Chapter

Natural Compounds in the Modulation of the Intestinal Microbiota: Implications in Human Physiology and Pathology

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Abstract

Clinical interest in the human gut microbiota has increased considerably, because of the increasing number of studies linking the human intestinal microbiota and microbiome to an ever increasing number of non-communicable diseases. Many attempts at modulating the gut microbiota have been made using probiotics and prebiotics. However, there are other avenues that are still little explored from a clinical point of view that appear promising to obtain modifications of the microbial ecology and biological activities connected to the microbiome. This chapter summarizes all *in vitro*, *in vivo* and clinical studies demonstrating the possibility to positively modulate the intestinal microbiota by using probiotics, foods (and prebiotics), essential oils, fungus and officinal plants. For the future, clinical studies investigating the ability to modify the intestinal microbiota especially by using foods, officinal and aromatic plants or their extracts are required. More knowledge in this field is likely to be of clinical benefit since modulation of the microbiome might support the therapy of most non-communicable diseases in the future.

Keywords: microbiota, microbiome, probiotics, essential oils, phytotherapy

1. The pivotal role of gut microorganisms in human health

Our knowledge of the relationship between human beings and the microorganisms we harbor in our gut has greatly increased in the past years, even if we are still far from having understood all their functions. We no longer consider these living entities as simply commensal, and we start to realize that humans are "super organisms" governed also by the microorganisms living inside us. There are approximately 100 trillion cells in the human body, and more than 90% of them are microbes. They make up the human microbiota, consisting of bacteria, fungi and even viruses, mainly located in the intestine where they are referred to as the intestinal microbiota.

The terms currently employed in this field are the following:

Microbiota, which refers to the communities of living microorganisms residing in a defined ecological niche.

Microbiome, which indicates the whole of the microorganisms, *i.e.* their genes (genome), their proteins (proteome) and their metabolites (metabolome) (even if the terms microbiota and microbiome are often used interchangeably).

Metagenomics, which is the analysis usually performed by next-generation sequencing techniques, of the genetic material of microorganisms obtained from a sample of the environment that is being studied, such as for example feces for the profiling of the fecal microbiota.

Dysbiosis, which is an alteration in the microbiota structure (as opposed to *eubiosis*), with negative implications for microbial metabolism and host physiology.

The first consideration that we have to do is that the microbial ecosystem of the intestine called gut microbiota, is one of the most dense communities that we know, surpassing for complexity those present in soil, subsoil and also oceans [1].

The second consideration is that the microbiota does not represent an inheritance dependent on our species or genes, but rather an environmental inheritance, mainly due to the type of environment to which we have been exposed in the first 3–4 years of our life [2]. This also implies that we can act during life with the aim of improving our microbiota (**Figure 1**).

The last one is that our gut microbiota and microbiome are strictly connected with our state of health or illness and, together with genetics and environment, certainly represent a discriminating point in predisposing us to the onset of some particular diseases rather than that of others. The gut microbiota is closely related to our metabolic balance as well as to the development and functioning of our immune system, as studies on germ-free animals have clearly shown. It is also closely connected with the intestinal and systemic endocrine system, and indirectly with the central nervous system, via the enteric nervous system, within what is commonly called the gut-brain axis [3].

These considerations must not make us think of the microbiota and microbiome as something fixed and stable in the course of our life. The aging of our organism physiologically leads to a change in the gut microbiota with a decrease in some specific populations, such as the short-chain fatty acid (SCFA)-producing families *Lachnospiraceae* and *Ruminococcaceae*, and an increase in potential or opportunistic pathogens, as enterobacteria. Aging is also accompanied by an increase in low-grade, chronic inflammation. This so-called "Inflammaging" impacts gut integrity and can be causally linked to age-related changes in the microbiota [4]. Inflammation and dysbiosis are always closely connected to each other and inevitably end up entering

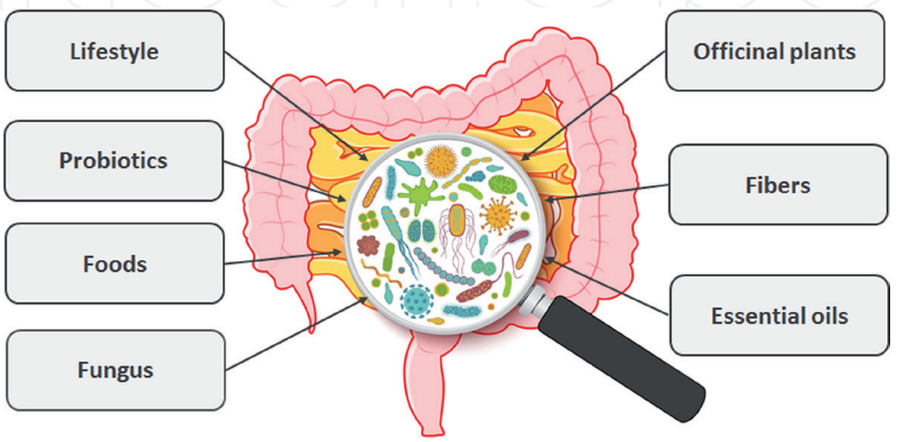

Figure 1.
The microbiota present in human gut is strictly connected with the whole organism state of health or illness. Major factors capable of modulating the gut microbiota in adults are represented in this picture.

into loops of which it is difficult or impossible to establish which of the two is the cause and which the consequence. Aging is also accompanied by increased intestinal permeability, impaired digestion, and disrupted nutrient absorption, each of which exhibits bidirectional interactions with the gut microbiome [5].

In addition to the physiological and irreversible increase in our biological age, there are other conditions that have a decisive impact on the composition and function of the intestinal microbiota. The first for importance and for the daily life with which it is implemented, is certainly our diet, which can cause, as we will see in the next paragraph, positive or negative changes in the microbiota. Another, often overlooked, condition is our lifestyle. Smoking and alcohol, for example, can negatively alter the microbiome [6], while regular physical activity seems to be capable of significantly improving it [7].

Finally, as we will analyze in the following paragraphs, there are many different pathologies, and consequent therapies, that can alter our intestinal microbiota, sometimes irreversibly. The most illuminating example concerns the transmissible pathologies of bacterial origin, encountered at an early age. The antibiotic therapies that often become necessary can, in the first 3 years of life, irreversibly alter the developmental trajectory of the intestinal microbiota leading, in the adult age, to a microbiota substantially different from that which would have developed in the absence of broad-spectrum antibiotic therapies [8]. On the contrary, antibiotic therapy in adults only reversibly alters the intestinal microbiota, which returns exactly to the starting point after the end of the therapy [9] Other intestinal pathogens, such as *Campylobacter jejuni*, can cause dysbiosis that persists even after the infection has been resolved, as it happens for example in the case of patients who develop post-infectious Irritable Bowel Syndrome (IBS) after enteritis caused by this bacterium [10].

However, we must not think that the pathologies correlated to alterations of the microbiota are essentially limited to the gastro-intestinal or metabolic ones. In recent years, many studies have linked alterations in the gut microbiome with a plethora of various diseases, including the neurodegenerative ones, such as Alzheimer's or Parkinson's [11]. Despite our limited mechanistic understanding of how the microbiota can predispose to neurodegenerative diseases, efforts to manipulate the microbiota through fecal microbiota transplantation, probiotic treatment, or other nutritional strategies, highlight the potential for microbial improvement in successfully preventing or decreasing the symptoms of these diseases, at least in laboratory animals [12]. It is therefore not surprising that some studies today are explicitly aimed at microbiome-targeted interventions for the prevention or treatment of neurodegenerative diseases.

To conclude this paragraph of premises, we can state that while conventional medicine aimed at maximum specialization, with branches such as organ and cellular medicine, on the other side of the pond the role of the intestinal microbiota has gradually assumed more and more importance, to remind us that our "super organism" is unique and that alterations of our gut microbial component, that is not even part of our cellular pool, can have a broad-spectrum negative impact on many if not all the organs and apparatuses that make up our organism. The microbiota well represents the complex relationships that exist between our health and the environment in which we are born and spend the first years of our life. A compromised environment, due to excessive sterilization or pollution, certainly has a strong impact on the structure of our microbiota in adulthood and, consequently, also on our state of health and well-being. Although fecal microbiota transplantation has opened new frontiers on the prevention and treatment of many pathologies, it is indisputably true that this community of microorganisms represents a central node in the functioning of all our organs and systems, and at the same time

it denotes a fundamental point of interaction between us and the environment in which we spend our lives.

2. Intestinal dysbiosis, immune system and related human pathologies

Intestinal dysbiosis is mainly characterized by lower bacterial diversity and it is often associated with an increase in bacterial species with pathogenic potential (*i.e.*, pathobionts) to the detriment of health-associated symbiotic commensals. Dysbiosis has been associated with several human pathologies, as demonstrated by several preclinical and clinical studies (**Figure 2**). Despite this evidence, it is almost never clear whether dysbiosis is a consequence of these diseases or if it is directly involved in their pathogenesis. Nevertheless, it is widely accepted that an imbalance of the intestinal microbiota may impact on diseases development and their clinical outcome. A consequence of intestinal dysbiosis is the loss of the barrier effect, followed by an impairment of the gut-associated immune system function. Mucosal barrier disruption leads to the release of pathogen-associated molecular patterns (PAMPs), triggers epithelial release of damage-associated molecular patterns (DAMPs), and finally causes the release of bacterial lipopolysaccharides (LPS) into the systemic circulation (metabolic endotoxaemia) [13]. The consequent activation of inflammatory pathways links intestinal dysbiosis to chronic inflammatory pathologies, autoimmune disorders and also cancer.

Moreover, together with the dysbiosis-related inflammation, the depletion of specific bacterial taxa involved in endocrine signaling may directly affect the function of different organs, and for these reasons dysbiosis has also been linked to metabolic, endocrine (e.g. thyroid-related) and also psychiatric disorders [14].

2.1 Dysbiosis in gastrointestinal disorders

A marked dysbiosis has been found to be associated with the main intestinal disorders, such as Inflammatory Bowel Diseases (IBD), Irritable Bowel Syndrome (IBS) and coeliac disease (CD). IBD are chronic inflammatory disorders characterized by the chronic activation of the immune system with an unbalanced production of inflammatory cytokines. Despite the pathogenesis of these diseases is unclear, there is evidence that, other than genetic and environmental factors,

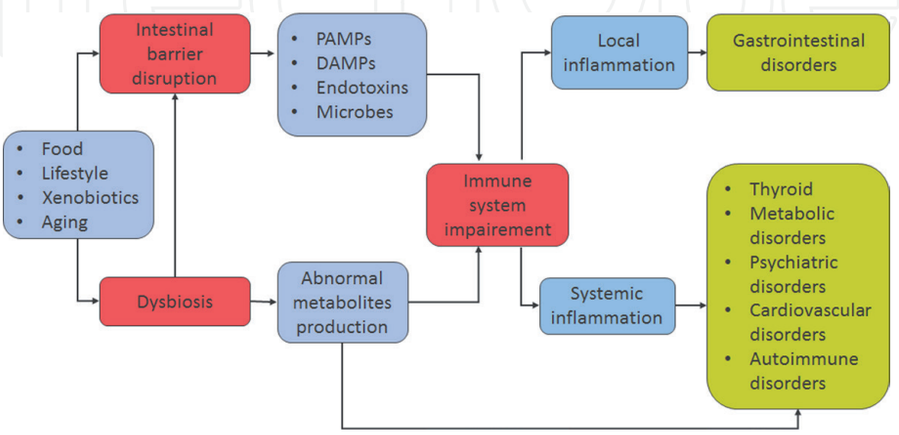

Figure 2.
Food, lifestyle, xenobiotics and aging are the main causes that can lead to dysbiosis and consequently to an alteration of the intestinal barrier function. These two conditions are linked immune system impairment and to the possible onset of many pathologies. PAMPs, pathogen-associated molecular patterns; DAMPs damage-associated molecular patterns (DAMPs).

an abnormal immune response against the microbial component of the gut may be involved in inflammation development and maintenance. It has been supposed that dysbiosis could trigger an aberrant activation of immune system in IBD patients, resulting in an unbalanced inflammatory cytokine production. In particular, compared to controls, the anti-inflammatory butyrate-producing species *Faecalibacterium prausnitzii* has been found to be reduced in both Crohn's disease and ulcerative colitis patients, with the latter also showing an increase in *Clostridium perfringens* and a decrease of *Eubacterium rectale*. Overall, members of the Proteobacteria phylum, such as *Enterobacteriaceae*, including *Escherichia coli*, are increased in patients with IBD compared to healthy individuals [15]. Also pouchitis, an inflammation involving the transition tissue (pouch) in patients with IBD who underwent proctocolectomy, is characterized by severe chronic dysbiosis [16, 17]. The involvement of the gut microbial component in these disorders is also supported by the efficacy of antibiotic therapy and probiotics, which are often used in order to manage inflammatory flares, especially in pouchitis, despite specific bacterial pathogens have never been found in these patients [18].

IBS is characterized by recurrent abdominal pain associated with a change in the bowel habits. IBS patients are divided into four subtypes: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), mixed diarrhea and constipation (IBS-M), and patients with non classifiable IBS symptoms (IBS-U) [19]. These patients are characterized by a lower microbial diversity compared to the healthy population, and also by increased proportions of Proteobacteria and Firmicutes members, such as *Veillonella*, *Lactobacillus* and *Ruminococcus*, and decreased relative abundance of *Bifidobacterium*, *Faecalibacterium*, *Erysipelotrichaceae*, and methanogens. Despite the etiology of this disease is unknown, the role of the gut microbiota component is supported by experiments on animals with induced dysbiosis, which showed abnormal intestinal behaviors similar to those typical of IBS patients [20]. Moreover, a number of studies have shown some improvement in IBS symptoms with antibiotic therapy, including rifaximin [21].

Coeliac disease (CD) is a well-characterized gut autoimmune disorder triggered by the interaction between the gut-associated lymphoid immune system and the undigested gluten peptides that translocate through the epithelial barrier into the lamina propria. About 30% of the world population is genetically predisposed to develop CD, but only a small amount (about 1% in developed countries) develops the disease, so a multifactorial etiology is supposed for this disorder. CD patient microbiota is characterized by an increased relative abundance of *Bacteroides*, *Prevotella* and *Escherichia*, and reduced amounts of bifidobacteria and lactobacilli. It has been supposed that this dysbiotic profile may contribute to the disease development by influencing the gluten peptide digestion, by stimulating dendritic cells and Treg lymphocytes and also by increasing intestinal permeability [22].

2.2 Dysbiosis in thyroid and autoimmune disorders

There is rising evidence that the intestinal microbiota compositional structure may impact on thyroid function, since microbial components can regulate iodine, selenium, iron and zinc uptake, and also enterohepatic cycling of thyroid hormones. Moreover, the microbiota may also impact on the bioavailability and metabolism of L-thyroxine and the anti-hyperthyroid drug propylthiouracil (PTU) [23]. The gut microbiota influences the synthesis of neurotransmitters, such as dopamine, which can inhibit thyroid-stimulating hormone (TSH) and modulate hypothalamus-pituary axis. It is therefore reasonable to affirm that intestinal dysbiosis may contribute to the abnormal immune activation in Hashimoto's thyroiditis (HT) [24] but also

in Grave's disease (GD), which is the second leading autoimmune thyroid disease. Studies on animals showed that microbiota transplant may increase the susceptibility to HT in rats. A proposed mechanism of action, is that *Lactobacillus* spp. and *Bifidobacterium* spp. may affect the synthesis of antibodies cross-reacting with thyreoperoxidase and thyroglobulin [25]. Notably, it has also been supposed that dysbiosis in HT patients may affect Treg cells modulation, a common feature shared with CD, which is often associated with thyroid disorders [26].

HT and GD evolve, respectively, in hypothyroidism and hyperthyroidism, with two distinct immunological patterns. HT is characterized by antibodies against thyreoperoxidase and thyroglobulin while GD is characterized by the presence of antibodies against TSH receptor. Nevertheless, in both disorders, anti-gliadin, anti-transglutaminase and anti-*Saccharomyces cerevisiae* antibodies have been detected, and both disorders are characterized by intestinal dysbiosis. A study conducted on 28 HT patients and 16 healthy controls showed an increase in the proportions of *Blautia*, *Roseburia*, *Ruminococcus*, *Romboutsia*, *Dorea*, *Fusicatenibacter* and *Eubacterium* group genera, and a decrease in *Fecalibacterium*, *Bacteroides*, *Prevotella* and *Lachnoclostridium* in HT [24]. Another study on 27 GD patients showed an increase of *Prevotellaceae* and *Pasteurellaceae* and a decreased amount of *Enterobacteriaceae*, *Veillonellaceae* and *Rikenellaceae* compared to healthy subjects [27].

2.3 Dysbiosis in metabolic disorders

Obesity, type-2 diabetes, metabolic syndrome and nonalcoholic fatty liver disease (NAFLD) are all metabolic disorders that manifest in comorbidity, and lead to an exacerbation of atherosclerosis and cardiovascular diseases [28]. These disorders are characterized by different microbial signatures, which may contribute to their chronicization. The intestinal microbiota has an active role in regulating host metabolism, indeed experiments on mice showed that conventionally raised mice had more total body fat than mice raised in germ-free condition, and that a fecal transplant in these mice was able to restore nutrient adsorption, metabolic function and body fat [29].

In obese subjects, a lower bacterial richness was detected, along with a predominance of "pro-inflammatory" taxa, such as *Ruminococcus gnavus* and *Bacteroides*, over the "anti-inflammatory" species *F. prausnitzii* [30]. Microbiome analysis in type 2 diabetes patients showed an altered pattern enriched in membrane transport of sugars and branched-chain amino acid transport, while depleted in butyrate synthesis, with a decrease of *Roseburia intestinalis* and *F. prausnitzii* [31]. Moreover, fecal transplant from lean donors to patients with metabolic syndrome showed to ameliorate their insulin resistance condition [32]. There is also evidence that overgrowth of SCFA-producing bacteria is directly correlated to an improvement of glycemic control, through regulation of glucagon-like peptide 1 [33]. In NAFLD patients, a microbial signature characterized by higher relative abundance of proteobacteria was detected, moreover there is a correlation between the microbiota composition and the degree of liver fibrosis. Patients with an advanced liver fibrosis showed a further increase of proteobacteria, particularly *E. coli*, and a decrease of Firmicutes [34].

Intestinal dysbiosis has also been found in subjects with a high risk for cardiovascular diseases compared to subjects with low risk. In particular, some bacterial genera, such as *Prevotella* and *Klebsiella*, seem to correlate with hypertension [35] Fecal transplantation from patients with overrepresentation of these two genera to germ-free mice led to an increase of blood pressure in experimental animals [36].

2.4 Dysbiosis in cancer

Intestinal microbiota disruption has been linked to the development of cancer, and different specific strains have been linked to the development of different tumors. In colorectal cancer (CRC) a particular strain of *Fusobacterium nucleatum* seems to be involved in CRC initiation and progression by activating different pathways leading to a rise of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α, and to the development of an immunosuppressive microenvironment and also to the induction of chemoresistance to 5-fluorouracil [37].

In hepatocellular cancer, the translocation of gut microbiota and its products via the portal vein seems to be a condition able to trigger inflammation and chronic liver disease that predisposes patients to the development of cancer [38].

Leukemia patients showed a marked dysbiosis. In acute lymphoblastic leukemia (ALL) patients, a lower microbial diversity has been found, along with an enrichment in *Enterococcaceae*, *Porphyromonadaceae* and other Bacteroidetes members, and a depletion in *Blautia*, Erysipelotrichales, *Lachnospiraceae* and Clostridiales members. In acute myeloid leukemia (AML), the abundance of *Staphylococcaceae* and *Streptococcaceae* represents a typical signature [39]. In lung cancer, a dysbiosis characterized by increased relative abundance in *Enterococcus*, *Bacteroides* and *Fusobacterium*, and a depletion in *Bifidobacterium* and other Actinobacteria components, *Dialister*, *Enterobacter*, *Escherichia-Shigella*, *Fecalibacterium* and *Kluyvera* has been detected [40].

In non-small cell lung cancer (NSCLC) patients, a depletion of butyrate producers such as *F. prausnitzii*, *Clostridium leptum*, Clostridial cluster I, *Ruminococcus* spp., Clostridial cluster XIVa, and *Roseburia* spp., has been described [41, 42].

2.5 Psychiatric disorders

There is evidence that psychiatric disorders such as schizophrenia (SCZ), autism spectrum disorders, mood disorders, and anxiety are linked to gut inflammation and that inflammatory status could be sustained by gut microbiota eubiosis breakdown [43]. Epidemiological studies link autoimmune and atopic disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and ankylosing spondylitis (AS) to affective, personality, and neurotic disorders [44].

A study conducted on Danish population demonstrated that individuals with SCZ have a 50% lifetime prevalence of autoimmune disorders. On the other hand, given a history of autoimmune disorders, the relative risk for SCZ increased by 45% [45].

An association between SCZ and RA, autoimmune thyroiditis, type 1 diabetes mellitus (T1DM), SLE, Guillain-Barre´ syndrome, psoriasis, multiple sclerosis (MS) and autoimmune hepatitis has been described [46]. Interestingly, all these diseases have been associated with CD and non–celiac gluten sensitivity, with a higher prevalence of immunological markers of CD among these patients [47].

Clinical and animal preclinical studies support the relationship between gut inflammation and mental disorders. Indeed, high levels of pro-inflammatory circulating cytokines such as IL-1b, IL-6, and TNF-α, have been found in patients suffering from SCZ. Moreover, immunomodulatory drugs have been used to effectively treat psychosis [43]. In patients with a high risk of psychosis, Clostridiales, Lactobacillales and Bacteroidales were found to be significantly higher than in healthy controls [48].

It has been hypnotized that the excessive rise of SCFA synthesis could be one of the causes of microglia activation. Studies on SCZ patients showed heterogeneous

results on the microbiota dysbiosis so, despite such a dysbiosis was always confirmed in these patients, it is difficult to link specific taxa to this disorder [43]. Anyway, fecal transplantation from SCZ patients to germ-free mice resulted in the development of SCZ-like behaviors in receiving mice, providing final evidence of the gut microbiota involvement in SCZ. An unbalanced microbiota was also detected in bipolar disorders and autism spectrum disorders, to underline that our gut microbiome may contribute, probably with varying importance, to most mental and stress-related disorders [43].

2.6 Neurodegenerative disorders

The implication of gut microbiota in neurodegenerative disorders has been widely investigated. Several clinical studies in Parkinson's disease (PD) patients showed modifications in the gut microbiota, characterized by a rise in the relative abundance of *Bifidobacterium*, *Lactobacillus* and *Verrucomicrobiaceae*, and a decrease in *Blautia*, *Coprococcus* and *Prevotellaceae* [49]. Interestingly, microbiota modifications are stable after the disease onset and some of these changes correlate with alterations in microbial metabolism of tryptophan and beta-glucuronide [50]. In addition to altered microbial metabolism, intestinal dysbiosis could be involved in PD development through immune-mediate pathways, since there is evidence that links GI inflammation to PD, maybe since inflammation may enhance alpha-synuclein aggregation [49].

For what concerns Alzheimer's disease (AD), animal experiments on mice with induced dysbiosis and on germ-free mice showed that microbiota manipulation can impact on disease severity and cognitive impairments. LPS seems to be involved in fibrillogenesis of β-Amiloid (Aβ), and some bacterial species, such as *E. coli*, *Bacillus subtilis*, *Salmonella* Typhimurium, *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* may generate functional amyloid, contributing to the pathogenesis of AD through the accumulation of proteinaceous misfolded Aβ oligomers and fibrils [51].

3. Probiotics for the modulation of human microbiota: Effectiveness and limits

Since the second half of the 19th century, with Metchnikoff's studies on the possibility of using lactic acid bacteria to decelerate the process of self-intoxication and infection by intestinal microbes [52] probiotics have been recognized as a tool to modulate the gut microbiota while conferring benefits to health. Their economic value was recognized shortly thereafter, and their global market is estimated to reach USD 69.3 billion by 2023 [https://www.marketsandmarkets.com/PressReleases/probiotics.asp]. Nowadays, probiotics represent one of the most commonly consumed food supplements worldwide, being present in yogurt, cheese, ice cream, snacks and nutritional bars, breakfast cereals, infant formulas and more recently also added to cosmetic products. They are also marketed as lyophilized pills, and their consumption is widely supported by physicians, particularly gastroenterologists [53]. The administration of probiotics is indeed a more than feasible approach in clinical practice, compared for example to diet, despite its recognized role as a pivotal determinant of the structure and function of the gut microbiota, able to support homeostasis or *vice versa* to contribute to the susceptibility to disease [54], due to the sometimes modest effects of nutritional interventions and the difficult of enforcing and monitoring patient compliance. The mainstay of commercial supply is represented by *Lactobacillus* and *Bifidobacterium* species, along with *E.*

coli Nissle 1917, *Streptococcus thermophilus* and *Saccharomyces boulardii*, all with a long history of use, having Generally Recognized as Safe (GRAS) status in the US or being granted Qualified Presumption of Safety status by the European Food Safety Authority (EFSA). Being sourced from the gut or traditional fermented foods, they have been selected, in large part, for their technological properties, *i.e.* the ability to survive processing and to retain viability during the shelf-life of the product.

According to the International Scientific Association for Probiotics and Prebiotics consensus meeting in October 2013 [55], the framework "probiotics" must include microbial species that have been shown in properly controlled studies to confer health benefits. Probiotics are also new commensals and consortia that include defined strains from human samples, for which adequate evidence of safety and efficacy exists. On the other hand, live cultures, traditionally associated with fermented foods (with no evidence of health benefits), and undefined, fecal microbiota transplants must be kept outside this framework.

Probiotics may have several effects on the host, including certainly the modulation of the gut microbiota but also the metabolism of lactose with improved digestion or bile salts with various systemic effects, vitamin synthesis, direct and indirect pathogen antagonism, regulation of intestinal transit and alleviation of visceral pain, strengthening of the gut barrier, production of specific bioactives and neurological, immunological and endocrinological effects. As expected, some underlying mechanisms are observed across taxonomic groups, such as the inhibition of potential enteropathogens or the production of useful metabolites or enzymes, while others, especially those at the extra-intestinal level, are more likely to be strain specific. These effects can be contact-dependent and/or mediated by surface molecules, e.g. lipoteichoic acid, peptidoglycan, cell surface proteins, exopolysaccharide, pili or other appendages, or by secreted molecules, e.g. SCFAs and bacteriocins [56]. In light of this, it is not surprising that paraprobiotics and postbiotics have recently been proposed as an alternative with a longer shelf-life and enhanced safety, especially for compromised individuals, with the former being non-viable (intact or broken) microbial cells or crude cell extracts [57] and the latter microbial cell constituents and metabolites, which act as bioactive compounds with local and systemic effects [58].

With specific regard to the gut microbiota, probiotics may impact resident communities through at least three different mechanisms: trophic interactions (*i.e.* by stimulating growth through the supply of metabolites such as lactate, acetate or propionate, growth factors such as vitamins or exopolysaccharide, or other substrates), a direct alteration of fitness, through a decrease in pH, niche competition or bacteriocin production, or an indirect one via host, through changes in the gut environment (*i.e.* by stimulating the production of mucins, increasing the levels of secretory IgAs and inducing the secretion of defensins, which represent the first line of defense of the intestinal epithelium against microbial invasion) [59]. As expected, while these effects may be relevant in the context of dysbiosis, *i.e.* when the blooming of potential opportunistic pathogens and/or the depletion of health-associated (mainly SCFA-producing, oxidative stress-sensitive) taxa occur, there is no convincing evidence of consistent effects of probiotics on the gut microbiota of healthy subjects, *i.e.* on an eubiotic and resilient microbial ecosystem [60].

Among the main (although sometimes only suggested) prophylactic and therapeutic indications and claims of probiotics, we can certainly mention gastrointestinal diseases, including the prevention or treatment of acute, antibiotic-associated and *Clostridium difficile*–associated diarrhea, and the amelioration of IBD and IBS [56]. Recent reviews have also cautiously suggested a beneficial role of probiotics in preterm infants, especially in terms of preventing necrotizing enterocolitis and reducing the risk of late-onset sepsis [61]. Bearing in mind that probiotics efficacy

is mostly strain-dependent and generalizations are highly inappropriate, moderate to strong evidence is also available for the eradication of *Helicobacter pylori* and the prevention of adverse reactions to its therapy or post-surgical infections [62]. Contrasting data have instead been reported in the context of many other disorders, including for example respiratory infections and metabolic syndrome [63]. This confusing situation may arise from the heterogeneity of probiotic agents, dosage, duration and mode of administration, but also from other issues related to study design and reporting of results (not always transparent, easy to assess and rigorous), to the population (e.g. demographic characteristics) and environmental variables (e.g. habitual diet).

In this regard, the awareness that one size does not fit all is rapidly gaining ground. It is now a fact that distinct baseline features of the host (e.g. age and underlying medical condition) and its microbiota (taxa represented and functions performed), including varying environmental exposure (mainly diet), can actually lead to differing outcomes even with the same probiotic preparation. As discussed recently, this could for example be due to the fact that the individual configuration of the gut microbiota may be permissive or resistant to even transient colonization of probiotics [64]. Moreover, it has been shown that probiotics could even perturb rather than aid in the recovery process of the gut microbiota after antibiotic treatment [9]. It is therefore now clear not only that their validity is not to be considered absolute but also that, if not tailored, probiotic-based interventions could not be entirely risk-free.

Future directions will be the adoption of a mechanism-based approach, in which probiotic strategies are designed *ad hoc*, taking into account a series of "precision" aspects related to the host and its microbiota, *i.e.* with careful consideration of the subject to be treated and the medical goal to be achieved. It is also expected that future human trials will overcome other current caveats in the probiotics field, by ensuring an adequate sample size (based on power analysis), clearly defining endpoints, accounting for placebo effects and reporting adverse events, while ensuring strain-level resolution [56].

Alongside traditional probiotics, it should be mentioned that novel candidate microorganisms with potential health benefits have been discovered thanks to recent research on the composition and function of the gut microbiota, deeply accelerated by massive sequencing. These microorganisms are referred to as next-generation probiotics or live biotherapeutics [65], as they fit well within the US Food and Drug Administration definition of live biotherapeutic as "a biological product that contains live organisms, such as bacteria, is applicable to the prevention, treatment or cure of a disease or condition of human being and is not a vaccine". Unlike currently used probiotics, they are generally strict anaerobes and therefore present a number of manufacturing challenges, and they should undergo a formal regulatory approval process similar to drugs or any other medical intervention. Among them, we can list SCFA producers, e.g. *F. prausnitzii*, proposed for the treatment of inflammatory bowel disease and other inflammation-based disorders [66], or the mucus degrader, *Akkermansia muciniphila*, identified as a promising candidate for the treatment of obesity and related complications [67]. Interestingly, a very recent proof-of-concept exploratory study has demonstrated that 3-month daily oral administration of live or pasteurized *A. muciniphila* to overweight/obese insulin-resistant volunteers was safe and well tolerated, and associated with numerous metabolic improvements [68].

Alternatively, it has been thought to engineer GRAS organisms or commensals as a delivery vehicle for bioactive molecules or to express certain functionality. In this approach, the bacterial vehicle is known not to produce any virulence factors, it will be tolerated by the host and, if chosen carefully, may not even colonize the host.

As an example, some researchers have used *Lactococcus lactis* strains (normally not considered probiotics but GRAS food-derived bacteria) as vehicle to deliver a range of anti-inflammatory molecules, e.g. elafin, a serine protease inhibitor, to reduce colitis-related inflammation [69], trefoil factor 1, for the treatment of oral mucositis [70] and IL-10 to control allergen sensitivity [71]. Other research groups have used the common intestinal bacterium *Bacteroides ovatus* to express IL-2 [72] or TGF-beta 1 [73], and *E. coli* Nissle 1917, which was engineered to bind to the surface of cancer cells and secrete myrosinase, to convert dietary glucosinolates into isothiocyanates, such as sulforaphane, a well-known anti-cancer compound [74].

However, in addition to the limitations discussed above, it should be emphasized that for most of these next-generation probiotic candidates, the available evidence is currently mostly preclinical, *in vitro* or on an animal model. Therefore, rigorously planned large-scale randomized controlled trials together with *in vivo* and *in vitro* experimentation are strongly needed for efficacy and long-term safety assessment, and data-driven explanation of the mechanisms of action.

In the future it is expected that overcoming all these challenges in the probiotics field will improve the state of evidence, regulation of use and, finally yet importantly, public awareness, for a precision, informed use. The current limitations in the field and future strategies to be undertaken to overcome them are summarized in **Figure 3**.

4. Foods and their prebiotic activities for the modulation of the gut microbiota

Food is a primordial need for our survival and well-being. However, diet is not only essential to maintain human growth, reproduction and health, but it also modulates and supports the symbiotic microbial communities that colonize the digestive tract, the gut microbiota. Type, quality and origin of our food shape our gut microbes and affect their composition and function, impacting on host–microbe interactions. Macronutrients (fat, protein, carbohydrate) and micronutrients (vitamins, minerals, polyphenols) directly interact with gut microbes and are involved in the production of key metabolites such as SCFAs and vitamins. Moreover, dietary fiber impacts on gut microbial ecology, host physiology, and health.

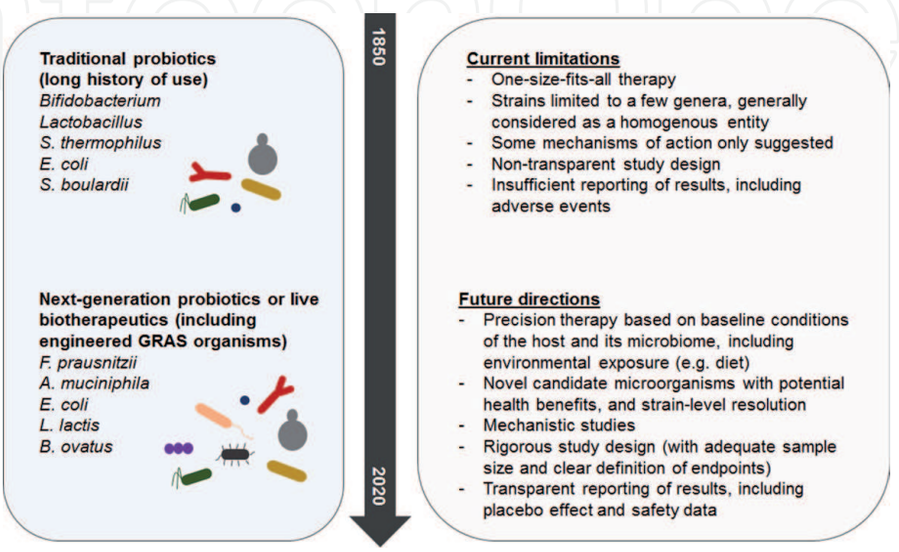

Figure 3.
From traditional to next-generation probiotics: Current limitations and future directions.

During or shortly after birth, the human gut is colonized by microbes. The fact that babies born spontaneously have higher bacterial counts in the gut at 1 month of age than those born by the cesarean section indicates that colonization of the gut by microbes starts and is improved during natural birth [75]. The growth and maintenance of a healthy gut microbiota is essential for the development of the immune system and continues during breastfeeding, a stage that seems essential to the individual's long-term health. Oligosaccharides found in breast milk encourage the growth of *Lactobacillus* and *Bifidobacterium*, which control the infant intestine, and this may improve or facilitate immune system development and help to prevent pathological conditions such as eczema and asthma. Functional maturation of the human microbiota, including the capacity to produce vitamins, increases during the early years of life [76].

A standard Western style diet offers about 50 g daily of potentially fermentable substrate, primarily dietary fiber, to the colonic microbiota. Non-starch polysaccharides are major components of dietary fiber and constitute 20–45% of the dry matter supplied to the colon. Simple sugars and oligosaccharides also account for another 10%, whereas starch (and starch hydrolysis products) supply less than 8% of dry matter. Some sugar alcohols also avoid the absorption of the small intestine and are minor dietary substrates for colonic microbiota [77]. Approximately 90% of dietary polyphenols (approximately 1 g/day) avoid digestion and absorption in the small bowel and can have a major effect on microbial composition and activities.

About 5–15 g of proteins and 5–10 g of lipids, mainly of dietary origin, pass daily through the proximal colon. Various other minor dietary constituents, including catechins, lignin, tannins and others, also nourish colonic microbes [78]. The action of all these macro and micronutrients is certainly synergistic and complex at the level of the intestinal microbiota, however in the following paragraphs we will analyze separately the effects of individual macro and micronutrients, trying not to lose the overall vision that is fundamental when it comes to microbial ecology.

4.1 Macronutrients and microbiota

Fats. The increase in dietary fats greatly changes the composition of the gut microbiota. Mice fed with high-fat diets (HFD, 40–80% of total caloric intake from fat) display phylum-level changes, with a decrease in Bacteroidetes and an increase in Firmicutes and Proteobacteria.

These changes have also been observed in weight gain-resistant mice, which implies a direct effect of dietary lipids on the microbiota. It has recently been found that microbes in the small intestine are highly susceptible to fat load and are essential for lipid digestion and absorption. These data suggest that the regional microbiota composition may have significant functional implications, and highlight the need for distinct microbiota and microbiome analysis along the gastrointestinal tract [79]. The lipid-mediated effects on the microbiota depend on the form and source of lipids. For example, mice fed with an isocaloric diet rich in long-chain saturated fats derived primarily from meat products showed greater insulin resistance and inflammation of the adipose tissue compared to mice fed with a high-fish oil diet. In addition, transgenic mice that constitutively generate n-3 polyunsaturated fatty acids (PUFAs) have higher phylogenetic diversity of the microbiome, which provides protection against the metabolic consequences of a high-saturated, high-sugar diet. One mechanism by which gut microbes can mediate the negative metabolic effects of high-fat intake could be by translocating LPS, a membrane toxin of gram-negative bacteria. An increase in circulating LPS after a high-fat meal has also been documented in humans, with amplified effects in obese people. Once in circulation, LPS induces a powerful inflammatory response by activating

Toll-like 4 receptor signaling, which has been involved in cardiovascular and metabolic disease development [80].

Inflammation appears to be the common denominator among the seemingly unrelated biological negative effects of fats on the gut microbiome, involving the immune system and n-3 PUFAs. It is currently accepted that inflammation plays a key role in the progression of several chronic diseases, such as atherosclerosis, inflammatory bowel disease, cancer, diabetes, and neurodegenerative syndromes [81]. Moreover, as described above, several evidence supports the role of n-3 PUFAs on the microbiota and on the regulation of inflammation and the immune system [82]. In addition, dietary n-3 PUFAs have been shown to reduce clinical colitis in IBD patients [83]. In clinical human studies, n-3 PUFA administration resulted in decreased Firmicutes/Bacteroidetes ratio, reduced relative abundance of Coprococcus and Facecalibacterium, and increased proportions of health-associated genera, i.e., Bifidobacterium, Lachnospira, Roseburia and Lactobacillus [84]. These data were consistent with those obtained in a subsequent study in which the authors also found a significant correlation between the plasma levels of n-3 PUFAs and the relative abundance of SCFA producers [85]. In addition, a diet supplemented with n-3 PUFAs has been able to prevent neuropsychiatric disorders and dysbiosis caused by social instability stress during adolescence, and these effects have been maintained through adulthood, supporting the concept that a healthy diet enriched in fish or n-3 PUFAs can have beneficial long-lasting effects and may help to prevent neuropsychiatric disorders [86]. Taken together, all these data allow us to hypothesize the existence of a strong link between n-3 PUFA intake, gut microbiome shaping and modulation of the immune system, with the ultimate objective of hampering the existing loop between bowel inflammation and gut dysbiosis.

In the fat dietary component, n-3 PUFAs can rightly be considered prebiotics. Therefore, the consumption of an n-3-rich diet is currently thought to be beneficial for microbiota health, even if the gut microbiome changes induced in humans by n-3 PUFA supplementation deserve further clinical investigations.

What we can conclude for the fat dietary component is that the lipid excess present in HFD diet is dangerous for the microbiota and, on the other hand, that a diet enriched in n-3 PUFAs protects the microbiota from possible alterations. However, n-3 PUFA sources, mainly fish, should not considered completely safe, considering the pollution of the sea and the growing presence of microplastics and xenobiotics in the trophic chain of marine animals. In particular, scientific data suggest that shellfish and other small marine organisms consumed with their intestine pose particular concern because they accumulate and retain microplastics. The biological effects of microplastics in human gut are poorly understood, but it has been supposed that in high amounts they could cause an alteration of the gut microbiome, with cascading effects on host physiology [87].

Proteins. That dietary proteins may affect the gut microbiota was first described in 1977. A pioneering study showed lower counts of *Bifidobacterium* and increased counts of *Bacteroides* and Clostridia in subjects eating a diet enriched in beef meat, compared with those eating a vegetarian diet [88]. With advances in metagenomics analysis, several studies have been able to investigate in depth the effects of dietary protein on the gut microbiota. These studies have evidenced a different effect depending on the protein source: animal or vegetarian. While the intake of animal meat proteins has been associated with a general worsening of the microbiota profile [89], vegetarian protein intake is overall positively associated with microbial diversity. For example, intake of pea protein extract has been reported to increase the proportions of the gut commensals *Bifidobacterium* and *Lactobacillus*. Pea protein intake was also observed to increase the levels of intestinal SCFAs, considered

to be important for several metabolic and immunological aspects, including the maintenance of the intestinal barrier. In contrast, counts of bile-tolerant anaerobes, such as *Bacteroides, Alistipes* and *Bilophila*, increase with animal-based protein intake [90]. Notably, different studies comparing high-animal protein diets and high-carbohydrate/fiber plant-based diets reported that the first dietary pattern can be effective for rapid weight loss but detrimental to microbiota health. In particular, the research showed that subjects following a high-protein/low-carbohydrate diet were depleted in *Roseburia* and *E. rectal*e in their gut microbiota with decreased butyrate levels in their feces [91]. Other studies confirmed decreased fecal SCFAs in Italian subjects eating a protein-rich diet. It has been proposed that high total protein intake, especially animal protein, could be associated with a significantly increased risk of IBD [89]. In addition, many microbial genera promoted by consumption of red meat have been related to increased levels of blood trimethylamine-N-oxide (TMAO), considered a pro-atherogenic marker of cardiovascular disease [92]. Finally, it is important to note that animal products-based diets are often high both in protein and fat, with potential synergistic negative effects on the human microbiota.

Carbohydrates. The effects of dietary carbohydrates on the gut microbiota are complex, since they can be classified based on three major components that are simple sugars, starches and fiber. Simple sugars such as sucrose, both alone and as part of a high-fat/high-sugar Western-style diet, can induce rapid remodeling of microbiota and metabolic dysfunction in laboratory animals and also in humans. Fibers should be considered as human indigestible carbohydrates. In a healthy microbiota, different bacterial genera possess fiber-degrading enzymes and thus use these indigestible carbohydrates as a primary source of energy. The term fibbers is widely used to classify such indigestible polymers, although this classification is problematic given that certain fibers are only partially degraded by intestinal microbes (such as cellulose), whereas other are readily fermentable (soluble fibers such as inulin). The metabolic effects of fiber are shown in **Figure 4**. Sonnenburg and his colleagues recently proposed the term 'microbiota-accessible carbohydrates' (MACs), to identify carbohydrates that are metabolically available to gut microbes [94]. MACs provide energy and a source of carbon for bacteria but also to the host. They can modify the microbiota structure by increasing the populations of fiber-degrading bacteria. This property of fibers warrants their additional classification as prebiotics, which by nature are non-digestible components of the diet that support selective growth of certain health-associated microbial populations, such as bifidobacteria. Examples of prebiotics include inulin, fructans, fructooligosaccharides, galactoligosaccharides, xyloligosaccharides and arabinoligosaccharides [95]. A diet low in prebiotic substances has been shown to reduce total abundance and diversity of gut bacteria. In particular, a diet rich in non-digestible carbohydrates most consistently increases intestinal bifidobacteria and lactic acid bacteria [96]. Other non-digestible carbohydrates, such as resistant starch and whole grain barley, also tend to increase the proportions of health-promoting SCFA-producing bacteria such as *Ruminococcus* and *Roseburia.*

Digestible carbohydrates are enzymatically degraded in the small intestine and contain starches and sugars such as glucose, fructose, sucrose and lactose. All these compounds release glucose into the bloodstream upon degradation, triggering an insulin response. Human subjects fed high levels of glucose, fructose and sucrose in the form of fruit, had increased relative abundance of bifidobacteria, with reduced *Bacteroides* [97]. Also, lactose supplementation was found to raise the fecal amounts of beneficial SCFA-producing bacteria in non-intolerant subjects [98].

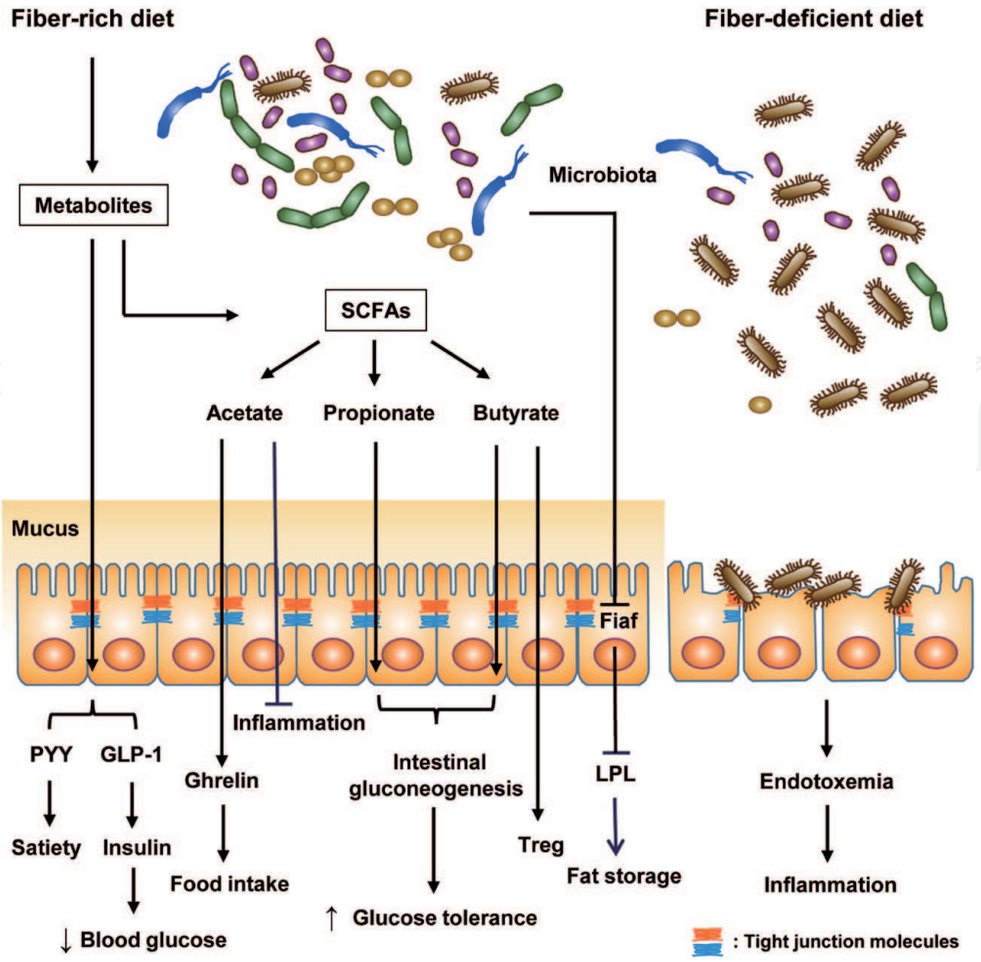

Figure 4.
Fiber intake impacts on host metabolism and immunity by affecting the gut microbiota. Under a fiber-rich diet, the gut microbiota metabolizes undigested dietary fiber into SCFAs (acetate, propionate, and butyrate), affecting host metabolism and immunity. Microbial metabolites from this process improve host metabolism. In particular, the secretion of peptide hormones, such as PYY and GLP-1, is promoted by microbial metabolites: PYY decreases appetite and GLP-1 lowers blood glucose level via promotion of insulin secretion. Among SCFAs, butyrate and propionate activate intestinal gluconeogenesis and improve systemic glucose profiles. Meanwhile, acetate promotes secretion of ghrelin, a hunger hormone, and increases food intake, consequently causing hyperphagia and obesity. Nevertheless, acetate has anti-inflammatory function like butyrate. Butyrate enhances gut barrier function of intestinal epithelial cells and increases regulatory T (Treg) cells. In addition, the gut microbiota suppresses expression of fasting-induced adipose factor (Fiaf), an inhibitor of LPL, promoting fat storage in adipocytes. Under fiber-deficient diet, mucus-degrading bacteria expand and impair the integrity of the mucus layer. Thereby, endotoxemia-induced metabolic inflammation ensues. SCFAs, short-chain fatty acids; PYY, peptide YY; GLP-1, glucagon-like peptide-1; LPL, lipoprotein lipase. From [93].

4.2 Micronutrients and microbiota

Vitamins. Diet is the primary source of vitamins, because our bodies cannot synthesize them to meet our daily needs, but certain vitamins, especially vitamin K and the B-group vitamins, are synthesized by some intestinal microbes. As a consequence, the abundance and diversity of intestinal microbiota components can modulate the metabolism and absorption of vitamins in the upper intestine [99].

The administration of retinoic acid (physiologically active vitamin A metabolite) in patients with norovirus infection significantly increased the abundance of *Lactobacillus* spp. Since *Lactobacillus* showed antiviral activity *in vitro*, it has been hypothesized that the intake of vitamin A and the consequent increase in the amount of *Lactobacillus* in the gut were partially responsible for norovirus inhibition [100]. Retinoic acid administration has also been shown to increase the relative abundance of *Allobaculum, Aggregatibacter, Bifidobacterium, Dialister* and

Enhydrobacter. Epidemiological studies have shown that norovirus infection rate and clinical symptoms decrease significantly with a sufficient supplementation of vitamin A [101]. In infants, supplementation of vitamin A showed to improve the Bacteroidetes/Bacteroidales population, and increase the relative abundance of *Bifidobacterium* and *Akkermansia* in feces [102].

Vitamin C is the most important antioxidant agent, and it must be obtained from dietary sources (mainly fruits and vegetables). This vitamin regulates the redox state and can considerably modulate the gut microbiota. In weaned piglets, vitamin C levels correlated positively with Firmicutes and negatively with Bacteroidetes relative abundances [103]. Vitamin D is thought to be a multifunctional vitamin involved in calcium homeostasis and in a list of systemic physiological functions that include the modulation of gut microbiota [104]. A randomized controlled trial showed that weekly vitamin D supplementation (50,000 ergocalciferol IU) over 12 months increased SCFA fecal levels and the relative abundance of SCFA-producing genera such as *Ruminococcus*, *Fecalibacterium* and *Dialister* [105].

Some vitamins of the B group have been shown to promote bacterial colonization of the gut, modulate bacterial virulence and participate in pathogen clearance [106]. However, they may also have a role in the growth of enteropathogens, such as *Salmonella* Typhimurium [107]. For example, different gut bacteria can synthesize vitamin B6, but dysbiosis could reduce the luminal level of vitamin B6 and facilitate gut colonization by enteropathogenic strains.

It is evident that there is a high and complex interaction between vitamins and the gut microbiota: some vitamins are produced by the microbiota itself and others, particularly liposoluble vitamins, are responsible for its modulation. On the other hand, some of these vitamins may also contribute to enhanced virulence and colonization of potential pathogenic microbes. These studies together suggest that vitamin supplementation could modulate the gut microbiota, but its effects depend on the level of vitamin in the host and the microbiota status. Further clinical trials should be carried out to understand the effects of multivitamin supplementation, in order to evaluate possible effects linked to over-supplementation.

Polyphenols. Dietary polyphenols are studied for their antioxidant properties. Popular foods with a rich content of polyphenols include fruits, nuts, vegetables, tea, cocoa, and wine. For example, the relative abundance of *Bacteroides* was reported to increase in subjects consuming pomegranate [108]. The consumption of cocoa-derived polyphenols has been associated with significant changes in the gut microbiome [109]. Fruit seed, wine and tea polyphenols were capable to positively modulate the human fecal microbiota by affecting the levels of pathogenic *Clostridium* species (*C. perfringens and C. histolyticum*) [110].

Conceptually, it is difficult to isolate the activity of polyphenols from the overall activity of the foods that contain them. Nevertheless, overall we can conclude that a diet rich in foods with high polyphenol content, can have positive effects on the human intestinal microbiota.

Food additives and xenobiotics. Another poorly understood area with potential implications for the human gut microbiota health is the impact of food additives and xenobiotics on the microbial ecology and intestinal homeostasis. Although Western diets typically attribute microbial and health consequences to macronutrient composition, several studies suggest that food additives may be driving the detrimental effects of these diets on the microbiota. For example, in the absence of other dietary manipulations in mice, two dietary emulsifiers, polysorbate-80 and carboxymethyl cellulose, induced obesity, intestinal inflammation, metabolic dysfunction and dysbiosis. The microbiota was both necessary and sufficient to explain all these effects as germ-free mice were protected from these detrimental effects, and the transfer of microbiota from emulsifier-treated mice was sufficient to

recapitulate the metabolic disruptions [111]. These results are particularly striking considering the wide range of foods containing emulsifiers (for example gluten-free and reduced-fat products, ice cream, and pickles), and that the doses used in this study reflect the human intake. Non-nutrient sweeteners (NNSs) have been linked to gut-associated metabolic alterations, in addition to emulsifiers. In experiments conducted in rodents and humans, NNS consumption induced glucose intolerance in a microbiota-dependent manner [112]. Nevertheless, literature data on the effects of NNSs on intestinal microbiota and microbiome sometimes are divergent, and this is also dependent on the fact that NNSs are a broad class of substances with high structural and functional variability. Additional human intervention studies examining the impact of individual NNSs on microbiota and microbiome are certainly needed.

Artificial sweeteners such as saccharin, sucralose and aspartame have been considered as options that might be used to replace natural sugar to prevent and control glucose dysmetabolism. However, recent evidence suggests that consumption of all types of artificial sweeteners may induce glucose intolerance. It is important to note that artificial sweeteners are thought to mediate this effect also by altering the gut microbiota. For example, it was noted that saccharin-fed mice had intestinal dysbiosis with increased relative abundance of *Bacteroides* and reduced *Lactobacillus reuteri* [112].

Even on the large category of xenobiotics it is very difficult if not impossible to generalize. Just as an example, analysis of the microbiome of children with Crohn's disease developed at a very young age showed that the most altered metabolic patterns in the gut microbiome were those related to xenobiotic metabolism [113].

4.3 Dietary patterns

Several popular diets have been studied for their ability to modulate the intestinal microbiota, including Western, ketogenic, omnivore, vegetarian, vegan and Mediterranean diets. The Western diet (high in animal protein and fat, low in fiber) has led in several studies to a marked decrease in microbial diversity and in some beneficial genera, such as *Bifidobacterium* and *Eubacterium* [114].

Ketogenic diets are characterized by a very low consumption of carbohydrates (5 to 10 percent of total caloric intake), sufficient to increase the production of ketone bodies. They were originally developed as a treatment for refractory childhood epilepsy, and the gut microbiota responses to a ketogenic diet seem to play a role in the effectiveness of this intervention in epileptic infants [41, 42]. In recent years, these diets are commonly adopted in order to obtain rapid weight loss and in some studies, they have been shown to improve longevity and reduce the onset of disease in experimental animals. Conversely, some human studies in which ketogenic diets were examined, suggest negative impacts on microbial ecology and gut health. These studies, however, were carried out in small cohorts with specific metabolic conditions, limiting the generalization to larger populations [115]

Vegan/vegetarian diets are both plant-rich diets associated with positive health outcomes and reduced risk of some diseases [116]. The beneficial effects of these diets on human health could also be linked to intestinal microbiota modulation. Plant-based foods are the primary source of dietary MACs, and it has been found that individuals who consume vegetarian or predominantly plant-based diets have a microbiota metabolically optimized for MAC fermentation. However, some intervention and cross-sectional studies have found only modest differences in microbiota composition between omnivores and vegetarians, and suggest that the effects of dietary patterns on the microbiota are greatest at the level of genus and species, but relatively minimal on broader compositional features such as diversity [117].

Despite the absence of a wide microbiota compositional shift, the species-level changes appear to be sufficient to alter metabolic outputs as SCFA production, which in vegetarians is typically increased. It is still unclear to what extent these microbiota-dependent metabolic outputs can mediate the beneficial effects of vegetarian diets.

Plant-based foods, in addition to supplying MACs, provide a diverse source of vitamins, polyphenols and other biologically active phytochemicals. Many phytochemicals may often reach the lower intestinal tract and have direct antimicrobial and anti-inflammatory effects in the intestine. Furthermore, microbial enzymes can modify phytochemicals into metabolites with increased bioactivity [118, 119]. So, microbiome-mediated changes in phytochemical bioavailability can be an additional mechanism underlying the beneficial effects of plant-based diets.

Several studies classify the Mediterranean diet as the most healthy and balanced human diet. It is characterized by a beneficial fatty acid profile, rich in both monounsaturated and polyunsaturated fatty acids, high polyphenols and other antioxidants and high fiber intake. Fruits, vegetables, cereals, legumes and nuts are at the basis of this diet, as well as consumption of fish and red wine [120]. The potential benefits of Mediterranean diet on the gut microbiota are linked to the increased levels of fecal SCFAs together with an increase of *Prevotella, Lactobacillus* and *Bifidobacterium*, and a decrease in *Clostridium* [92].

Even if there are different types of Mediterranean diet, as well as several ketogenic diets (e.g. normo- or iper-proteic) and even vegetarian diets (with or without eggs, with or without fish), what can be concluded in general about the effects of dietary patterns on the intestinal microbiota is that all those patterns which, for various reasons, tend to restrict the amount of vegetables, seem to be inadvisable. Thus the Western diet, which is poor in fruit and vegetables, and the ketogenic diets, which necessarily eliminate fruit for its carbohydrate content, appear to be diets with a probable negative impact on the intestinal microbial ecology. Despite this, comparative controlled clinical trials are needed to fully evaluate the possible short-term and long-term effects of these dietary patterns on the gut microbiome.

5. Use of fungus and officinal plants for the modulation of the intestinal microbiota and immune system

Microbiota and its multiple connections, already described in the previous paragraphs, remind us that every human being is an unrepeatable and unique Psycho-Neuro-Endocrine-Immuno-Somatic-Environmental unit that is constantly dynamic and interactive in its parts [121]. From this perspective, the gastrointestinal system should be evaluated and treated as a neuro-immuno-endocrine-visceral-microbial interface of the human body. The modulation of the gut microbiota and, consequently, of the immune system is a key function of this complex network. Any disorder of the gastrointestinal tract, be it functional or with organic inflammatory basis, involves cells belonging to multiple tissues, including the sphere of the microbiota, and is therefore continuously reflected at the systemic level.

Consequently, even medicinal plants can, indeed should, act at multiple levels of the organism through direct and indirect actions that certainly, with various types of mechanisms, involve the Intestinal Immune System (IIS) and the intestinal microbiota. The action of fungi and medicinal plants is exerted on the gastrointestinal system through the immunomodulating, antioxidant and protective properties of the microbiota. Furthermore, the protection of the biofilm and the intestinal barrier, in the structuring of which the microbiota directly and actively participates,

also fall within these therapeutic actions. These effects on the intestinal barrier and on the gastrointestinal system can obviously also have systemic consequences.

Several medicinal plants and fungi are described in the scientific literature as being able to act positively on various acute and chronic inflammatory disorders of the gastrointestinal system, most of these are also part of the medical tradition of one or more regions of the world.

Medicinal mushrooms that have been used in most preclinical and clinical studies are *Hericium erinaceus*, *Inonotus obliquus* (called Chaga), *Ganoderma lucidum* (called Reischi), and *Auricularia auricula*. Instead, the most used medicinal plants for the gut are *Boswellia serrata*, *Pistacia lenticus*, and *Aloe* in its various species. With a mainly European traditional use, we find *Olea europea* (olive tree), *Angelica arcangelica*, *Achillea millefolia*, *Cichorium intybus* and finally *Cetraria islandica*, which belongs to the lichen species.

In this brief discussion, we will limit ourselves to analyzing the scientific literature supporting possible therapeutic use of some of these fungi and these plants, in the modulation of intestinal inflammation and dysbiosis, the two components that are always associated in almost all pathologies of the gastrointestinal tract.

5.1 Microbiota-modulating fungi

Hericium erinaceus represents the most used fungus for all the disorders of the gastrointestinal system. Also known as Lion's Mane Mushroom, is an edible fungus, which has a long history of usage in traditional Chinese medicine for the protection of mucous membranes, gastric ulcers, acute and chronic gastritis and nervous degeneration [122–124].

The drugs used are the fruiting body and/or the mycelium in aqueous or hydroalcoholic or alcoholic extracts titrated and standardized in one or more of the following components: polysaccharides and beta-glucans (with anti-inflammatory and antibacterial action), alpha-glucans, diterpenes and triterpenes and polyphenols [125, 126].

The most studied activities of this fungus relate to its immunomodulatory effects on the gut, its anti-inflammatory systemic activity, but also its prebiotic activities on the intestinal microbiota [127].

A single protein, called HEP3, isolated from *H. erinaceus* and administered to rats treated with trinitrobenzenesulfonic acid (TNBS) to induce experimental colitis similar to IBD, was capable of restoring the microbiota diversity in treated rats. In particular, treatment with *H. erinaceus* single protein increased the amounts of Actinobacteria and Tenericutes, reduced those of Bacteroidetes and Firmicutes, and was able to restore a healthy-like ecological structure [128, 129]. The effectiveness of HEP3 was also confirmed in other animal models of colitis [130]. We would like to underline the general concept that it is always advisable to use titrated extracts with greater complexity than single proteins, as these extracts can keep the phytocomplexes and mycocomplex intact, and *in vivo* could have synergistic actions on multiple targets. Also, raw extracts of *H. erinaceus* were tested in an IBD animal model as whole polysaccharide, alcoholic extract or whole extract. Results indicate that all these formulations were capable of positively modulating the microbiota, but while the polysaccharide extract seems to play a major prebiotic role, the alcoholic extract and whole extract showed major bactericidin-like effects [128, 129].

Similar results were obtained in a model of dextran sulfate sodium (DSS)-induced colitis in mice. DSS treatment resulted in increased relative abundances of Verrucomicrobia and Actinobacteria and decreased amounts of Bacteroidetes in fecal samples, compared to the control group. Treatment of colitic mice with dry power of fermented *H. erinaceus* mycelium reversed most changes, including the increased levels of *A. muciniphila*. Collectively, these results showed that *H.*

erinaceus effectively modulate the gut microbiota of colitic animals, restoring a microbiota composition similar to that of healthy mice [131].

Inonotus obliquus commonly known as Chaga is a parasitic fungus mainly of Birch (*Betulaceae*) trees with numerous biological properties [132, 133]. Commonly used as a folk remedy in Russia and other northern European countries for various disorders affecting the digestive system, it is now widely studied for its numerous potential applications in the medical field. The most used formulations are powder, aqueous extract and hydroalcoholic extract. These can be titrated in polysaccharides, beta-glucans, alpha-glucans and polyphenols. Even the fungus *I. obliquus* has been successfully used to counteract the effects of DSS-induced colitis in mice [134]. Its major effects seem to be the modulation of IIS [Won et al., 2011]. Nevertheless, its polysaccharides showed a positive regulatory effect on the microbiota in animal models of colitis. In a chronic pancreatitis mice model, the compromised microbiota profile was partially restored by *I. obliquus* polysaccharides administration, which was able to increase microbiota diversity and richness and also to improve mouse clinical conditions [93].

Ganoderma *lucidum* (in Japanese called Reishi) is considered, in the Far East, the mushroom of immortality due to the countless biological activities it would be able to promote. It is a mushroom with a woody texture and a bitter taste, which grows preferably on oaks and chestnut trees. The main traditional use in China and Japan is aimed to counteract allergic and inflammatory status [136] but its traditional use also covers hypertension, liver and cardiovascular problems. Recent studies have identified more than 400 bioactive molecules present in this mushroom. Some of these were identified for the first time in this specie, and consequently named as ganoderiol, ganolucidinic acids, and ganodermantriol [137].

The most studied activities of this mushroom are the immunostimulatory effect exerted on the gut but also at systemic level. However, there is also evidence of prebiotic activity on the microbiota, although this could be secondary to a direct effect on immune system components. Its powerful immunomodulatory effects led to extend its field of use also to the therapy of tumors, a topic which, however, goes beyond the themes of this chapter [138]. In DSS-induced colitis in rats, *G. lucidum* β-glucans increased SCFA-producing bacteria such as *Ruminococcus*, and reduced pathobionts such as *Escherichia* and *Shigella* in both the small intestine and cecum [139]. In mice fed a HFD, which showed increased body weight, gut and systemic inflammation and insulin resistance, treatment with *G. lucidum* mycelium reversed the HFD-induced gut dysbiosis, decreasing the Firmicutes-to-Bacteroidetes ratio and the Proteobacteria levels. Moreover, Reishi treatment reduced metabolic endotoxemia by restoring the intestinal barrier integrity. These anti-obesity effects were transmissible via fecal transfer from *G. lucidum*-treated mice to untreated HFD-fed mice, demonstrating that the leading mechanism of action of *G. lucidum* was linked to the modulation of the microbiota. High molecular weight polysaccharides (>300 kDa) present in *G. lucidum* were capable of producing similar microbiota modulation and anti-obesity effects [140]. Similar results were obtained in a rat model of type 2 diabetes, in which *G. lucidum* treatment reduced the relative abundance of harmful bacteria, such as *Aerococcus*, *Ruminococcus*, *Corynebacterium* and *Proteus*, and increased the levels of *Blautia*, *Dehalobacterium*, *Parabacteroides* and *Bacteroides*. Microbiome analysis indicated that Reishi treatment could also restore the microbial metabolism of amino acids, carbohydrates, inflammatory substances and nucleic acids, altered by the obesity status [141, 142]. Taken together, these results indicate that *G. lucidum* and particularly its high molecular weight polysaccharides may be effectively used as prebiotic agents to prevent gut dysbiosis and obesity-related metabolic disorders, at least in obese rodents.

In a mouse model of pancreatitis, induced by diethyldithiocarbamate (DDC), polysaccharides from *G. lucidum* were capable of positively modulating the gut microbiota, by decreasing the relative abundance of Bacteroidetes and increasing that of Firmicutes. At the genus level, supplementation of Reishi polysaccharides increased the relative abundance of beneficial bacteria, such as Lactobacillales, *Roseburia* and *Lachnospiraceae*. These results confirmed that also the therapeutic mechanism on chronic pancreatitis might be dependent on the restoration of a eubiotic intestinal microbiota layout [16, 17].

Finally, it should be emphasized that even if all these mushroom preparations can be easily found for free sale, and even if they do not seem to have side effects, it is a good practice to never use them in self-prescriptions as their direct interactions with drugs, or their effects on detoxifying enzymes such as CYP, have not yet been studied or poorly known. For example, Chaga extract inhibited platelet aggregation in mice. It may also have synergistic effects when used with anticoagulant/antiplatelet drugs, but the clinical relevance in humans is not known [143]. Chaga may also interact with hypoglycemic agents drugs, since it has demonstrated to possess hypoglycemic activity in animals [144, 145]. A single case-report described oxalate nephropathy as a side effect associated with the ingestion of Chaga mushroom powder (4–5 teaspoons daily for 6 months), in a 72-year-old Japanese woman with liver cancer [146].

Chaga effects on detoxifying enzymes such as CYP have not yet been studied. Reishi may increase the risk of bleeding, interfering with anticoagulants/antiplatelets drugs [147]. Reishi can also enhance immune response and this effect should be taken into account in patients on immunosuppressive therapy. Finally, at least *in vitro*, Reishi polysaccharides inhibited many different CYP enzymes [148].

5.2 Microbiota-modulating plants

Cichorium intybus is a perennial herbaceous plant whose rhizome and roots are traditionally used in Europe to treat gastrointestinal disorders [149]. It has been tested in farmed broiler chickens in order to improve productive performance, and showed to induce changes in ileal microbiota consisting of lower counts of *E. coli* and higher counts of *Lactobacillus*. These effects were associated with improved growth performance. Dietary chicory powder supported ileal microbiota ecology probably by acting as a prebiotic, since it has a high content in soluble fibers, particularly inulin [150]. In mice fed with different chicory genotypes, all preparations were capable of modifying the fecal microbiota by modulating the Firmicutes/Bacteroidetes ratio and some bacterial genera, such as *Alloprevotella*, *Blautia*, *Alistipes*, and *Oscillibacter*, with a variable effect depending on the chicory genotype. In addition to microbiota changes, some modifications in the release of satiety hormones, and as a consequence appetite, were also observed in mice treated with *C. intybus* [151].

Boswellia serrata is an arboreal plant that forms an aromatic resin also known as frankincense. The *B. serrata* resin has been used as supplementation in rabbit diets at different dosages to observe variations in the cecal microbiota. Substantial changes in microbial cecal populations were found in rabbits treated with *B. serrata*, with a significant decrease of total bacterial counts and in particular a decrease of *Salmonella enteritidis* and *E. coli* if compared to the control untreated rabbit group. These results could be ascribed to the high polyphenol content in *B. serrata* and to the presence of Boswellic acid that holds a powerful anti-microbial effect [152, 153].

Pistacia lentiscus is a shrub or small evergreen tree that produces a resin called Chios mastic gum, used as a natural food supplement. The effect of *P. lentiscus* was investigated in mice with obesity, nonalcoholic steatohepatitis (NASH), and liver

fibrosis induced by HFD. Treatment with *P. lentiscus* promoted a partial but significant recovery of microbiota diversity associated with a decrease in Bacteroidia and an increase in Proteobacteria relative abundance [154].

Olea europaea is an evergreen fruit tree found traditionally in the Mediterranean area. *O. europaea* extra virgin oil (EVO) obtained from its fruits, olives, is able to induce higher gut microbiota biodiversity and promote the growth of beneficial commensal bacteria, as showed by studies on humans and animals [155]. Nevertheless, the traditional use of *O. europaea* as officinal plants is limited to its leaf preparations. *O. europaea* leaf extract administered to obese mice was capable of improving the gut microbiota by partially restoring the amounts of Actinobacteria, Bacteroidetes and Verrumicrobia. Also, the relative abundance of *Akkermansia* spp. was restored, suggesting a possible effect on intestinal barrier function in treated mice [156].

As for *Angelica arcangelica*, *Achillea millefolia*, *and Cetraria islandica,* officinal plants that are traditionally used to treat intestinal dysbiosis and inflammation, there are no scientific studies so far published to support their positive action on the intestinal microbiota and microbiome. This does not mean that these medicinal plants are not effective in modulating the gut microbial ecology, but only that the documented scientific evidence of their supposed therapeutic activities are still very poor.

6. Aromatic plants end essential oils (EOs) as bowel "Eubiotics"

6.1 Intestinal microbiota modulation exerted by essential oils and aromatic plants

Aromatic plants are a wide group of herbs with characteristics aroma due to the presence of high amounts of volatile compounds known as EOs. Consequently, aromatic plants have always constituted a characteristic aspect of the gastronomic traditions. In recent years, the use of these aromatic plants has been replaced, especially in countries with high per capita income, with artificial flavors that allow the elaboration of more sophisticated aromas that in many cases are kept secret by the food industry, to avoid plagiarism. This replacement is certainly part of the transition from the traditional cuisines to the so-called western diet, the process called westernization of the diet that has taken place in many countries, parallel to the increase in the incidence of many intestinal diseases related to alterations of the gut microbioma, such as Inflammatory Bowel Diseases (IBD) [157]. EOs have multitarget effects on the intestine due to their antioxidant, anti-inflammatory but also antimicrobial properties directed on the bacterial, yeasts, fungi and viruses components of the human microbioma [158]. The antibacterial activity of EOs depends on the concentration that they reach into the gut, but also on the species of bacteria that they encounter. In fact, some OEs have more marked effects (i.e. lower Minimum Inhibitory Concentrations or MICs) for bacterial species considered pathogenic, while showing less activity (i.e. higher MICs) towards components of the microbiota such as bifidobacteria and lactobacilli [159]. This multitarget positive effects of EOs on the intestinal microbiota, different from those obtained with the use of probiotics and prebiotics, has not found a definition in the literature yet. Hence, we propose here for the first time the term "eubiotic" activity since EOs restore the intestinal microbiota back to a physiological state of eubiosis, when a dysbiosis has been established into the gut.

6.2 Eubiotic proprieties of EOs on gut microbiota of animals and humans

There is no doubt that EOs are able to modulate the intestinal microbioma for their antimicrobial activities, which is one of the reasons why nature has selected these complex mixtures of active molecules with evolution. EOs may have "eubiotic" effects thanks to their capability to control and modulate bacterial growth, acting both as bacteriostatic or bactericidal agents [160]. In fact, due to their lipophilic properties, EOs can penetrate membranes, and damage bacterial cells structure making their membranes more unstable and permeable. Membrane disruption may also lead to bacterial death caused by the significant leak of ions and other essential cytosolic components. These EO effects are generally more pronounced on Gram positive bacteria respect to Gram negative ones [161]. However, it has been demonstrated that EOs can also affect bacterial cell wall in Gram-negative bacterial strains [162]. Despite this, there are very few clinical studies of their eubiotic activity on humans, while the scientific data obtained on animals bred for human consumption or on experimental animal models are numerous and really convincing.

In broiler chickens, EOs have been widely adopted to improve intestinal microbiota and, as a consequence, to boost the growth performances of farmed animals. For example, the effects of liquidambar essential oils (LEO) isolated from Turkish sweet gum leaves (*Liquidambar orientalis* Mill.) were a decreased *Escherichia coli* counts in jejunum, associated with an increased weight of chickens after 42 days of treatment [163]. Feeding broilers with a trade mark EO mixture containing thymol (*Thymus vulgaris*), eugenol (*Cinnamomum* spp.), and piperine (*Piper* spp.) resulted in an increase in Lactobacillus and a decrease in *E. coli* counts in ileal microbiota, associated with an increased food conversion ratio [164]. In another study, the administration of a commercial EO mixture, containing cinnamaldehyde, isophorone and eugenol significantly increased the relative abundance of phyla Bacteroidetes and decreased the abundance of phyla Firmicutes in cecal microbiota of chickens, with an increase in the relative abundance of genus of *Alistipes*, *Rikenellaceae, Roseburia,* and *Anaeroplasma*. This microbioma changes was confirmed by more than hundred different metabolites detected in cecum of EO treated animals, probably linked to their improved growth performances [141, 142].

Broiler chicken is not the only farmed animal treated with EOs for the purpose of modifying microbiota and reduce the susceptibility to infection by pathogenic bacteria. In farmed rainbow trout, the treatment with a mixed EO (containing eucalyptus, oregano, thyme and sweet orange EOs) caused significant microbiota changes in alpha and beta diversity, increasing also their growth performance and the final product quality. [165]. In farmed pigs, oral administration of a EO mixture (containing cinnamon and oregano EOs) caused a significant decrease of infections caused by two porcine diarrhetic enterotoxigenic *E. coli* strains [166].

Two different essential oils were tested on farmed ducks, again in order to improve their growth performance and also to replace the use of antibiotics in animal farming. One consisted of oregano oil, the second of thyme and cinnamon oil. Both of these EO preparations were able to decrease the cecal populations of coliforms and lactose-negative enterobacteria, demonstrating also in these animals an eubiotic effect of these OE on the gut microbiota [167].

Even on farmed crustaceans, a blend of organic acids and essential oils was tested for the improvement gut microbiota and disease resistance of Pacific white shrimps. Results demonstrated that this mixture was capable to enhance microbiota diversity and richness, increasing the abundance of Firmicutes and reducing the abundance of Proteobacteria. Also, a significant increase in the abundance of *Lactobacillus* was observed in shrimp gut [125, 126].

All these studies as a whole demonstrate without doubt the eubiotic potential of orally administered EOs. Furthermore, they clearly demonstrate that dosages effective for modulating the microbiota are free of toxic effects on animals. Nevertheless, it remains rather difficult to understand which components of EOs are most active for modulating the microbiota, because of their natural complexity and their use in mixtures. For these reasons, several studies have explored the eubiotic properties of EO single molecules. The most studied was certainly geraniol, for its interesting antimicrobial potential. Geraniol antibacterial activity seems to be linked to his property to destabilize bacterial cell wall and damage transmembrane efflux pumps [168]. Despite being absorbed very quickly and in an active manner by the small intestine mucosa, geraniol is reported to positively modulate the colitis-associated dysbiosis when administered by oral route by using a controlled delivery system based on microencapsulation [169]. In mice but also in humans, geraniol has demonstrated to act as an excellent modulator of intestinal microbiota, capable to boost populations of butyrate-producer bacteria such as *Collinsella* and *Faecalibacterium*, normally reduced in the dysbiotic human intestinal flora of IBS patients [157]. It is interesting to note how geraniol antibacterial activities is quite selective for pathogenic bacteria and do not involve commensal species [159]. For these reasons, geraniol can be considered as an efficient eubiotic for the human gut microbiota.

Another interesting EO molecule with antibacterial activities is eugenol (2-Methoxy-4-(prop-2-en-1-yl) phenol), the major compound present in clove oil, but also found in many other EOs. Eugenol has demonstrated antimicrobial activities based on a non-specific permeabilization of the bacterial membrane with depletion of adenosin triphosphate (ATP), an energy moiety necessary for bacterial metabolism and survival [170]. This effect has been observed against gut pathobionts such as *E. coli*, *Listeria monocytogenes* and *Lactobacillus sakei* at the relatively low concentration of 10 mM [171]. In mice, orally administrated eugenol improved the secretion of the intestinal mucus, creating a thicker intestinal layer associated with positive changes of the mucosal-microbiota ecology. In particular, eugenol inhibited the intestinal adherence of *Citrobacter rodentium*, a mice pathogen that shares several biochemical features with *Clostridium difficile* in humans [172]. It would be really interesting to use eugenol in a clinical study aimed at the eradication of *C. difficile*: the results could be surprising.

Cinnamaldehyde (2E-3-Phenylprop-2-enal) is a phenylpropanoid naturally present in the plant of the genus *Cinnamon*. Cinnamaldehyde is one of the most studied EO molecule and it has been already approved as antimicrobial food preservative [173]. Antibacterial effects of cinnamaldehyde have been demonstrated by using many different bacterial models, but only few studies evaluated its impact on the whole intestinal microbiota. *In vitro*, cinnamaldehyde was capable to inhibit the growth of potentially pathogenic bacteria such as *S. aureus, E. cloacae, A. baumannii* and *L. monocytogenes* [174] and it was able to kill a pathogenic strand of *E. coli* at very low concentrations (0,05% v/v) [175]. One of the proposed antibacterial mechanisms of cinnamaldehyde inhibition of *E. coli* growth was the inactivation of its acetyl-CoA carboxylase enzyme [176]. Other studies showed that cinnamaldehyde antimicrobial activity has a broad spectrum of action, being effective against many different intestinal pathobionts such as *Enterococcus faecalis, Enterococcus faecium, E. aerogenes Salmonella enterica* and *Clostridium perfringens* [177]. *In vivo*, only few studies have been conducted on cinnamaldehyde, perhaps because of its strong aggressiveness towards the mucosal epithelia. Nevertheless, in animal experimental colitis, the oral administration of cinnamon EO (approx. 70% in Cinnamaldehyde) at 10 mg/Kg or 15 mg/Kg lead to an improvement of the ecological biodiversity of the intestinal microbiota. Short-chain fatty acids (SCFA)-producing bacteria

family, such as Bacteroidaceae, were increased while intestinal *Helicobacter* and *Bacteroides* were reduced [178].

Other molecules, such as thymol do not seem to show eubiotic effects in the gut, being non-selective and affecting all the intestinal bacteria and therefore behaving like a broad-spectrum antibiotics, depleting the microbiota even when administered at low dosages with a negative impact also on commensal bacteria [159].

Carvacrol, a major component of oregano EO, showed to inhibit bacterial adhesion, invasion and biofilm development in cultured intestinal cells [144, 145]. In farmed broiler, treatment with carvacrol-rich EO was tested to control the pathogenic bacteria spreading inside the farms. Results of these studies demonstrated that carvacrol reduced the microbial counts of *E. coli* and different *Salmonella* species in the small intestine of farmed chicken [144, 145]. Moreover, carvacrol administration to broiler chickens, was capable to eliminate the intestinal presence of *Campylobacter spp*. after 21 days of oral daily administration at 120–300 mg/Kg. This effect was associated to the enhanced growth of *Lactobacillus*, that were found to be increased in chicken microbiota, after carvacrol administration. For its eubiotic activity, this molecule is today the most used in organic chicken farming [179].

Limonene (1-Methyl-4-(prop-1-en-2-yl) cyclohex-1-ene) is a cyclic monoterpene present in high amount in EO of citrus fruit peels that has widely demonstrated antimicrobial and eubiotic effects *in vivo*. In mice, daily oral administration of 160 mg (8.000 mg/Kg) of limonene-rich orange EO modulated the gut microbiota by enhancing the relative abundance of *Lactobacillus* genus and of *Bifidobacterium* population [180]. Despite the low toxicity of limonene, it should be noted that these eubiotic effects were obtained only with high dosages of this EO.

Eucalyptol (1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane) is a cyclic ether and a monoterpenoid. It is the major compound in *Eucalyptus* EO, but it can be also found in many other officinal plants. *Eucalyptus* EO has extraordinary antimicrobial activities and has shown to be effective against a plethora of bacteria species and among them *S. aureus*, *E. coli, Bacillus subtilis, Klebsiella pneumonia, Salmonella enteritidis* and *P. aeruginosa* [181]. Nevertheless, *Eucalyptus* EO also contains high amounts of other antimicrobial components besides eucalyptol, therefore not all of *Eucalyptus* EO antibacterial activity can be ascribed to the presence of eucalyptol. However, literature data regarding eucalyptol eubiotic activity are very limited, and new studies focused on this interesting compound are needed.

Menthol (5-Methyl-2-(propan-2-yl)cyclohexan-1-ol) is a chiral alcohol and the main molecule present in cornmint and peppermint EOs. It has been well known for its use in foods as a cooling and minty-smell aroma. Many *in vitro* studies focused on its antibacterial activities [182]. Nevertheless, studies on the use *in vivo* of menthol alone, to modulate the gut microbiota, are lacking.

6.3 EOs in the modulation of gut mycobiome

Fungi were reported to represent about 0,1% of all the microorganisms present in the gastrointestinal tracts. Maybe also for this reason, despite the presence of fungi in the intestine has been known for many years, in depth studies of the human mycobiome were only recently performed [183]. Together with bacteria, fungi contribute to the modulation of the intestinal immune system [184]. Many of them have a clear pathogenic potential even if, physiologically, they are commensals in our bodies. Only in some specific conditions their overgrowth can lead to well-known mycosis. The best known fungal pathogen of humans is certainly *Candida albicans,* which is a normal component of the gut mycobiota but may causes candidiasis in case of its intestinal and vaginal overgrowth [185]. An altered intestinal

mycobiota has also been observed in other human pathological conditions, such as IBS [186], inflammatory bowel disease (IBD) [187] and also autism-spectrum disorders and Rett syndrome [188].

EOs antimycotic activities are characterized by a broad spectrum of actions [189]. *C. albicans* has been one of the main target for studies focusing the antifungal effect of EOs and their single molecules. The antifungal activities of EO obtained from *Thymus vulgaris*, *Citrus limonum*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Ocimum basilicum*, and *Eugenia caryophyllus* have been evaluated against clinical isolates of *C. albicans* and *C. glabrata.* All of these EOs exhibited both fungistatic and fungicidal activity towards these two *Candida* species, but cinnamon oil demonstrated the highest activity [190]. Since the most represented active compounds of *Cinnamomum* EO is cinnamaldehyde, many studies have been addressed to analyze in depth its activity against *C. albicans* [191].

Limonene has shown to possess strong antifungal properties [192] and in particular an excellent anti-*Candida* activity. A recent study analyzed the efficacy of this compound against the growth of *C. albicans* isolates, whose growth was completely inhibited at doses ranging between 5 mM and 20 mM [193].

Mentha EOs have demonstrated good antimycotic activities against different fungi genus, including *Candida* [194]. Menthol and (+)-carvone are the major components of peppermint EO and both exhibited strong antifungal activity *in vitro* [195] and mycobiome modulation activities *in vivo* [196].

Thymus vulgaris EO has also shown to be effective against fungi pathobionts capable to infect humans. A study on Dermatophyte, fungi that can cause superficial infections of the skin, and on *Aspergillus*, fungus genera that can cause respiratory infections, reported MIC values for *Thymus vulgaris* EO ranging from 0.16 to 0.32 µl/ml. Higher MIC values, between 0.32 and 0.64 µl/ml, were reported for *Candida spp*. The antifungal activity of this EO has been attributed to its two major components: thymol and carvacrol, that accounted respectively 26% and 21% of *Thymus vulgaris* EO [196]. Both these phenolic compounds seem to act by disrupting the fungal cell membranes [161].

Clove EO has been traditionally used in dentistry for its anesthetic and antimicrobial activities [197]. Its anti-fungal action has been attributed to eugenol, the major clove oil molecule. A recent study indicated that Clove EO, at concentrations that ranged between 0,03% and 0.25% (v/v), inhibited the biofilm formation in many *Candida* species, grown on different substrates [198]. For what the mechanism of action concerns, eugenol was able to cause permanent injury to the cell membranes of *C. albicans* and morphological alterations to its cell wall [161, 199]. Although these studies suggest that the eubiotic effect of EOs and their individual chemical compounds may also be extended to the mycobiome, there is currently no conclusive evidence showing that the improvement in microbial ecology linked to the use of these compounds can also involve the fungal component of the human microbiota.

EOs have also been shown to have strong antiviral activities, which could affect the gut virome, which is an integral part of the human microbiota [200]. To date, no study has been performed to understand the impact of EOs on the intestinal virome. The main physiological viral component of the gastrointestinal tract is represented by prophages or phages [201]. The bacteriophage component is mainly composed by temperate virus of the Caudovirales order, but most of the detected viral sequences in human gut virome could not be attributed to known viruses [202] and to date it is estimated that the number of virus in human stools is up to 10^9 per gram [203]. Despite it is clear that EOs may impact on the intestinal virome composition by modulating all the microbiota components, it could be really difficult to understand the direct impact of EOs on the intestinal viruses and the consequences of this modulation on the intestinal ecology.

7. Conclusions

The scientific data present in the literature undoubtedly demonstrate that some EOs and some of their components are able to positively modulate the human intestinal microbiota, acting in a differentiated way on pathobiontic microorganisms, without altering or even improving the component of microorganisms defined as healthier commensals. This selective antimicrobial activity is certain for the bacterial component of the intestinal microbiota, conceivable for fungi, but at the moment completely unknown for viruses. It is therefore possible to define with certainty an eubiotic activity for some EOs and some of their components, such as for example geraniol, eugenol, cinnamic aldehyde and limonene, which can properly be considered as eubiotics. Finally, it is interesting to note that the antibacterial activities of these compounds are always multitarget and that for this reason the bacteria are unable to develop resistance. These data associated with the low toxicity of these compounds (by oral administration), suggests that these EOs may be part of a long-term therapy aimed at restoring an eubiotic and resilient microbial ecosystem.

Acknowledgements

The authors thank Dr. Alberto Sardo for illuminating us on the infinite potential of essential oils.

Conflicts of interest

The authors declare no conflict of interest.

Funding

This research received no external funding.

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