https://mibiogen.gcc.rug.nl/, accessed on 7 June 2023) [45]. This dataset included genome-wide genotype data and 16S rRNA fecal microbiome profiles obtained from 18,340 individuals across 24 cohorts. We included only individuals of European descent (n = 13,266) in our study. The taxonomic resolution of the fecal microbiome in this research extended to the genus level. A total of 119 bacterial genera were extracted. We used independent SNPs ($r^2 \leq 0.01$) as genetic tools and selected 116 genera ($p < 1 \times 10^{-5}$) for subsequent analyses, excluding those with fewer than three SNPs.

The second part of the GWAS exposure data on fecal and blood metabolites came from five different studies (Supplementary Table S3). The IVs for the fecal butyric acid metabolic pathway (PWY-5022) originated from the GWAS conducted in the Dutch Microbiome Project by Lopera-Maya EA et al. [46]. The IVs for circulating acetic acid were derived from a pooled GWAS comprising 14 European cohorts encompassing 24,925 individuals, as reported by Kettunen J et al. [47]. IVs associated with circulating propionate and TMAO were extracted from a cohort of 822 participants in the Chronic Renal Insufficiency Cohort (CRIC) study conducted by Rhee EP et al. [48]. IVs concerning indole and its derivatives (circulating indole-3-propionate and 3-indole glyoxylic acid) were acquired from GWASs on blood metabolomics conducted within the KORA and TwinsUK European cohorts by Shin et al. [49] and the Canadian Longitudinal Study on Aging (CLSA) cohort by Chen Y et al. [50], respectively. Circulating cholic acid, taurocholic acid, and LPS (proxied by 3-hydroxymyristate [44]) IVs were also obtained from the study by Chen Y et al. [50]. The selection criteria for all IVs were $r^2 \leq 0.01$ and $p < 1 \times 10^{-5}$.

There were three genetic outcome-traits in this study. GWAS summary statistics for AITD were acquired from the Icelandic and UK biobanks (https://www.ukbiobank.ac.uk/, accessed on 7 June 2023) [6], encompassing 755,406 individuals (30,234 patients and 725,172 control participants) of European descent. The phenotype 'AITD' was specifically characterized as encompassing GD, HT, other forms of hypothyroidism, and/or treatment with thyroxine, excluding known non-AITD and drug-induced thyroid conditions [6]. The GWAS summary data for GD and HT were obtained from a recent large-scale meta-analysis of 220 phenotypes [51], which drew on data from three prominent biobanks: BioBank Japan (https://biobankjp.org/en/, accessed on 7 June 2023), UK Biobank (https://www.ukbiobank.ac.uk/, accessed on 7 June 2023), and FinnGen (https://www.finngen.fi/en, accessed on 7 June 2023). We selected only subjects of European ancestry for inclusion in the analysis (GD: 1678 patients and 456,942 control participants; HT: 15,654 patients and 379,986 control participants). The described methods of assessment and diagnostic criteria for GD and HT are shown in Supplementary Method S1.

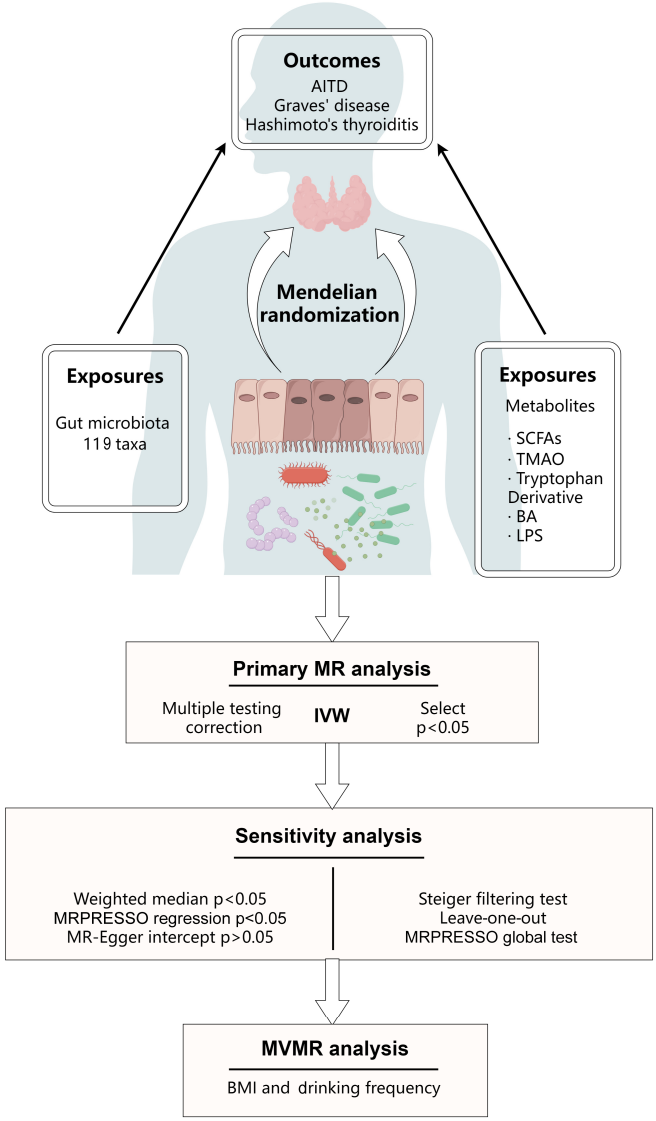

Figure 1. An overview of the Mendelian randomization design used to investigate the causal effect. AITD, autoimmune thyroid disease; BA, bile acid; IVW, inverse-variance weighting; LPS, lipopolysaccharide; MR, mendelian randomization; MRPRESSO, Mendelian randomization pleiotropy residual sum and outlier; MVMR, multivariate mendelian randomization; SCFAs, short-chain fatty acids; TMAO, trimethylamine *N*-oxide.

All relevant GWAS data were approved by the respective institutional ethics review boards, and no additional ethical review was required for the published data of this MR study.

2.2. Mendelian Randomization

Before delving into the results, we aligned the exposure and outcome data by deducing the forward strand alleles utilizing allele frequency data and excluding palindromic genetic variants (kb > 10,000 and $r^2 < 0.001$) [52]. Additionally, we assessed the potency of the genetic instruments for all SNPs using the F-statistic derived as (β^2/se^2), followed by an MR analysis focusing on IVs with an F-statistic surpassing 10 [53].

Our primary analytical approach centered around employing the inverse-variance weighting (IVW) method [54]. IVW calculates the weighted average of each IV's effect estimate, using the square of its standard error as the weight, to estimate the overall effect of exposure on the outcome. We used Cochran's Q test for heterogeneity, and when heterogeneity existed, we used the multiplicative random-effects model of IVW [54]. When

$p < 0.05$ for the IVW method, we used the weighted median [55] and Mendelian randomization pleiotropy residual sum and outlier (MRPRESSO) [56] regression for sensitivity analysis to strengthen its robustness. The weighted median approach, based on the median effect of the IVs rather than the mean, reduces bias in the presence of pleiotropic IVs. MRPRESSO offers an advanced method for detecting and correcting pleiotropy, performing a global test and identifying outliers among the IVs; outliers are then adjusted or removed to improve estimation accuracy. To gauge pleiotropy, we employed the intercept term of the MR–Egger regression and the MRPRESSO global test [56]. In addition, we performed "leave-one-out (LOO)" analysis [56], sequentially excluding each IV to verify result stability. To exclude IVs more closely associated with the outcome than with the exposure, we executed an additional Steiger test [57], which compares the explanatory power of the instruments for exposure versus the outcome. If an instrument explains more of the exposure than the outcome, this supports the assumed causal direction. More detailed information on these MR methods is provided in Supplementary Method S2.

Vujkovic-Cvijin et al. identified BMI and the frequency of alcohol consumption as influential factors affecting the composition of the host gut microbiota [58]. Therefore, we performed multivariate MR (MVMR) analyses. Furthermore, Cochran's Q test and the MR-Egger intercept were employed to examine study heterogeneity and detect horizontal pleiotropy, respectively. In instances where heterogeneity arose within the IVW method, we employed the weighted median approach to assess the impact of BMI and alcohol consumption frequency on causal inference. Detailed information regarding the origins and characteristics of the GWAS data for BMI and alcohol consumption frequency can be found in Supplementary Table S3.

2.3. Statistical Analysis

We performed a total of $(116 + 9) \times 3 = 375$ primary IVWs. To reduce the risk of false positives, we applied the FDR (using the Benjamini–Hochberg method) multiple-test correction (using a 5% false discovery rate). We considered a significant causal association between exposure and outcome when $p < 0.05$ and FDR < 0.05 and a suggestive association when $p < 0.05$ but FDR ≥ 0.05 [59]. We conducted the data analysis using R (version 4.1.2) and applied the R packages "TwoSampleMR" [57] (version 0.5.6), "MendelianRandomization" (version 0.9.0) [60], and "MRPRESSO" [56] (version 1.0).

3. Results

The objective of this study was to test the hypothesis that the gut microbiota and its associated metabolites play a causal role in AITD. Participants were exclusively from European populations, and there was no significant overlap between the samples used for exposures and outcomes. We only included exposures with more than three independent SNPs ($r^2 < 0.01$ and $p < 1 \times 10^{-5}$) and an F-statistic > 10. All pooled datasets that were used to obtain study exposures and outcomes are presented in Supplementary Tables S4 and S6.

3.1. Impact of Gut Microbiota Genus Abundance on AITD, GD, and HT

Out of the 348 primary IVW analyses, only 22 passed a significance test with a threshold of $p < 0.05$ (Figure 2 and Supplementary Table S5). An increase in the abundance of seven genera (*Anaerofilum*, *Candidatus Soleaferrea*, *Lachnospiraceae UCG0101*, *Marvinbryantia*, *Ruminiclostridium5*, *Ruminococcaceae NK4A214* group, and *Tyzzerella3*) showed potential causal associations with AITD, while five genera (*Defluviitaleaceae UCG011*, *Eubacterium rectale* group, *Family XIII AD3011* group, *Gordonibacter*, and *Oscillospira*) exhibited potential protective effects against AITD. Two genera, *Bifidobacterium* and *Ruminococcus torques* group, were found to have potential pathogenic effects on GD, while *Coprobacter* and *Flavonifractor* had potential protective effects against GD. *Anaerostipes* and *Turicibacter* had potential causal effects on HT, and *Akkermansia*, *Butyrivibrio*, *Prevotella7*, and *Ruminococcaceae UCG011* had potential protective effects against HT. However, these associations no longer existed after FDR (Benjamini–Hochberg) correction (Supplementary Table S5).

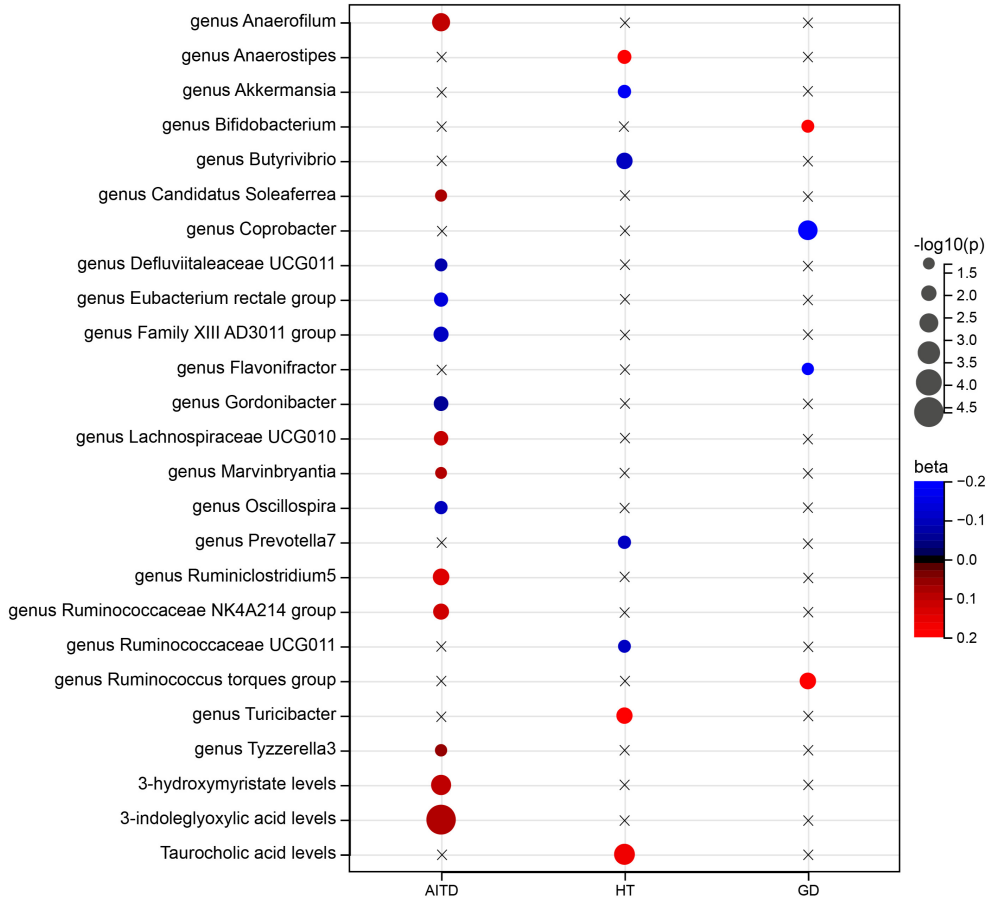

Figure 2. Balloon plot of association of gut microbiota and associated metabolites with three AITD outcomes. Significance of associations of 25 exposures with one or more outcomes is depicted (IVW: $p < 0.05$). Associations at $p > 0.05$ are depicted with crosses. Beta means magnitude of potential causal effect of gut microbiota traits or metabolites on thyroid disease. Beta > 0 indicates that changes in gut microbiota may increase risk of thyroid disease, while beta < 0 suggests that gut microbiota may have protective effect on thyroid health.

3.2. Impact of Gut Microbiota Metabolites on AITD, GD, and HT

Out of the 24 primary analyses conducted, only three had statistically significant results ($p < 0.05$) (Figure 2 and Supplementary Table S7). The levels of 3-hydroxymyristate and 3-indoleglyoxylic acid were identified as potential causative factors for AITD, while taurocholic acid levels were identified as potential causative factors for HT (Figure 2 and Supplementary Table S7). After correcting for FDR, only a significant positive causal relationship between levels of circulating 3-indoleglyoxylic acid and AITD remained. The risk of AITD increased by 9% for each standard deviation increase in 3-indoleglyoxylic acid levels (OR = 1.09, 95% CI = 1.05 to 1.14, $p = 2.43 \times 10^{-5}$, FDR = 0.009) (Figure 2 and Supplementary Table S7).

3.3. Sensitivity Analysis, Pleiotropy Test, and MVMR Analysis

To check the robustness of the results, we performed sensitivity analyses for the above 25 exposures and outcomes with potential causality (IVW: $p < 0.05$). Weighted median and MRPRESSO regressions were used, with each test making different assumptions regarding potential pleiotropy. The estimates from the different tests were consistent, supporting the existence of robust causality. Pleiotropy was also assessed using the MR-Egger intercept and the MRPRESSO Global test. Seven associations were retained after excluding nonrobust results (weighted median $p > 0.05$, MRPRESSO $p > 0.05$, MR-Egger intercept $p < 0.05$, MRPRESSO Global test < 0.05) (Figure 3 and Supplementary Table S8), and all IVs were

subjected to a Steiger filter test, which showed that the IVs used in the analysis did not have reverse causal relationships with the outcomes (Supplementary Tables S4 and S6). Our analysis revealed a strong positive correlation between circulating 3-indoleglyoxylic acid levels and AITD, which was consistent across multiple analytical approaches. The weighted median approach yielded an OR of 1.06 (95% CI: 1.01 to 1.12, $p = 0.025$), while the MRPRESSO approach yielded an OR of 1.09 (95% CI: 1.05 to 1.14, $p = 0.001$). Additionally, LOO analysis confirmed the robustness of all SNPs (Supplementary Figure S1).

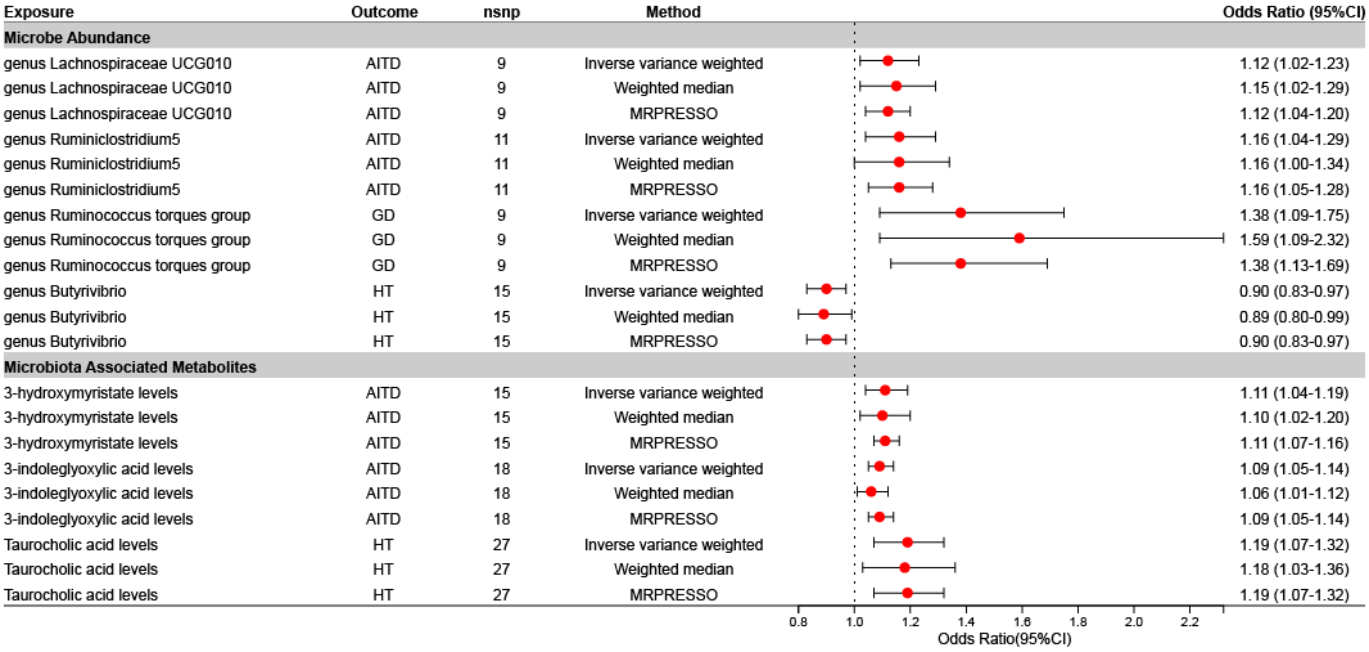

Figure 3. The forest plots illustrate the relationship between the seven exposures and outcomes with robust agreement across robust MR analysis methods. Dots depict the odds ratios (OR). Horizontal bars depict the 95% confidence intervals (CIs). The OR is used to quantify the potential causal effect of specific exposures on the risk of outcomes. OR > 1 indicates that the gut microbiota and associated metabolite characteristics are associated with an increased risk of thyroid disease; OR < 1 suggests that the gut microbiota and associated metabolite characteristics may have a protective effect against thyroid disease. AITD, autoimmune thyroid disease; GD, Graves' disease; HT, Hashimoto's thyroiditis; MRPRESSO, Mendelian randomization pleiotropy residual sum and outlier.

Further MVMR analysis with BMI and frequency of alcohol consumption as confounders showed that circulating 3-indoleglyoxylic acid levels increased the risk of AITD and were not confounded by BMI and alcohol consumption (with BMI: OR = 1.11, 95% CI = 1.02 to 1.21, $p = 0.017$; with alcoholic drinks per week: OR = 1.13, 95% CI = 1.04–1.22, $p = 0.003$) (Figure 4). Similarly, the potential association of the *Ruminococcus torque* group with GD (with BMI: OR = 1.37, 95% CI = 1.04 to 1.81, $p = 0.027$; with alcoholic drinks per week: OR = 1.58, 95% CI = 1.24 to 2.01, $p = 2.44 \times 10^{-4}$), and the potential causal role of circulating taurocholic acid level on HT (with BMI: OR = 1.21, 95% CI = 1.08 to 1.35, $p = 0.001$; with alcoholic drinks per week: OR = 1.22, 95% CI = 1.04 to 1.43, $p = 0.014$) were also unaffected by BMI and frequency of alcohol consumption, which may warrant further investigation.

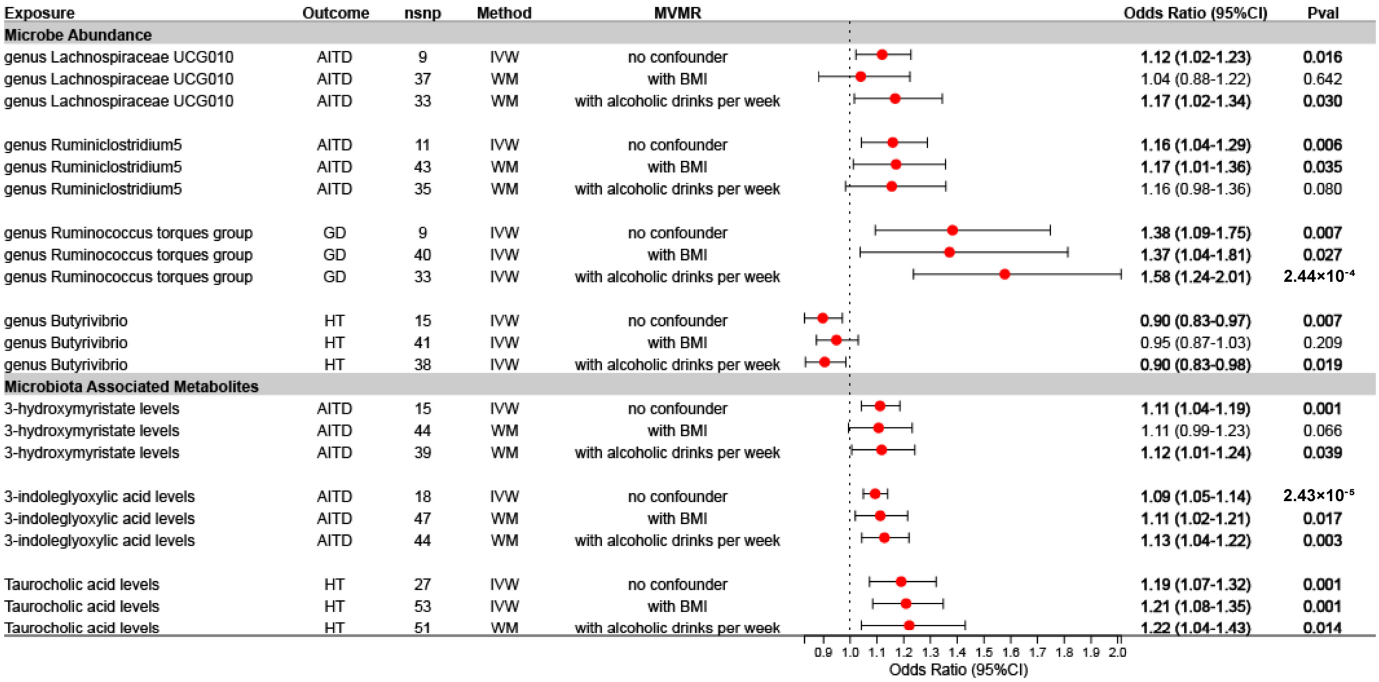

Figure 4. The MVMR results before and after correcting for BMI and alcohol consumption frequency. Dots depict the odds ratio (OR). Horizontal bars depict the 95% confidence intervals (CIs). The OR is used to quantify the potential causal effect of specific exposures on the risk of outcomes. OR > 1 indicates that the gut microbiota and associated metabolite characteristics are associated with an increased risk of thyroid disease; OR < 1 suggests that the gut microbiota and associated metabolite characteristics may have a protective effect against thyroid disease. No confounders, no adjustments were made; with BMI, MVMR adjusted for BMI as a confounding factor; with alcoholic drinks per week, MVMR adjusted for the frequency of alcohol consumption. AITD, autoimmune thyroid disease; BMI, body mass index; GD, Graves' disease; HT, Hashimoto's thyroiditis; IVW, inverse-variance weighting; MVMR, multivariate mendelian randomization; WM, weighted median. Bold, $p < 0.05$.

4. Discussion

We performed a comprehensive two-sample MR analysis of 119 gut microbiota exposures and nine metabolite exposures on three AITD outcomes in order to estimate the causal relationship. We found that genetically determined elevated levels of 3-indoleglyoxylic acid were associated with an increased risk of AITD in a European population and were not affected by BMI and frequency of alcohol consumption. In addition, the ability of the *Ruminococcus torques* group to potentially increase the risk of GD, and the potential pathogenic role of circulating taurocholic acid level on HT, were also not affected by BMI and frequency of alcohol consumption.

The strong familial and heritable nature of AITD development prompts attention to important susceptibility genes common to the development of AITD [61,62]. The abnormal expression of immune-related genes resulting from the polymorphism of some thyroid-related immune regulatory genes can lead to the collapse of the immune system and the development of disease [61]. Some immune-related susceptible genes, such as FOXP3, CTLA-4, PTPN22/LYP, FCRL3, etc., have been found in both GD and HT [61]. These genes affect thyroid autoimmunity at various stages [61]. The partial consistency of genetic susceptibility and the similarity in the pathogenesis of GD and HT allow some genetic epidemiological studies to integrate the genetic information of AITD together and conduct subsequent analyses [6,63]. Our MR analysis is based on previously reported integrated GWAS data and large blood and fecal histology studies.

The influence of the gut microbiota, a novel environmental factor, on thyroid disease appears to be less direct due to biological distance limitations. Based on the gut–thyroid axis hypothesis, the role of gut flora-related metabolites in the circulation is particularly im-

portant [8]. Tryptophan derivatives are an important gut microbiota metabolite for immune regulation [64]. Tryptophan can be catabolized by aromatic amino acid aminotransferases (ArAT) into various indole derivatives [65]. These indole derivatives are known ligands for the immunoregulatory transcription factor aryl hydrocarbon receptor (AhR), which interacts with transcription factors such as NF-κB, estrogen receptor, etc. [66], and is capable of activating regulatory feedback loops highly relevant to immunoregulation [67]. 3-indoleglyoxylic acid is a derivative of indole-3-acetic acid [42,68]. A recent study on microbiology revealed that 3-indoleglyoxylic acid enhances autoimmunity and promotes IL-17 production by T cells in vitro [42]. Furthermore, there may be a causal relationship between 3-indoleglyoxylic acid and multiple sclerosis [42]. In our study, through MR analysis, we found that circulating 3-indoleglyoxylic acid in plasma showed a significant pathogenic role in AITD and was not disturbed by BMI or frequency of alcohol consumption. The role played by 3-indoleglyoxylic acid in AITD and even in autoimmune diseases should be explored in depth.

In addition to metabolites, we also evaluated a wide range of gut microbiota genera. In the primary IVW analysis, there may have been 22 genera potentially associated with AITD; however, after correction for multiple testing, all of these associations disappeared. Although the *Ruminococcus torques* group was able to potentially increase the risk of GD, in the LOO analysis, the potential causal association of *Ruminococcus torques* group with GD disappeared after excluding rs10904297 (Supplementary Figure S2). This suggests that the potential causal association of *Ruminococcus torques* group with GD was driven by a single SNP (rs10904297); therefore, robust conclusions cannot yet be drawn. Molecular simulations have shown that certain *Bifidobacterium* and *Lactobacillus* strains may induce AITD through a cross-reactive antigen-mimetic mechanism due to their amino acid sequence homology with human thyroid peroxidase and thyroglobulin [69]. In our study, the IVW method revealed that the same *Bifidobacterium* nominally increased the risk of GD, which somewhat supports the conclusions of molecular modeling. However, in our study, no genetically determined increase in *Lactobacillus* abundance was found to be associated with any type of AITD risk. In addition, it has been shown that some gut microbiota, such as *Yersinia pestis* and *Helicobacter pylori*, produce antigenic cross-reactive substances with the thyroid-stimulating hormone receptor, which affects thyroid autoimmunity [70]. Unfortunately, the 119 genera analyzed in this study did not include these pathogenic bacteria, and their causal relationship with AITD could not be further verified. The many discrepant genera previously reported in observational studies may be influenced by many confounding factors, such as diet, race, alcohol consumption, and BMI, which are difficult to control for. The MVMR analyses we performed can compensate for this lack of control for confounding factors. In addition, we selected participants with European ancestry only to meet the requirements of MR analysis and to avoid the influence of mixed race on the relationship between gut microbiota and disease. In conclusion, invalid results accounted for a large proportion of our MR analyses of gut microbiota and AITD. There may be some publication bias in the large number of positive results presented in recently published research articles [71] and the causal relationship between flora and AITD may be overestimated if not analyzed with correction for multiple testing. It is also possible that altered gut flora abundance is a consequence rather than a cause of AITD. The ability to explore the direction of causality is precisely the outstanding advantage of MR analysis for problem solving.

Our article also has some limitations. First, although our MR analysis included 119 genera and 9 metabolites, some genera and metabolites were still not included. Second, the threshold for SNP selection in our analysis did not reach the strict threshold level ($p < 5 \times 10^{-8}$), but instead used the threshold commonly used in gut microbiota and metabolite research ($p < 1 \times 10^{-5}$). However, we removed all weak IVs. Third, in addition to BMI and alcohol consumption, environmental factors and even seasons have important effects on gut flora, but we have not yet been able to exclude the influence of these factors by analytical means. In future clinical studies on the gut microbiota, dietary intake can

be assessed through food records or questionnaires, while detailed lifestyle factors (e.g., physical activity, sleep quality, and stress levels) are evaluated and adjusted for in the analysis. Consistency in the season of microbiota sampling should also be maintained. Additionally, longitudinal studies can investigate seasonal fluctuations in the gut microbiota, and statistical methods may be used to minimize seasonal impact on microbiota composition. Fourth, we only examined the gut microbiota at the genus level, as this was the smallest classification identified in the original GWASs. Since higher levels, such as phylum or family, are more likely to include both increasing and decreasing genera, a mixed result would be expected. Further technical and data improvements, such as identification to the species or even subspecies level, are needed for future studies. Finally, although we selected the European population for our study to minimize bias due to ethnicity, this also limits the extrapolation of our results.

Although there are limitations in the IV assumptions in MR analysis, and there may be residual confounding or other alternative causal pathways, this study provides a good reference for future investigations between the gut microbiota and AITD in clinical practice. In addition, our study shows, for the first time, that the level of metabolites associated with the gut microbiota may be a causative factor for AITD to some extent. Intervention strategies targeting specific gut bacteria, such as tryptophan-metabolizing bacteria and *Ruminococcus torques*, may hold promise for targeted biotherapy in AITD management. Future research could further elucidate the gut–thyroid axis by exploring temporal changes in the gut microbiota of AITD patients and examining how the key microbial composition and associated metabolite levels evolve over the course of the disease. Additionally, future work could focus on analyzing the impact of other environmental factors, such as diet and lifestyle, on the gut microbiota to address potential confounders and strengthen causal conclusions. These approaches can meaningfully expand the translational impact of gut microbiota research on the management of AITD.

5. Conclusions

In conclusion, our MR analysis suggests a possible pathogenic effect of circulating 3-indoleglyoxylic acid on AITD, highlighting the impact of tryptophan derivatives on AITD. Additionally, the *Ruminococcus torques* group may increase GD risk, and elevated taurocholic acid levels may play a pathogenic role in HT. Further research is needed to confirm these findings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16223898/s1, Method S1: Methods of assessment and diagnostic criteria for diseases; Method S2: Detailed description of Mendelian randomization methods; Figure S1: Plots of leave-one-out analysis for MR estimates of causal effect of 3-indoleglyoxylic acid on AITD; Figure S2: Plots of leave-one-out analysis for MR estimates of causal effect of *Ruminococcus torques* group on GD; Table S1: STROBE-MR checklist of recommended items to address in reports of Mendelian randomization studies; Table S2: Description of nine gut microbiota-associated metabolite selection rationale; Table S3: Details of data sources included in mendelian randomization analysis; Table S4: Harmonized datasets for each gut microbiota abundance trait exposure and outcome; Table S5: Results of IVW analysis and sensitivity analysis; Table S6: Harmonized datasets for each metabolite trait exposure and outcome; Table S7: Results of IVW analysis and sensitivity analysis; Table S8: Results of robust MR analysis; Table S9: Results of MVMR analysis and sensitivity analysis. References [71–73] are cited in Supplementary Materials.

Author Contributions: Conceptualization, W.T., Z.S. and C.Z.; methodology, C.Z.; software, C.Z.; validation, C.W.; formal analysis, C.Z.; resources, C.Z.; data curation, C.W.; writing—original draft preparation, C.Z.; writing—review and editing, W.T. and Z.S.; visualization, C.Z.; supervision, C.W.; project administration, Z.S. and C.W.; funding acquisition, Z.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All relevant GWAS data were approved by the respective institutional ethics review boards, and no additional ethical review was required for the published data of this MR study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: The datasets supporting the conclusions of this article are available on the GWAS catalog website (https://www.ebi.ac.uk/gwas/, accessed on 7 June 2023), and in the MiBioGen consortium (https://mibiogen.gcc.rug.nl/, accessed on 7 June 2023), UK Biobank (https://www.ukbiobank.ac.uk/, accessed on 7 June 2023), BioBank Japan (https://biobankjp.org/en/, accessed on 7 June 2023), and FinnGen (https://www.finngen.fi/en, accessed on 7 June 2023). The references for these GWAS meta-analyses are shown in Supplementary Table S3.

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References

1. Hayter, S.M.; Cook, M.C. Updated assessment of the prevalence, spectrum and case definition of autoimmune disease. *Autoimmun. Rev.* **2012**, *11*, 754–765. [CrossRef] [PubMed]
2. Chen, Y.; Zhu, C.; Chen, Y.; Wang, N.; Li, Q.; Han, B.; Zhao, L.; Chen, C.; Zhai, H.; Zhang, L.; et al. Are Thyroid Autoimmune Diseases Associated with Cardiometabolic Risks in a Population with Normal Thyroid-Stimulating Hormone? *Mediat. Inflamm.* **2018**, *2018*, 1856137. [CrossRef] [PubMed]
3. Lin, J.F.; Wang, J.R.; Wang, J.Q.; Li, J.M. The detection of up-regulated anti-thyroid antibodies and autoimmune thyroid diseases in patients with autoimmune encephalitis: A retrospective study of 221 patients. *Neurol. Sci.* **2022**, *43*, 3901–3910. [CrossRef] [PubMed]
4. Tańska, K.; Gietka-Czernel, M.; Glinicki, P.; Kozakowski, J. Thyroid autoimmunity and its negative impact on female fertility and maternal pregnancy outcomes. *Front. Endocrinol.* **2022**, *13*, 1049665. [CrossRef]
5. Antonelli, A.; Ferrari, S.M.; Corrado, A.; Di Domenicantonio, A.; Fallahi, P. Autoimmune thyroid disorders. *Autoimmun. Rev.* **2015**, *14*, 174–180. [CrossRef]
6. Saevarsdottir, S.; Olafsdottir, T.A.; Ivarsdottir, E.V.; Halldorsson, G.H.; Gunnarsdottir, K.; Sigurdsson, A.; Johannesson, A.; Sigurdsson, J.K.; Juliusdottir, T.; Lund, S.H.; et al. FLT3 stop mutation increases FLT3 ligand level and risk of autoimmune thyroid disease. *Nature* **2020**, *584*, 619–623. [CrossRef]
7. Zhang, C.; Wang, H.; Teng, W.; Shan, Z. The Relationships among the Urinary Iodine Concentration, Selenium Intake, and Thyroid Antibodies in Adults, Including the Interaction between Iodine and Selenium: National Health and Nutrition Examination Survey 2007–2012. *Nutrients* **2024**, *16*, 3443. [CrossRef]
8. Knezevic, J.; Starchl, C.; Tmava Berisha, A.; Amrein, K. Thyroid-Gut-Axis: How Does the Microbiota Influence Thyroid Function? *Nutrients* **2020**, *12*, 1769. [CrossRef]
9. Belkaid, Y.; Hand, T.W. Role of the microbiota in immunity and inflammation. *Cell* **2014**, *157*, 121–141. [CrossRef]
10. Ivanov, I.I.; Frutos Rde, L.; Manel, N.; Yoshinaga, K.; Rifkin, D.B.; Sartor, R.B.; Finlay, B.B.; Littman, D.R. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* **2008**, *4*, 337–349. [CrossRef]
11. Hosseinkhani, F.; Heinken, A.; Thiele, I.; Lindenburg, P.W.; Harms, A.C.; Hankemeier, T. The contribution of gut bacterial metabolites in the human immune signaling pathway of non-communicable diseases. *Gut Microbes* **2021**, *13*, 1882927. [CrossRef] [PubMed]
12. Kim, C.H. Complex regulatory effects of gut microbial short-chain fatty acids on immune tolerance and autoimmunity. *Cell Mol. Immunol.* **2023**, *20*, 341–350. [CrossRef] [PubMed]
13. Chan, M.M.; Yang, X.; Wang, H.; Saaoud, F.; Sun, Y.; Fong, D. The Microbial Metabolite Trimethylamine *N*-Oxide Links Vascular Dysfunctions and the Autoimmune Disease Rheumatoid Arthritis. *Nutrients* **2019**, *11*, 1821. [CrossRef] [PubMed]
14. Su, X.; Gao, Y.; Yang, R. Gut microbiota derived bile acid metabolites maintain the homeostasis of gut and systemic immunity. *Front. Immunol.* **2023**, *14*, 1127743. [CrossRef] [PubMed]

15. Vatanen, T.; Kostic, A.D.; d'Hennezel, E.; Siljander, H.; Franzosa, E.A.; Yassour, M.; Kolde, R.; Vlamakis, H.; Arthur, T.D.; Hämäläinen, A.M.; et al. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* **2016**, *165*, 842–853. [CrossRef]
16. Gong, B.; Wang, C.; Meng, F.; Wang, H.; Song, B.; Yang, Y.; Shan, Z. Association Between Gut Microbiota and Autoimmune Thyroid Disease: A Systematic Review and Meta-Analysis. *Front. Endocrinol.* **2021**, *12*, 774362. [CrossRef]
17. Ishaq, H.M.; Mohammad, I.S.; Guo, H.; Shahzad, M.; Hou, Y.J.; Ma, C.; Naseem, Z.; Wu, X.; Shi, P.; Xu, J. Molecular estimation of alteration in intestinal microbial composition in Hashimoto's thyroiditis patients. *Biomed. Pharmacother. Biomed. Pharmacother.* **2017**, *95*, 865–874. [CrossRef]
18. Zhao, F.; Feng, J.; Li, J.; Zhao, L.; Liu, Y.; Chen, H.; Jin, Y.; Zhu, B.; Wei, Y. Alterations of the Gut Microbiota in Hashimoto's Thyroiditis Patients. *Thyroid. Off. J. Am. Thyroid. Assoc.* **2018**, *28*, 175–186. [CrossRef]
19. Chen, J.; Wang, W.; Guo, Z.; Huang, S.; Lei, H.; Zang, P.; Lu, B.; Shao, J.; Gu, P. Associations between gut microbiota and thyroidal function status in Chinese patients with Graves' disease. *J. Endocrinol. Investig.* **2021**, *44*, 1913–1926. [CrossRef]
20. Jiang, W.; Yu, X.; Kosik, R.O.; Song, Y.; Qiao, T.; Tong, J.; Liu, S.; Fan, S.; Luo, Q.; Chai, L.; et al. Gut Microbiota May Play a Significant Role in the Pathogenesis of Graves' Disease. *Thyroid. Off. J. Am. Thyroid. Assoc.* **2021**, *31*, 810–820. [CrossRef]
21. Cornejo-Pareja, I.; Ruiz-Limón, P.; Gómez-Pérez, A.M.; Molina-Vega, M.; Moreno-Indias, I.; Tinahones, F.J. Differential Microbial Pattern Description in Subjects with Autoimmune-Based Thyroid Diseases: A Pilot Study. *J. Pers. Med.* **2020**, *10*, 192. [CrossRef] [PubMed]
22. Yang, M.; Zheng, X.; Wu, Y.; Zhang, R.; Yang, Q.; Yu, Z.; Liu, J.; Zha, B.; Gong, Q.; Yang, B.; et al. Preliminary Observation of the Changes in the Intestinal Flora of Patients with Graves' Disease Before and After Methimazole Treatment. *Front. Cell. Infect. Microbiol.* **2022**, *12*, 794711. [CrossRef] [PubMed]
23. Chang, S.C.; Lin, S.F.; Chen, S.T.; Chang, P.Y.; Yeh, Y.M.; Lo, F.S.; Lu, J.J. Alterations of Gut Microbiota in Patients with Graves' Disease. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 663131. [CrossRef] [PubMed]
24. Nicola, J.P.; Nazar, M.; Mascanfroni, I.D.; Pellizas, C.G.; Masini-Repiso, A.M. NF-kappaB p65 subunit mediates lipopolysaccharide-induced Na(+)/I(−) symporter gene expression by involving functional interaction with the paired domain transcription factor Pax8. *Mol. Endocrinol.* **2010**, *24*, 1846–1862. [CrossRef] [PubMed]
25. Sponziello, M.; Scipioni, A.; Durante, C.; Verrienti, A.; Maranghi, M.; Giacomelli, L.; Ferretti, E.; Celano, M.; Filetti, S.; Russo, D. Regulation of sodium/iodide symporter and lactoperoxidase expression in four human breast cancer cell lines. *J. Endocrinol. Investig.* **2010**, *33*, 2–6. [CrossRef]
26. Kasaikina, M.V.; Kravtsova, M.A.; Lee, B.C.; Seravalli, J.; Peterson, D.A.; Walter, J.; Legge, R.; Benson, A.K.; Hatfield, D.L.; Gladyshev, V.N. Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2011**, *25*, 2492–2499. [CrossRef]
27. Zavros, A.; Andreou, E.; Aphamis, G.; Bogdanis, G.C.; Sakkas, G.K.; Roupa, Z.; Giannaki, C.D. The Effects of Zinc and Selenium Co-Supplementation on Resting Metabolic Rate, Thyroid Function, Physical Fitness, and Functional Capacity in Overweight and Obese People under a Hypocaloric Diet: A Randomized, Double-Blind, and Placebo-Controlled Trial. *Nutrients* **2023**, *15*, 3133. [CrossRef]
28. Gong, B.; Meng, F.; Wang, X.; Han, Y.; Yang, W.; Wang, C.; Shan, Z. Effects of iodine intake on gut microbiota and gut metabolites in Hashimoto thyroiditis-diseased humans and mice. *Commun. Biol.* **2024**, *7*, 136. [CrossRef]
29. Tian, X.; Zeng, Y.; Tu, Q.; Jiao, Y.; Yao, S.; Chen, Y.; Sun, L.; Xia, Q.; Luo, Y.; Yuan, L.; et al. Butyrate alleviates renal fibrosis in CKD by regulating NLRP3-mediated pyroptosis via the STING/NF-κB/p65 pathway. *Int. Immunopharmacol.* **2023**, *124*, 111010. [CrossRef]
30. Su, X.; Zhao, Y.; Li, Y.; Ma, S.; Wang, Z. Gut dysbiosis is associated with primary hypothyroidism with interaction on gut-thyroid axis. *Clin. Sci.* **2020**, *134*, 1521–1535. [CrossRef]
31. Mendoza-León, M.J.; Mangalam, A.K.; Regaldiz, A.; González-Madrid, E.; Rangel-Ramírez, M.A.; Álvarez-Mardonez, O.; Vallejos, O.P.; Méndez, C.; Bueno, S.M.; Melo-González, F.; et al. Gut microbiota short-chain fatty acids and their impact on the host thyroid function and diseases. *Front. Endocrinol.* **2023**, *14*, 1192216. [CrossRef] [PubMed]
32. Li, Y.; Luo, B.; Tong, B.; Xie, Z.; Cao, J.; Bai, X.; Peng, Y.; Wu, Y.; Wang, W.; Qi, X. The role and molecular mechanism of gut microbiota in Graves' orbitopathy. *J. Endocrinol. Investig.* **2023**, *46*, 305–317. [CrossRef] [PubMed]
33. Shi, L.; Guo, M.; Shi, C.; Gao, G.; Xu, X.; Zhang, C.; Fu, J.; Ni, Y.; Wang, F.; Xue, X.; et al. Distinguishing benign and malignant thyroid nodules using plasma trimethylamine *N*-oxide, carnitine, choline and betaine. *J. Cancer Res. Clin. Oncol.* **2024**, *150*, 142. [CrossRef] [PubMed]
34. Wang, Z.; Yang, Y.; Xing, Y.; Si, D.; Wang, S.; Lin, J.; Li, C.; Zhang, J.; Yin, D. Combined metabolomic and lipidomic analysis uncovers metabolic profile and biomarkers for papillary thyroid carcinoma. *Sci. Rep.* **2023**, *13*, 17666. [CrossRef] [PubMed]
35. Greenland, S. An introduction To instrumental variables for epidemiologists. *Int. J. Epidemiol.* **2000**, *29*, 1102. [CrossRef]
36. Skrivankova, V.W.; Richmond, R.C.; Woolf, B.A.R.; Davies, N.M.; Swanson, S.A.; VanderWeele, T.J.; Timpson, N.J.; Higgins, J.P.T.; Dimou, N.; Langenberg, C.; et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomisation (STROBE-MR): Explanation and elaboration. *BMJ Clin. Res. Ed.* **2021**, *375*, n2233. [CrossRef]
37. Boef, A.G.; Dekkers, O.M.; le Cessie, S. Mendelian randomization studies: A review of the approaches used and the quality of reporting. *Int. J. Epidemiol.* **2015**, *44*, 496–511. [CrossRef]

38. Su, X.; Yin, X.; Liu, Y.; Yan, X.; Zhang, S.; Wang, X.; Lin, Z.; Zhou, X.; Gao, J.; Wang, Z.; et al. Gut Dysbiosis Contributes to the Imbalance of Treg and Th17 Cells in Graves' Disease Patients by Propionic Acid. *J. Clin. Endocrinol. Metab.* **2020**, *105*, 3526–3547. [CrossRef]
39. Ratajczak, W.; Rył, A.; Mizerski, A.; Walczakiewicz, K.; Sipak, O.; Laszczyńska, M. Immunomodulatory potential of gut microbiome-derived short-chain fatty acids (SCFAs). *Acta Biochim. Pol.* **2019**, *66*, 1–12. [CrossRef]
40. Velasquez, M.T.; Ramezani, A.; Manal, A.; Raj, D.S. Trimethylamine *N*-Oxide: The Good, the Bad and the Unknown. *Toxins* **2016**, *8*, 326. [CrossRef]
41. Arioz, B.I.; Tastan, B.; Tarakcioglu, E.; Tufekci, K.U.; Olcum, M.; Ersoy, N.; Bagriyanik, A.; Genc, K.; Genc, S. Melatonin Attenuates LPS-Induced Acute Depressive-Like Behaviors and Microglial NLRP3 Inflammasome Activation Through the SIRT1/Nrf2 Pathway. *Front. Immunol.* **2019**, *10*, 1511. [CrossRef] [PubMed]
42. Montgomery, T.L.; Eckstrom, K.; Lile, K.H.; Caldwell, S.; Heney, E.R.; Lahue, K.G.; D'Alessandro, A.; Wargo, M.J.; Krementsov, D.N. Lactobacillus reuteri tryptophan metabolism promotes host susceptibility to CNS autoimmunity. *Microbiome* **2022**, *10*, 198. [CrossRef] [PubMed]
43. Zheng, D.; Liao, H.; Chen, S.; Liu, X.; Mao, C.; Zhang, C.; Meng, M.; Wang, Z.; Wang, Y.; Jiang, Q.; et al. Elevated Levels of Circulating Biomarkers Related to Leaky Gut Syndrome and Bacterial Translocation Are Associated with Graves' Disease. *Front. Endocrinol.* **2021**, *12*, 796212. [CrossRef] [PubMed]
44. Weil, D.; Pais de Barros, J.P.; Mourey, G.; Laheurte, C.; Cypriani, B.; Badet, N.; Delabrousse, E.; Grandclément, E.; Di Martino, V.; Saas, P.; et al. Circulating levels of 3-hydroxymyristate, a direct quantification of endotoxaemia in noninfected cirrhotic patients. *Liver Int.* **2019**, *39*, 106–114. [CrossRef]
45. Kurilshikov, A.; Medina-Gomez, C.; Bacigalupe, R.; Radjabzadeh, D.; Wang, J.; Demirkan, A.; Le Roy, C.I.; Raygoza Garay, J.A.; Finnicum, C.T.; Liu, X.; et al. Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* **2021**, *53*, 156–165. [CrossRef]
46. Lopera-Maya, E.A.; Kurilshikov, A.; van der Graaf, A.; Hu, S.; Andreu-Sánchez, S.; Chen, L.; Vila, A.V.; Gacesa, R.; Sinha, T.; Collij, V.; et al. Effect of host genetics on the gut microbiome in 7,738 participants of the Dutch Microbiome Project. *Nat. Genet.* **2022**, *54*, 143–151. [CrossRef]
47. Kettunen, J.; Demirkan, A.; Würtz, P.; Draisma, H.H.; Haller, T.; Rawal, R.; Vaarhorst, A.; Kangas, A.J.; Lyytikäinen, L.P.; Pirinen, M.; et al. Genome-wide study for circulating metabolites identifies 62 loci and reveals novel systemic effects of LPA. *Nat. Commun.* **2016**, *7*, 11122. [CrossRef]
48. Rhee, E.P.; Surapaneni, A.; Zheng, Z.; Zhou, L.; Dutta, D.; Arking, D.E.; Zhang, J.; Duong, T.; Chatterjee, N.; Luo, S.; et al. Trans-ethnic genome-wide association study of blood metabolites in the Chronic Renal Insufficiency Cohort (CRIC) study. *Kidney Int.* **2022**, *101*, 814–823. [CrossRef]
49. Shin, S.Y.; Fauman, E.B.; Petersen, A.K.; Krumsiek, J.; Santos, R.; Huang, J.; Arnold, M.; Erte, I.; Forgetta, V.; Yang, T.P.; et al. An atlas of genetic influences on human blood metabolites. *Nat. Genet.* **2014**, *46*, 543–550. [CrossRef]
50. Chen, Y.; Lu, T.; Pettersson-Kymmer, U.; Stewart, I.D.; Butler-Laporte, G.; Nakanishi, T.; Cerani, A.; Liang, K.Y.H.; Yoshiji, S.; Willett, J.D.S.; et al. Genomic atlas of the plasma metabolome prioritizes metabolites implicated in human diseases. *Nat. Genet.* **2023**, *55*, 44–53. [CrossRef]
51. Sakaue, S.; Kanai, M.; Tanigawa, Y.; Karjalainen, J.; Kurki, M.; Koshiba, S.; Narita, A.; Konuma, T.; Yamamoto, K.; Akiyama, M.; et al. A cross-population atlas of genetic associations for 220 human phenotypes. *Nat. Genet.* **2021**, *53*, 1415–1424. [CrossRef] [PubMed]
52. Abecasis, G.R.; Altshuler, D.; Auton, A.; Brooks, L.D.; Durbin, R.M.; Gibbs, R.A.; Hurles, M.E.; McVean, G.A. A map of human genome variation from population-scale sequencing. *Nature* **2010**, *467*, 1061–1073. [CrossRef] [PubMed]
53. Pierce, B.L.; Ahsan, H.; Vanderweele, T.J. Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. *Int. J. Epidemiol.* **2011**, *40*, 740–752. [CrossRef] [PubMed]
54. Burgess, S.; Thompson, S.G. Interpreting findings from Mendelian randomization using the MR-Egger method. *Eur. J. Epidemiol.* **2017**, *32*, 377–389. [CrossRef] [PubMed]
55. Hartwig, F.P.; Davey Smith, G.; Bowden, J. Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. *Int. J. Epidemiol.* **2017**, *46*, 1985–1998. [CrossRef]
56. Verbanck, M.; Chen, C.Y.; Neale, B.; Do, R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* **2018**, *50*, 693–698. [CrossRef]
57. Hemani, G.; Tilling, K.; Davey Smith, G. Orienting the causal relationship between imprecisely measured traits using GWAS summary data. *PLoS Genet.* **2017**, *13*, e1007081. [CrossRef]
58. Vujkovic-Cvijin, I.; Sklar, J.; Jiang, L.; Natarajan, L.; Knight, R.; Belkaid, Y. Host variables confound gut microbiota studies of human disease. *Nature* **2020**, *587*, 448–454. [CrossRef]
59. Storey, J.D.; Tibshirani, R. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9440–9445. [CrossRef]
60. Patel, A.; Ye, T.; Xue, H.; Lin, Z.; Xu, S.; Woolf, B.; Mason, A.M.; Burgess, S. MendelianRandomization v0.9.0: Updates to an R package for performing Mendelian randomization analyses using summarized data. *Wellcome Open Res.* **2023**, *8*, 449. [CrossRef]
61. Lee, H.J.; Li, C.W.; Hammerstad, S.S.; Stefan, M.; Tomer, Y. Immunogenetics of autoimmune thyroid diseases: A comprehensive review. *J. Autoimmun.* **2015**, *64*, 82–90. [CrossRef] [PubMed]

62. Banga, J.P.; Schott, M. Autoimmune Thyroid Diseases. *Horm. Metab. Res.* **2018**, *50*, 837–839. [CrossRef] [PubMed]
63. Li, G.H.; Tang, C.M.; Cheung, C.L. COVID-19 and Thyroid Function: A Bi-Directional Two-Sample Mendelian Randomization Study. *Thyroid. Off. J. Am. Thyroid. Assoc.* **2022**, *32*, 1037–1050. [CrossRef] [PubMed]
64. Agus, A.; Planchais, J.; Sokol, H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. *Cell Host Microbe* **2018**, *23*, 716–724. [CrossRef] [PubMed]
65. Gutiérrez-Vázquez, C.; Quintana, F.J. Regulation of the Immune Response by the Aryl Hydrocarbon Receptor. *Immunity* **2018**, *48*, 19–33. [CrossRef]
66. Vogel, C.F.; Sciullo, E.; Li, W.; Wong, P.; Lazennec, G.; Matsumura, F. RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol. Endocrinol.* **2007**, *21*, 2941–2955. [CrossRef]
67. Vogel, C.F.; Matsumura, F. A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochem. Pharmacol.* **2009**, *77*, 734–745. [CrossRef]
68. Arora, P.K.; Bae, H. Identification of new metabolites of bacterial transformation of indole by gas chromatography-mass spectrometry and high performance liquid chromatography. *Int. J. Anal. Chem.* **2014**, *2014*, 239641. [CrossRef]
69. Kiseleva, E.P.; Mikhailopulo, K.I.; Sviridov, O.V.; Novik, G.I.; Knirel, Y.A.; Szwajcer Dey, E. The role of components of Bifidobacterium and Lactobacillus in pathogenesis and serologic diagnosis of autoimmune thyroid diseases. *Benef. Microbes* **2011**, *2*, 139–154. [CrossRef]
70. Wang, Z.; Zhang, Q.; Lu, J.; Jiang, F.; Zhang, H.; Gao, L.; Zhao, J. Identification of outer membrane porin f protein of Yersinia enterocolitica recognized by antithyrotopin receptor antibodies in Graves' disease and determination of its epitope using mass spectrometry and bioinformatics tools. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 4012–4020. [CrossRef]
71. Burgess, S.; Dudbridge, F.; Thompson, S.G. Combining information on multiple instrumental variables in Mendelian randomization: Comparison of allele score and summarized data methods. *Stat. Med.* **2016**, *35*, 1880–1906. [CrossRef] [PubMed]
72. Hemani, G.; Zheng, J.; Elsworth, B.; Wade, K.H.; Haberland, V.; Baird, D.; Laurin, C.; Burgess, S.; Bowden, J.; Langdon, R.; et al. The MR-Base platform supports systematic causal inference across the human phenome. *Elife* **2018**, *7*, e34408. [CrossRef] [PubMed]
73. Skrivankova, V.W.; Richmond, R.C.; Woolf, B.A.R.; Yarmolinsky, J.; Davies, N.M.; Swanson, S.A.; VanderWeele, T.J.; Higgins, J.P.T.; Timpson, N.J.; Dimou, N.; et al. Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) Statement. *JAMA* **2021**, *326*, 1614–1621. [CrossRef] [PubMed]

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