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GUT MICROBIOME BASED THERAPEUTICS

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A Word from the Guest Editors

Over the past decade, we have had the delightful opportunity to step into the amazing field of microbiota research. The human body is inhabited by quadrillions of microbes, collectively referred to as the human microbiota, comprising bacteria, archaea, viruses, and eukaryotes in various commensal, mutualistic, or pathogenic interactions with the host, and playing an important role in maintaining general health and wellbeing.

Rapid advances in DNA sequencing, metagenomics and bioinformatics, along with improvements in culturing methodologies, have opened up opportunities for a new era in microbiota research, particularly related to novel therapeutic approaches based on the gut microbiota modulation and development of novel probiotics, postbiotics and prebiotics addressing specific consumer needs and issues.

According to the FAO/WHO, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Lately, the probiotics research field has been greatly advanced, creating vast knowledge that helps to understand the role of microbiota in human health, as well as to encourage the development of novel strategies for microbiota modulation. Recently, the trend of using commensal bacteria as probiotics to restore healthy gut homeostasis in a natural way opened the door to a new kind of probiotics, commonly referred to as Next-Generation Probiotics, based on the cultivation of gut commensal bacteria.

In addition, metabolic by-products, dead microorganisms, or other microbial-based, nonviable products, the so-called postbiotics, have been found to have a great potential to benefit host health. The terms "non-viable probiotics", "paraprobiotics", "ghostbiotics", "heat-inactivated probiotics" or, most commonly, "postbiotics", refer to inanimate microorganisms and/or their components that confer health benefits.

Last but not least, prebiotics, described as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health", can modulate the composition of intestinal microbiota, serving as food for gut microbes. The degradation of prebiotics liberates products such as short-chain fatty acids that are released into blood circulation, consequently affecting not only the gastrointestinal tracts but also other distant organs.

To disseminate what we have learned on this exciting journey to pharmacists, other health professionals, and the wider academic public, we accepted the invitation to edit the special issue entitled "Gut microbiome based therapeutics", collecting the latest knowledge related to new microbiota-based approaches in the prevention and treatment of various diseases.

Guest Editors

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Reč gostujućih urednika

Tokom protekle decenije imali smo divnu priliku da zakoračimo u neverovatno polje istraživanja mikrobiote. Ljudsko telo naseljeno je kvadrilionima mikroba objedinjenih pod imenom „ljudska mikrobiota“, u čiji sastav ulaze bakterije, arheobakterije, virusi i eukariotski mikroorganizmi u različitim komensalnim, mutualističkim ili patogenim interakcijama sa domaćinom, koji igraju važnu ulogu u održavanju opšteg zdravlja i blagostanja ljudi.

Ubrzani razvoj metoda sekvenciranja DNK, metagenomike i bioinformatike, zajedno sa poboljšanjima u metodologijama kultivacije bakterija, otvorili su mogućnosti za novu eru u istraživanju mikrobiote, posebno u oblasti novih terapijskih pristupa zasnovanih na modulaciji crevne mikrobiote i razvoju novih probiotika, postbiotika i prebiotika koji rešavaju specifične potrebe i pitanja potrošača.

Prema FAO/WHO, probiotici su definisani kao „živi mikroorganizmi koji kada se daju u adekvatnim količinama imaju pozitivan zdravstveni efekat na domaćina“. U poslednje vreme je oblast istraživanja probiotika uveliko napredovala, što je rezultovalo akumulacijom velike količine znanja koje pomaže da se razume uloga mikrobiote u ljudskom zdravlju, kao i da se podstakne razvoj novih strategija za modulaciju mikrobiote. Nedavno je trend upotrebe komensalnih bakterija kao probiotika za uspostavljanje crevne homeostaze na prirodan način otvorio vrata novoj vrsti probiotika nazvanih „probiotici sledeće generacije“, koji su zasnovani na kultivaciji komensalnih bakterija iz creva.

Pored toga, utvrđeno je da metabolički nusproizvodi, mrtvi mikroorganizmi ili drugi neživi proizvodi mikroorganizama, nazvani „postbiotici“, mogu da imaju izuzetan pozitivan zdravstveni efekat na domaćina. Termini kao što su „neživi probiotici“, „paraprobiotici“, „gostbiotici“, „probiotici inaktivirani toplotom“, ili, najčešće, „postbiotici“, odnose se na nežive organizme i/ili njihove komponente koje imaju pozitivne zdravstvene efekte na domaćina.

Na kraju, ali ne i najmanje važno, prebiotici, opisani kao „nesvarljivi sastojci hrane koji blagotvorno utiču na domaćina tako što selektivno stimulišu rast i/ili aktivnost jedne ili ograničenog broja bakterija u debelom crevu, i na taj način poboljšavaju zdravlje domaćina“, mogu da modulišu sastav crevne mikrobiote, služeći kao hrana za crevne bakterije. Degradacijom prebiotika nastaju produkti, kao što su kratkolančane masne kiseline, koji se oslobađaju u krvotok, što posledično utiče ne samo na gastrointestinalni trakt, već i na druge udaljene organe.

Kako bismo preneli znanja stečena tokom ovog uzbudljivog putovanja farmaceutima, drugim zdravstvenim radnicima i široj akademskoj javnosti, prihvatili smo poziv da uređujemo specijalno izdanje pod naslovom „Terapeutici zasnovani na crevnoj mikrobioti“, gde smo se potrudili da na jednom mestu objedinimo sva do sada akumulirana znanja vezana za nove terapeutske pristupe u prevenciji i lečenju raznih bolesti putem modulacije crevne mikrobiote.

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The Microbiome-Gut-Brain Axis in Multiple Sclerosis

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Abstract

The microbiome-gut-brain axis (MGBA) represents a close two-way relationship between the gut and the central nervous system (CNS) mediated by the immune system, the enteric nervous system (ENS), the vagus nerve, and the gut microbiome. Gut microbes, including bacteria, fungi, and viruses, can communicate with the CNS and modulate the physiology of the brain in health and disease, which marks them as an important MGBA factor. It is becoming increasingly evident that gut microbiome dysbiosis is implicated in the onset and severity of different neurodegenerative and psychiatric diseases including multiple sclerosis (MS). MS is a chronic disease of the CNS associated with different genetic and environmental risk factors. Neuroinflammation and demyelination in the brain and the spinal cord are hallmark features of MS. The accumulating evidence shows that the MGBA, although a relatively new concept, has an important role in MS. Therefore, the purpose of this article is to review recent research on the gut-brain connection in MS, and to highlight MS-associated gut microbiota constituents and the role of bacterial metabolites in MS.

Key words: gut microbiome, multiple sclerosis, gut-brain axis, bacterial metabolites, dysbiosis

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Microbiome in the gut-brain axis

There has been an epidemic of various neurodegenerative and autoimmune diseases, strongly associated with the modern lifestyle. Among them, neurodegenerative disorders are a huge burden on society, impairing the health and the quality of life of affected people and their families, as well as impacting society as a whole. Neurodegenerative diseases are a heterogeneous group of disorders characterized by the progressive degeneration of the structure and function of the central (CNS) or peripheral nervous system (PNS), with the most prevalent ones being dementia (more than 55 million people worldwide with Alzheimer disease [1] and Parkinson's Disease [2]), amyotrophic lateral sclerosis, synucleinopathies, Huntington disease and related polyglutamine diseases, prion disease, traumatic brain injury, chronic traumatic encephalopathy, stroke, spinal cord injury (3), and multiple sclerosis (MS; 2.8 million people globally [4]). Most of the neurodegenerative disorders in children and adults are considered multifactorial diseases prompted by environmental factors in genetically susceptible individuals (5). A number of preclinical and clinical studies indicate that patients affected by neurodegenerative diseases have gastrointestinal (GI) dysfunction, accompanied with alterations in the diversity and composition of gut microbiota and the microbiome-gut-brain axis (MGBA) as one of the common denominators (6, 7, 8, 9, 10). Gut microbiota is a term that refers to the bacteria, archea, fungi, viruses and protozoans residing in the gut, while gut microbiome includes microorganisms and their genetic material and metabolites (11). The MGBA is a term intended to describe the interactions between the host and gut microbiota, together with the effects within these interactions that have an impact on the CNS (Figure 1). Over the past decade, the MGBA has become appreciated as bidirectional communication between gut microbiome and the CNS, exerting a profound influence on neural development, neuroinflammation, activation of stress response, neurotransmission, and modulation of complex behaviours (12). Gut microbiota regulates host production of different molecules with known neuromodulatory properties, including endocannabinoids, neuropeptides and biogenic amines (13). Of these, the hormone and neurotransmitter serotonin (5-hydroxytryptamine (5-HT)) is expressed highly in the GI tract and regulated by gut microbiota, particularly the spore-forming bacteria dominated by families *Clostridiaceae* and *Turicibacteraceae* (14). Several bacterial taxa were found to be commonly disturbed in various neurodegenerative diseases, most of which are "anti-inflammatory" short-chain fatty acids (SCFA)-producing bacteria (15). In particular, *Firmicutes* (*Fecalibacterium*, *Anaerostipes*, and *Turicibacter*), *Bacteroidetes* (*Prevotella*, *Parabacteroides*), *Actinobacteria* (*Adlercreutzia* and *Collinsella*), *Lachnospiraceae* were found to be disturbed in the animal model of MS (9). Interestingly, besides changes in the gut microbiota composition, changes in metabolic pathways were observed as well, where SCFAs, major end-products of bacterial fermentation, decreased anxiety- and depressive-like behaviour in mice (16, 17). In addition, gut microbiota is an important regulator of γ-aminobutyric acid (GABA) and host tryptophan (TRP) metabolism along the kynurenine pathway, which both have implications for depressive disorder (18, 19). However, it is unclear how gut microbiota dysbiosis can trigger potential immunological changes in the

CNS in the presence of the blood–brain barrier (BBB), as well as how members of gut microbiota influence the MGBA. The gut microbiome can potentially influence these central processes through modulation of the immune system, production of neurotransmitters, through the regulation of gut barrier permeability, the increase of circulating lipopolysaccharide (LPS), alteration of neuroendocrine (hypothalamic-pituitary-adrenal [HPA] axis) and neural (e.g. vagus afferents, enteric nervous system) pathways (13, 20, 21). Decreased microbiota diversity seems to be one of the most consistent findings in gut microbiome dysbiosis, repeatedly associated with the modern lifestyle and autoimmune diseases, including gut microbiota from neurological patients (10). Recently, the gut microbiome has been shown to have a direct influence on the brain by modulating the immune system. Some evidence suggests that dysbiosis and increased gut permeability allow the translocation of bacteria or their metabolites from the lumen and induction or exacerbation of the immune response (e.g. production of pro-inflammatory cytokines tumor necrosis factor α [TNF-α], interleukin (IL)-6 and IL-1β). Peripheral inflammation, which may increase BBB permeability, is causatively implicated in the pathogenesis of neurological disorders (22). Furthermore, progress in MS treatment was achieved by interventional therapies on gut microbiota diversity and the metabolic traits of the microbiome, as well as by the use of probiotics in the treatment of experimental autoimmune encephalomyelitis (EAE), the animal model of MS (23–25). Currently, there is not enough evidence supporting the beneficial effects of gut microbiome manipulation in neurodegenerative and psychiatric diseases. Thus, further clinical and preclinical investigations are needed to specifically identify and counteract MGBA dysregulation.

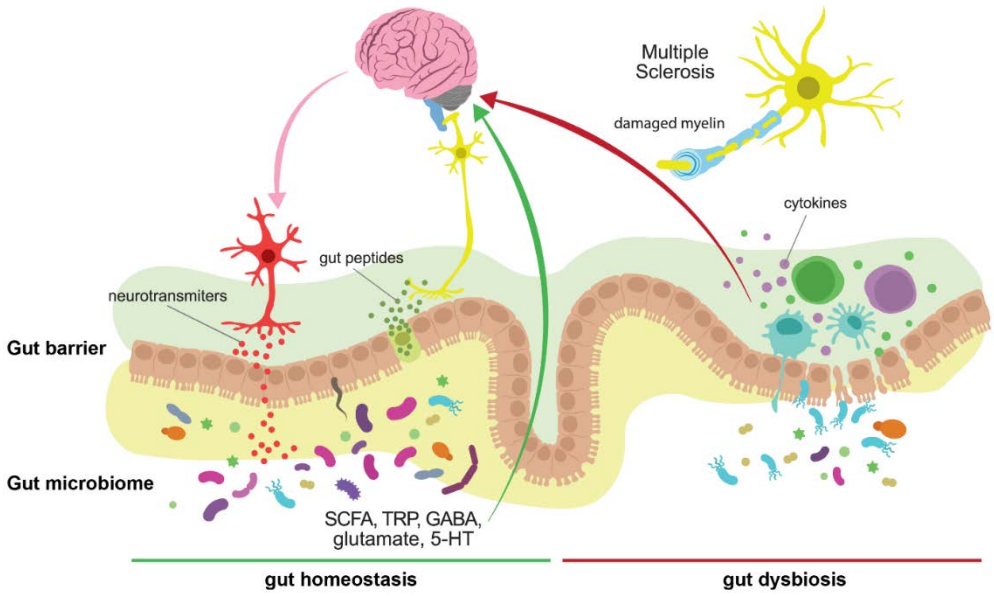

Figure 1. **The microbiome-gut-brain axis in the context of multiple sclerosis. SCFA, short chain fatty acid; TRP, tryptophan; 5-HT, 5-hydroxytryptamine.**

Slika 1. **Osovina crevo-mozak u kontekstu multiple skleroze. SCFA, masne kiseline kratkog lanca; TRP, triptofan; 5-HT, 5-hidroksitriptamin.**

The underlying pathology of multiple sclerosis

MS is a complex neurological disorder characterized by chronic autoimmune inflammation, demyelination, and neurodegeneration within the CNS (26). The exact etiology of MS remains elusive, but it is widely accepted that a combination of genetic predisposition, environmental factors, and dysregulated immune responses contributes to its development. It has been estimated that around 2.8 million people live with MS across the globe, with women being diagnosed more often than men (4). MS often occurs in early adulthood, between the ages of 20 and 40. MS can, however, arise in children and elderly individuals, though less frequently (27). Females are more likely than males to develop MS. The female-to-male ratio fluctuates but is considered to be around 3:1, indicating that the risk of developing MS is about three times higher for women compared to men (28). MS clinical manifestations might vary greatly from person to person. Fatigue is one of the most prevalent and debilitating symptoms of MS, and it can have a substantial impact on everyday living. Sensory symptoms include numbness, tingling, and burning sensations, which are most commonly felt in the limbs. Motor symptoms can include weakness, muscle spasms, and coordination issues. Optic neuritis, a common early sign of visual disruption in MS, may manifest as impaired vision, eye discomfort, or even temporary vision loss. Challenges in balance and coordination can restrict mobility and walking for individuals with MS. Cognitive irregularities, such as memory problems and reduced concentration, may arise in some cases, alongside mood fluctuations, depression, and anxiety, which are frequently observed in those affected by the condition (29).

MS comes in several subtypes, including the most common relapsing-remitting MS (RR-MS) and three other subtypes: secondary progressive MS (SP-MS), primary progressive MS (PP-MS), and progressive-relapsing MS (PR-MS) (30). RR-MS is characterized by instances of relapse or exacerbation, during which new symptoms or a worsening of existing ones occur, followed by periods of partial or total recovery (remission). Individuals frequently enjoy periods of stability in between relapses. Some people progress from RR-MS to SP-MS after a period of time. During the course of SP-MS, symptoms worsen gradually and steadily, with or without periodic relapses and remissions, and disabilities tend to accumulate more steadily. The PP-MS subtype is less prevalent than RR-MS, and it is distinguished by a progressive and constant escalation of disability from the outset of symptoms, with no discernible relapses and remissions. PR-MS is a less common subtype in which people have a continuous progression of disability from the start, but also have apparent relapses or exacerbations along the way (31). Our current understanding of MS has largely been shaped by research conducted on the experimental model for human inflammatory demyelinating diseases, such as EAE. EAE is a complex condition in which the interaction of several immunopathological and neuropathological pathways results in pathological hallmarks of MS: inflammation, demyelination, axonal loss, and gliosis (32).

Here we discuss recent findings about the MS underlying pathophysiology, with an accent on the role of the MGBA in this complex disabling neurological disease.

Role of genetic and environmental factors

An important factor in the onset and development of MS is the interaction between environmental stimuli and genetic predisposition (33). Research by Brynedal and colleagues (34) has confirmed the significance of genetic variations in MS development, particularly in the Human Leukocyte Antigen (HLA) region, which influence immune responses and increase susceptibility to MS. The influence of the HLA complex is not uniform. The HLA-DRB115*:01 variant from the class II repertoire appears as a powerful factor, with a strong association with increased MS risk. With an odds ratio (OR) of roughly 3, the role of HLA-DRB115*:01 in enhancing MS susceptibility is clear. On the other hand, the class I variant HLA-A*02 plays the opposite role, with an OR of around 0.6 associated with MS protection, which emphasizes its potential to protect against the disease (35). In addition to MHC molecules, various non-MHC genes and genetic variants have been linked to susceptibility or protection against MS. Among these are IL2RA (Interleukin-2 receptor alpha), CD58 (Lymphocyte function-associated antigen 3, LFA-3), CD226 (DNAX accessory molecule-1, DNAM-1), CYP27B1 (Cytochrome P450 27B1), TNFRSF1A (Tumor necrosis factor receptor superfamily member 1A), IRF8 (Interferon regulatory factor 8), IL7R (Interleukin-7 receptor), and TYK2 (Tyrosine kinase 2). These genes play distinct roles in immune system regulation, activation, and response. Immune function can be affected by genetic differences within these genes, thereby increasing the likelihood of MS development (36). These genetic factors, however, do not solely determine disease susceptibility; environmental factors such as vitamin D deficiency, infections, and smoking have also been implicated. An early study by Munger and colleagues (37) underscores the importance of vitamin D in regulating immune responses and its potential role in modulating MS risk. Later research on supplementation and sun exposure has validated the function of vitamin D in lowering the risk of MS (38, 39). Vitamin D impact extends beyond risk reduction, as higher levels correlate with diminished axonal damage, highlighting its broader neuroprotective potential (40). Additionally, the work of Santiago and colleagues (41) emphasizes the intricate link between Epstein-Barr virus (EBV) infection and MS risk, suggesting a potential role for viral persistence in triggering autoimmune responses. Individuals with MS have been linked to increased antibodies targeting EBV nuclear antigen 1 (EBNA1) a specific section (amino acids 385-420) (42). Nested case-control research revealed that nearly all EBNA1-negative people had progressed to EBNA1 antibody positive prior to the beginning of MS (43). The pivotal studies, undertaken in recent years, have significantly advanced our understanding of the link between obesity and MS risk. Large-scale cohort investigations have decisively established a robust correlation between obesity during adolescence and an elevated risk of MS in the future, particularly among females (33). The association between smoking and MS risk was first proposed in small studies, with an OR of 1.5, and was later confirmed in a large case-control study (33). Importantly, smoking and MS risk have a dose-response connection, with cumulative smoking exposure corresponding with increased susceptibility, and even passive smoking enhanced the risk of MS (44). More recently, gut microbiome dysbiosis has been

recognized as a key environmental factor leading to the development of MS, which will be discussed further below.

Immunological Dysregulation and Inflammatory Responses

MS is an autoimmune disorder driven by dysregulated immune responses against self-antigens within the CNS (45). MS involves an autoimmune response against myelin components in the CNS. Some of the myelin proteins implicated in MS include myelin basic protein (MBP), a major component of the myelin sheath and one of the primary targets of the immune response in MS, proteolipid protein (PLP), another key myelin protein that can be targeted by the immune system in MS, and myelin oligodendrocyte glycoprotein (MOG), found on the surface of myelin sheaths (46). Our understanding of the underlying immunopathophysiology of MS has evolved, revealing the central involvement of various immune cell types in both the PNS and CNS. This complex network of interactions between immune cells, such as peripheral T cells, B cells, and myeloid cells, as well as resident CNS cells like microglia and astrocytes, is crucial to the pathophysiology of the disease. These immune responses lead to the secretion of inflammatory mediators that recruit inflammatory cells to the CNS across damaged BBB, resulting in neuronal demyelination and CNS inflammation (47).

$CD4^+$ T cells, specifically T helper cells (Th)1 and Th17 cells, are key players in the MS development (48). The pro-inflammatory milieu in MS involves the production of cytokines such as TNF-α, IL-12, IL-6, IL-23, and IL-1, all of which influence Th1 and Th17 cell development (49). Th1 cells play a central role in the disease's progression, orchestrating a cascade of immune responses that contribute to the characteristic demyelination and neuroinflammation seen in MS (50). Th1 cells are characterized by their secretion of pro-inflammatory cytokines, most notably interferon-gamma (IFNγ). Within the context of MS, these cells are implicated in driving the immune response towards a pro-inflammatory profile. IFN-γ in particular has been linked to the activation of immune cells such as astrocytes and microglia in the CNS, which exacerbates the inflammatory environment (51). The aberrant activation of Th1 cells in MS is intricately linked to antigen presentation by antigen-presenting cells (APC)s, including B cells and myeloid cells such as macrophages and dendritic cells. These APCs present CNS-specific antigens to Th1 cells, fueling their activation and the subsequent immune response targeted at CNS tissue (52).

Th17 cells, which produce IL-17, and $CD8^+$ T cells are implicated in direct injury of astrocytes, oligodendrocytes, and neurons. These immune cells can also indirectly cause tissue damage by activating other immune cells, such as macrophages (53). Th17 cells collaborate with Th1 cells to induce a pro-inflammatory environment in the CNS. IL-17, best known for its function in extracellular bacterial and fungal defense, has a strong effect on astrocytes. By synergizing with other cytokines, Th17 cells amplify the secretion of proinflammatory cytokines (such as IL-6, Granulocyte Macrophage Colony-Stimulating Factor [GM-CSF], and TNF-α), chemokines, and effector proteins, contributing to immune pathology and neuroinflammation (48). This synergistic effect

enhances neuroinflammation and tissue damage, contributing to the clinical manifestations of MS.

$CD8^+$ T cells are notably more abundant in both white and grey matter demyelinating lesions and closely correlate with axonal damage. Their activation and response, as well as epitope spreading, play a significant role in MS pathogenesis (54). Immune cells in MS lesions lead to myelin loss, oligodendrocyte damage, and axon damage, all of which contribute to neurological disability. $CD8^+$ T cells carry cytolytic granules containing perforin and granzyme molecules that are polarized toward demyelinated axons and will release them to kill oligodendrocytes and neurons (48). When these lesions are inflamed, the body activates immune-modulating systems to suppress the immune response and initiate repair processes, which can result in partial remyelination and clinical improvement (55). However, in the relapsing form of the disease, despite these repair attempts, over 80% of patients experience disease progression (48).

A potential cause of aberrant effector T cell activation in MS is the inadequacy in the function of regulatory T (Treg) cells, coupled with the resistance of CNS-specific effector T cells to Treg cell-mediated regulation. Abnormalities in circulating Treg cells, including decreased expression of FOXP3, have been observed and are implicated in MS. These regulatory cells are crucial for maintaining immune homeostasis (56).

Building upon our understanding of MS pathogenesis, B cells have emerged as significant contributors, and therapies directed at B cells have displayed potential. Notably, pro-inflammatory B cells, particularly $CD27^+$ GM-CSF-expressing memory B cells, are more prevalent in the bloodstream of MS patients. These B cells play a crucial role in driving abnormal Th1 and Th17 cell responses by secreting cytokines such as TNF-α and IL-6, ultimately provoking pro-inflammatory responses in myeloid cells, predominantly through GM-CSF (57).

Neurodegeneration and Remyelination Impairment

While inflammation and demyelination are hallmark features of MS, the disease also encompasses neurodegenerative processes that contribute to irreversible neurological deficits (58). Lesions occur in both white matter and grey matter and are typically found throughout the CNS, including the brain, optic nerve, and spinal cord (26). In the early stages of MS such as Clinically Isolated Syndrome (CIS) and RR-MS, characterized by active demyelinating lesions, there are active areas in the brain with a lot of antigen-specific immune cells like $CD8^+$ T cells and $CD20^+$ B cells (59). These areas also have activated microglia, macrophages, containing myelin debris, and large, reactive astrocytes (60). However, as MS progresses to SP-MS and PP-MS, these active areas become less common. Instead, there are areas with fewer active cells and clear signs of damage, but they are not actively getting worse (61, 62). There are also other types of damaged areas, like chronic active plaques, which are more common in people with longer-lasting MS. These have a different pattern of cell activity. Slow expanding lesions are also seen in people with SP-MS, and they show very slow damage to the brain's

protective covering, with fewer cells involved, but still causing damage over time (26). Gray matter damage in MS patients begins early in the disease and can be more extensive in those with PP-MS and SP-MS, involving more than 60% of the cortex of the brain in severe cases (63). Grey matter lesions can also appear in deep brain structures and the grey matter of the spinal cord, where they are more widespread than in the white matter (64). These grey matter lesions often form in the cortical sulci and in deep invaginations of the brain and are linked to inflammation in the brain's meninges (65). Interestingly, these grey matter lesions are different from the more common white matter lesions seen in MS, tend to have less disruption of the BBB, less swelling, and fewer infiltrating activated microglia and macrophages (66). Additionally, they can lead to the loss of nerve connections, brain cells, and synapses (67). Remyelination, a critical repair mechanism, is impaired in MS due to factors such as the presence of inhibitory molecules and insufficient oligodendrocyte precursor cell recruitment (68). Understanding these complex issues is essential for creating ways to enhance remyelination and slow disease progression.

Intestinal microbiota biomarkers of multiple sclerosis

Although genetic predisposition is important and may play a significant role in the MS onset, growing evidence suggests that interactions between gut microbiome and immune system are crucial for the development of MS (7, 69).

Among microbiome members, researchers are mostly focused on the gut bacteria, with little attention given to the contribution of fungi, parasites or viruses to MS development and severity. Although fungal components make up a smaller proportion of the gut microbiome, fungi have a significant impact on human health (70). Probably the first report on this subject was made by Truss in 1981, where he reported amelioration of symptoms in several MS patients following nystatin treatments (71). A recent case-control observational study showed that people with MS have higher fungal alpha diversity and increased relative abundances of *Saccharomyces* and *Aspergillus* genera when compared to healthy subjects (72). Another study reported the presence of antibodies against *Candida* in the cerebrospinal fluid of MS patients (73). Interestingly, one study reported increased fungal to bacterial ratio in RR-MS patients (74). Further research is also needed in order to better understand the role of gut mycobiome in MS, which should not be underestimated.

The role of viruses in MS development is proposed based on the findings of viral genetic material and antiviral antibodies in the cerebrospinal fluid and blood of patients with MS (75). Besides EBV, human herpesvirus 6 (HHV-6) has been linked to MS and is notably more prevalent within MS plaques when compared to EBV in both MS and non-MS brain white matter. Intriguingly, reactivation of HHV-6 has been observed during clinical relapses in MS (76).

In addition to microbiome, emerging evidence suggests that members of microbiome such as certain parasites, particularly helminths, and protozoa, may confer protective effects in the context of MS. Multiple studies have lent support to the notion

that parasitic infections, such as those caused by *Toxoplasma gondii* and *Schistosoma mansoni*, demonstrate a protective effects in humans (77) and in the C57BL/6J mice model of MS (78). Notably, a study involving *Trichinella spiralis* reveals that infection with L1 stage muscle larvae (TSL1) is associated with a reduction in CNS inflammation in EAE induced Dark Agouti rats (79). Consequently, parasites are recognized as potential risk-reduction factors in the development of MS.

However, a number of studies have provided evidence that alternations in bacteriobiota, the bacterial component of the gut microbiome, are associated with MS development and severity. An early study by Goverman and colleagues found that transgenic mice expressing MBP-specific T cell receptors (MBP-TCR) develop MBP-Complete Freund's adjuvant (CFA) induced EAE when housed in non-sterile conditions, while they remained healthy under specific pathogen-free (SPF) conditions (80). Another study demonstrated that C57BL/6 mice maintained under germ free (GF) conditions after immunization with MOG/CFA exhibited attenuated symptoms of EAE, and that colonization with segmented filamentous bacteria (SFB) promoted EAE development (81). In contrast to this study, Berer and colleagues demonstrated that monocolonization with SFB of GF SJL/J mice expressing MOG-TCR was not effective in the promotion of EAE, and colonization with conventional commensal microbiota prompted EAE development (82). In addition, these authors showed that without induction, EAE spontaneously occurred in SPF-bred animals, while GF-bred animals remained EAE-resistant (82). Importantly, transplantation of an MS patient’s gut microbiota into EAE-induced GF mice resulted in increased EAE activity and severity compared to mice colonized with healthy donors’ gut microbiota (83).

Several studies have revealed connections between antibiotic-induced microbial reduction and EAE development (84, 85). Early studies showed that a short-term oral antibiotics treatment one week prior to immunization leads to gut microbiota alternations associated with a decreased Th17 level in mesenteric lymph nodes and EAE amelioration (84), as well as proinflammatory cytokines depletion, anti-inflammatory cytokines increase, and Treg cell-dependent reduction of disease severity in a $PLP_{139–151}$/$MOG_{35–55}$ induced EAE model (85). Interestingly, our previous results suggested that antibiotics exposure during the prenatal and neonatal period of EAE-susceptible Dark Agouti (DA) rats has long term effects, reflected in increased disease severity after immunization later in their lives, even though gut microbiota was restored (9). Today we know that gut microbiome can affect the host’s immune system, BBB integrity and function, and autoimmune demyelination (86).

Over the years, researchers have uncovered certain bacterial taxa that are associated with both MS and EAE (Figure 2). Several studies reported a significantly increased relative abundance of *Akkermansia* genus in MS patients (83, 87, 88). Considering that commensal species *Akkermansia muciniphila* is involved in mucin turnover in the gut and production of acetate and propionate, one study suggests that its increased abundance in MS is probably a consequence of the disease (89). This is something to keep in mind when studying host-microbiota interactions, because modulation in the gut microbiome

can be either a cause or a consequence of disease. Another species with increased relative abundance in MS is *Acinetobacter calcoaceticus,* known for its role in molecular mimicry of MBP and MOG and reduction of Treg proportion in peripheral blood mononuclear cells (PBMC) *in vitro* (83, 90). Moreover, relative abundances of two genera from family *Lachnospiraceae*, *Blautia*, and *Dorea*, are found to be increased in faecal samples of MS patients (91). A study by Schepici and colleagues showed an increased abundance of the genus *Streptococcus* in MS patients, which is in line with results by other authors (92, 93). Our previous results show increased prevalence of the genus *Romboutsia* and family *Peptococcaceae* in EAE-induced DA rats (94). Besides commonly MS-associated bacteria, several studies have reported an increased abundance of the archeal genus *Methanobrevibacter* in MS patients (88).

On the other hand, the relative abundance of several genera such as *Prevotella*, *Bacteroides*, *Parabacteroides*, *Collinsella*, *Adlercreutzia*, *Lactobacillus*, *Clostridium*, *Anaerostipes*, *Butyricicoccus*, and *Faecalibacterium* decreased in MS patients compared to healthy controls (88, 91, 92). It is interesting that, among these MS negatively associated bacteria, the *Prevotella*, *Parabacteroides*, *Lactobacillus* and *Butyricicoccus* genera are well-known SCFA producers (95–97), while *Faecalibacterium prausnitzii* species are reported to be the main butyrate producers in the gut (98).

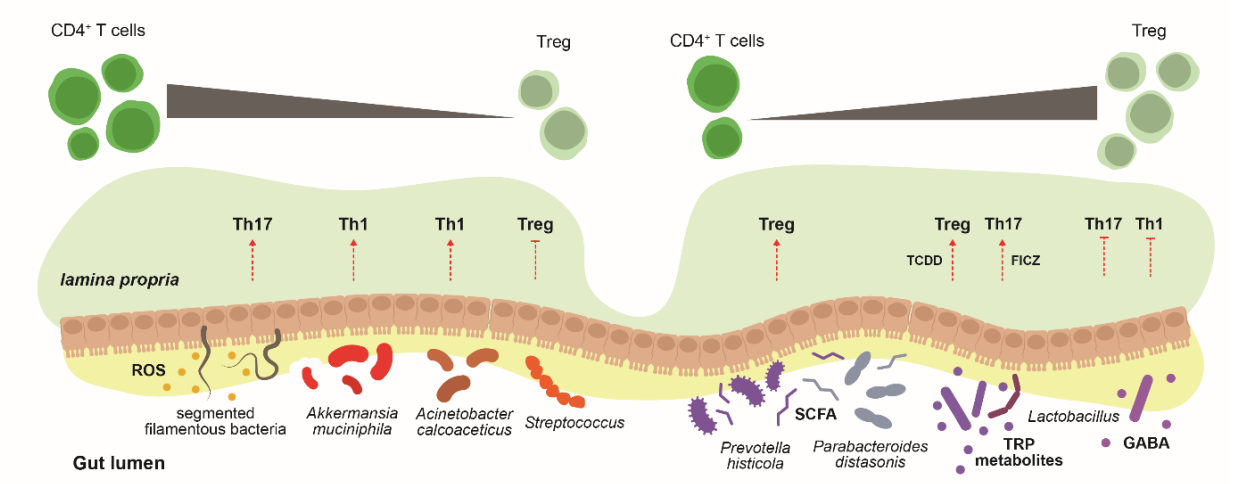

Figure 2. Gut microbiota and its metabolites in multiple sclerosis. ROS, reactive oxygen species; Th, T helper cell; Treg, regulatory T cell; SCFA, short chain fatty acid; TRP, tryptophan; GABA, γ-aminobutyric acid; TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin; FITZ - 6-formylindolo(3,2-b)carbazole.

Slika 2. Mikrobiota creva i njeni metaboliti kod multiple skleroze. ROS, reaktivne kiseonične vrste; Th, pomoćničke T ćelije; Treg, regulatorne T ćelije; SCFA, masne kiseline kratkog lanca; TRP, triptofan; GABA, γ-aminobuterna kiselina; TCDD - 2,3,7,8-tetrahlorodibenzo-p-dioksin; FITZ - 6-formilindolo(3,2-b)karbazol.

Gut microbiota-related metabolites in multiple sclerosis

Short-chain fatty acids play a major role in multiple sclerosis

SCFAs are small organic molecules with important physiological properties in the context of gut microbiome-host interactions. They are the products of fermentation of complex indigestible polysaccharides facilitated by certain bacterial species in the host colon. The most abundant SCFAs in the MGBA are acetic acid, propionic acid, and butyric acid (99). A myriad of properties have been ascribed to these structurally simple molecules, from being important energy sources for the colon epithelium to exhibiting potent immunomodulatory effects, in addition to strengthening the intestinal barrier (100). Besides gut barrier protection, SCFAs can cross the BBB as signal molecules, regulate its permeability and modulate GBA (101). Several studies reported a reduction of SCFAs level in fecal samples of RR-MS patients (102, 103). Acetate, propionate and butyrate levels in particular were observed to be significantly lower or even depleted in MS patients (102). Relative fecal SCFA levels in patients have also been shown to correlate with the severity of disease, expressed in terms of the level of MS-induced disability, resulting in lower relative abundance of butyric and caproic acids and higher relative abundance of acetic acid in patients with higher Expanded Disability Status Scale scores (EDSS). Reduced SCFA levels have also been observed in sera of these patients (104). Among gut microbiome members, it is known that different bacterial taxa are involved in SCFAs production from dietary fibers, such as genera *Prevotella* (105), *Butyricimonas* (106), *Bifidobacterium, Veillonella* and species *Faecalibacterium prausnitzii*, *Eubacterium hallii* and *Phascolarctobacterium succinatutens* (107). For example, *Prevotella* deficiencies are claimed to be unique features of the gut microbiota of MS patients, and a direct correlation has been observed between the abundance of the *Prevotella* genus in the gut and the levels of fecal acetate and propionate (88, 91, 102). Many studies associated fecal and serum levels of SCFAs with MS progression and immune cell differentiation in MS patients (102, 104, 108). Thus, significant negative correlations between the *Streptococcus* and *Prevotella* genera abundance and peripheral Treg (pTreg) and Th17 cells have been revealed respectively (Figure 2). Finally, total SCFA levels have been shown to positively correlate with the proportion and function of pTreg cells (102, 104).

The effects of SCFA supplementation as a therapeutic approach for treating MS have been studied. Propionic acid supplementation in particular was shown to have immunomodulatory and neuroprotective effects (104). These disease-ameliorating properties of propionic acid, when taken as an oral supplement, stem from the enhanced IL-10 mediated suppressive function of Treg cells. It additionally led to a decreased proportion of Th1 and Th17 cells, and an increased presence of pTreg cells. A decrease in relapse rates and a stabilization of disability were also observed in long-term supplementation. Keeping in mind the fact that propionic acid is mainly produced by gut bacteria, these results suggest a significant impact of the gut microbiome on the pathophysiology of MS.

Similar results have been achieved with oral supplementation of *Prevotella histicola*, a known producer of propionic acid among other SCFAs. A recent study has shown that the administration of this bacterium can lead to the suppression of EAE in mice, as evidenced by a decrease in Th1/Th17 cells, an increase in Treg cells, tolerogenic dendritic cells and suppressive macrophages, and reduced demyelination in the CNS (109).

Protective role of intestinal γ-aminobutyric acid in multiple sclerosis

GABA is an inhibitory mediator of the CNS and its levels are decreased in MS patients (110, 111). Certain bacteria in the gut are capable of producing GABA, granting the gut microbiome a particularly potent role in regulating both the CNS and PNS (112). *Lactobacillus* species have emerged as especially relevant gut microbiome constituents when it comes to the GABA-mediated regulation of the host's nervous system, with the most important GABA producer in the gut being *Lactobacillus brevis* (113). Our previous results showed that oral administration of *L. brevis* BGZLS10-17 alleviates EAE symptoms in DA rats (114). Finally, the neuroprotective properties of GABA are assumed to stem from its immunosuppressive potential, as suppression of APCs by GABA through decreased MAPK signaling leads to a dampening of the inflammatory immune response to myelin antigens (115).

Role of tryptophan derivatives and bile acids in multiple sclerosis

L-tryptophan is an essential amino acid found in foods like meat and legumes, and it is subject to biotransformation in the gut, both by the host and the gut microbiota (116). TRP and end products of its transformation are transported out of the gut into circulation. TRP metabolism is a complex network of metabolic pathways, but it can be roughly grouped into three branches: the kynurenine pathway, the serotonin pathway and the indoles pathway (117). Most of the production of the metabolites of the first two pathways is attributed to the host, while gut bacteria produce most of the indole and indole derivatives. Many of these metabolites can act as aryl hydrocarbon receptor (AhR) ligands, granting them immuno- and neuroprotective properties.

MS has been associated with altered levels of TRP metabolites (118). Quinolinic acid (QA) and kynurenic acid (KA) have both been shown to be elevated in MS, with QA exhibiting neurodegenerative and neuroinflammatory effects, and KA potentially playing a neuroprotective role (119). Both metabolites have been detected in increased levels in MS patients, but with QA being increased significantly more than KA, leading to accumulation of damage to the nervous system.

TRP metabolites shed light on the importance of gut microbiota. Higher levels of TRP byproducts containing indole, primarily generated by gut bacteria, have been linked to milder disease symptoms. Additionally, having more genes related to TRP breakdown in the gut microbiota is connected to a reduced risk of experiencing disease relapses (118). Certain indole derivatives, like indole-3-propionic acid (IPA), have been shown to improve intestinal epithelial barrier integrity by promoting tight junction formation (120).

Secondary bile acids are another type of important bacterially derived metabolites that have been linked to MS. Decreased serum levels of certain secondary bile acids have been reported both in mouse models of EAE and in MS patients (121, 122). A decrease in the abundance of *Clostridium* cluster XIVa species in MS patients accompanies this change in secondary bile acids levels (123). MS-specific neuroprotection mediated by secondary bile acids and/or their producers in the gut is an ongoing topic of research, and the link mostly extends to statistical correlations.

Conclusion

Considering further directions in the gut microbiome-MS association research, it is important to note that the differences in levels of bacterially derived metabolites and microbiota composition between MS patients and healthy controls are not universal across different geographic locations and further depend on factors such as diet, body-mass index, sex, age and ethnicity (88, 91, 103). Efforts are underway to accumulate data with these important considerations in mind, in order to come to more relevant conclusions, which will help determine the true role of bacterially derived metabolites in MS pathophysiology. Importantly, experimental models should be developed to investigate the potential causative relationship between metabolites levels and gut microbiota composition and MS pathogenesis. Finally, an emerging group of probiotics commonly referred to as neurobiotics, which could be used in treatment of neurodegenerative disorders, might revolutionize the treatment of specific psychiatric and neurodegenerative disorders.

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Mikrobiom-crevo-mozak osovina kod multiple skleroze

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Kratak sadržaj

Mikrobiom-crevo-mozak osovina (MGBA) predstavlja blisku dvosmernu vezu između creva i centralnog nervnog sistema (CNS) posredovanu imunskim sistemom, enteričnim nervnim sistemom (ENS), nervom vagusom i mikrobiomom creva. Posredstvom metabolita koje produkuju, mikroorganizmi creva, uključujući bakterije, gljive i viruse, komuniciraju sa CNS-om i tako utiču na funkcije mozga, zbog čega je mikrobiota creva prepoznata kao veoma važan faktor održavanja homeostaze MGBA. Takođe, veliki broj podataka ukazao je na povezanost disbioze mikrobioma creva i nastanka i težine simptoma različitih neurodegenerativnih i psihijatrijskih bolesti, uključujući multiplu sklerozu (MS), autoimunsku bolest nervnog sistema. MS je hronična bolest CNS-a povezana sa više genetskih faktora, kao i sa različitim sredinskim faktorima i životnim navikama. Najvažnija obeležja MS su neuroinflamacija i demijelinizacija u mozgu i kičmenoj moždini, a veliki broj istraživanja je ukazao i na specifične mikrobijalne markere ove bolesti. Cilj ovog rada je da pruži pregled najvažnijih podataka o povezanosti promena u sastavu i funkciji mikrobiote creva i patoloških promena karakterističnih za MS.

Ključne reči: mikrobiom creva, multipla skleroza, crevo-mozak osovina, metaboliti bakterija, disbioza

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Impact of gut microbiota on immune reactions relevant to lung pathologies

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Abstract

Bacterial microbiota of the gastrointestinal tract is known to prevent the invasion of pathogenic microorganisms and regulate intestinal permeability, digestion, metabolism, and immune response. It affects function, homeostasis, and disease outcomes in the gastrointestinal tract and extra-intestinal sites such as the lungs. This review summarizes the currently available knowledge regarding the gut-lung axis. The association of bacterial composition and/or dysbiosis in the gut with asthma, chronic obstructive lung disease, cystic fibrosis, recurrent respiratory tract infections, and lung cancer in humans is highlighted, as well as data obtained from animal models of pulmonary inflammation, which indicated that modulation of immune system activity lies at the base of this interaction. Additionally, the potential use of prebiotics, probiotics, and postbiotics in the treatment of lung inflammation is presented.

Key words: gut bacterial microbiota, gut-lung axis, lung inflammation

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Introduction

Although the term microbiota includes different microorganisms such as bacteria, viruses, fungi, and protozoa distributed over different body surfaces in humans and animals, bacterial microbiota of the gastrointestinal tract (GIT) is the most studied. There is a large amount of data regarding gut bacterial microbiota composition and their role in preventing the invasion of pathogenic microorganisms, intestinal permeability, digestion, metabolism, and immune response (1). The impact of gut microbiota composition and dysbiosis on homeostasis in the gastrointestinal tract and its relationship with various diseases in the GIT has been extensively studied. In recent years, the impact of gut microbiota on distal sites such as the brain (2), skin (3), or lungs (4) has been shown, leading to the coining of terms such as the gut-brain, gut-skin, or gut-lung axis. These new concepts investigate mechanisms by which bacterial microbiota in the GIT affects function, homeostasis, and disease in extra-intestinal sites. Examining the interaction between the gut and lungs might be interesting as these organs have the same embryonic origin (both alveolar and intestinal epithelia develop from the endoderm, and have physical, chemical, and physiological barrier functions), have specific microbiota, and are part of the common mucosal immune system. Additionally, due to their same embryonic origin, both the gastrointestinal and respiratory systems share an entrance (oral cavity) through which microorganisms from the external environment gain access to the host.

The respiratory system (and the lungs), besides gas exchange as its main physiological role, protects individuals from harmful substances present in the air (such as particles, pollen, dust, bacteria, viruses, etc.) by the production of mucus and the activity of cilia. Various xenobiotics to which lungs are continuously exposed might affect their function, resulting in many conditions and disorders of which some are minor and temporary, while others are chronic and more severe. The most common lung disorders include asthma, chronic obstructive lung disease (COPD), cystic fibrosis (CF), lung cancer, bacterial (*Mycobacterium tuberculosis*), viral (respiratory syncytial virus/RSV, influenza virus, severe acute respiratory syndrome coronavirus 2/SARS-CoV-2) or fungal (*Aspergillus fumigatus*) infections. The immune system is relevant for the development and/or progression of each mentioned disease. For example, childhood asthma develops in susceptible (atopic) individuals following an encounter with various environmental allergens that results in the activation of the T helper (Th) 2 response (production of interleukin (IL)-4, IL-5, IL-13), migration of the eosinophils to the lungs and production of immunoglobulin (Ig) E (5). The development of COPD is mainly associated with the immune response to chronic inhalation of cigarette smoke, characterized by an increased number of immune cells (macrophages, neutrophils, lymphocytes and dendritic cells) in the lungs, impaired macrophage function (reduced phagocytosis), increased production of reactive oxygen and nitrogen species, and increased proinflammatory response (interferon (IFN)-γ, and IL-17) (6). Inflammation (migration of neutrophils to the lungs, high production of cytokines and chemokines, etc.), in addition to the production of more viscous mucus resulting in impaired

mucociliary clearance, is noted in CF patients (7). Immune cells (Th lymphocytes, macrophages, dendritic cells and natural killer cells) are also important for lung tumor pathogenesis, and the production of proinflammatory cytokines by Th1 cells and increased cytolytic response contribute to the limitation of tumor progression (8). The activation of the immune response in the lungs is vital for the elimination of pathogens from this organ, but the characteristics of the response depend on the pathogen (9-11).

Bacterial microbiota in the gastrointestinal tract can affect immune reactions in the lungs, but on the other hand, pulmonary inflammation might cause gut dysbiosis (4). In this review, we presented only one aspect of bidirectional communication between the gut and lungs, limited to the papers investigating how bacterial microbiota composition in the gut affects inflammation in the lungs. Results from epidemiological studies are included to show an association of gut dysbiosis with human diseases, although from these studies it is generally not clear whether gut dysbiosis precedes the disease or is its consequence (except for asthma). Evidence from experimental models in which gut dysbiosis exists prior to lung inflammation (germ-free or antibiotic treated animals) or in which microbiota was targeted (by oral application of prebiotics, probiotics or postbiotics) indicate that the modulation of immune system activity is the main mechanism of the gut to lung axis.

Association of gut bacteria with lung diseases

The first indices of the gut-lung axis are co-occurrences of pulmonary abnormalities with inflammatory bowel disease (12). Currently, bacteria in the gastrointestinal tract have been associated with asthma (13-18), COPD (19-22), CF (23), recurrent respiratory tract infection (24), and lung cancer (25-28).

Asthma is a chronic lung disease affecting people of all ages that often starts in childhood, and increased risk for developing asthma in childhood is associated with less mature gut microbiota in the first year of life (13, 18). Early life application of antibiotics that results in decreased alpha diversity indices of gut microbiota (13) and a lower abundance of *Faecalibacterium prausnitzii*, *Roseburia*, *Ruminococcus bromii* and *Clostridium perfringens* (13), or reduction in *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* (14), were noted in asthmatic children. Early life colonization with *Bacteroides fragilis* (at 3 weeks of age) (15) and *Clostridium difficile* (1 month) (16) might contribute to the development of asthma. Other factors that can impact gut microbial colonization are also associated with asthma. For example, a higher risk of asthma at the age of six years was noted in children born by cesarean section that results in lower alpha diversity (at age one month and a year) and different microbial composition (differences were most obvious at early time points) when compared with vaginal delivery (17). However, children born by cesarean section have a high risk of asthma only if their gut microbiota remains less mature up to the first year of life. In another study that examined bacterial microbiota at different time points, the occurrence of asthma at the age of 5 years was related to different microbial compositions between healthy and asthmatic children born to asthmatic mothers at age 1 (18). Asthma in these

children is a consequence of the increased abundance of *Veillonella* and lower abundance of *Roseburia, Alistipes,* and *Plavonifactor*. Apart from microbial composition, the relevance of the metabolic activity of bacteria in this disease was also recognized. Asthma was shown to be connected with a lower level of lipopolysaccharide biosynthesis (14), decreased concentration of acetate (14), increased levels of histamine synthesis (29), and a higher level of 3-ketoshinganine (at 3-6 months of age) but a lower linoleic acid (at age one year) (30). In one study examining the impact of cesarean section on asthma development, a higher risk of asthma in children born by cesarean section (compared to naturally born infants) was associated with lower levels of metabolites (tryptophan, bile acid, and phenylalanine) early following birth (31).

Chronic obstructive pulmonary disease is an inflammatory chronic lung disease characterized by airflow blockage and breathing-related problems. A comparison of gut microbiota in COPD patients during the period of stable disease with healthy controls revealed differences in bacterial composition between the two groups, with a higher abundance of *Streptococcus, Rothia, Romboutsia,* and *Intestinibacter*, but a lower abundance of *Bacteroides*, *Roseburia,* and *Lachnospira* in COPD patients (19). Bacterial microbiota is correlated with disease severity as well (20). Although no differences were noted in alpha diversity and composition between patients with different stages of diseases (GOLD recommendations), the relative abundance of *Veillonella, Corynebacterium, Romboutsia* and *Aerococcus* was higher in patients with stages 3 and 4 of the disease, while *Megasphaera* was the lowest in patients with stage 1 disease (20). Associations were found between gut microbiota and better lung function in a patient population with a higher abundance of some *Streptococcus* and *Lachnospiraceae* species and a lower abundance of *Desulfovibrio*. Gut microbiota in COPD patients can affect disease progression, as a decline in lung function was correlated with an increase in alpha diversity indices, a decrease in the abundance of Firmicutes, and an increase in *Stentrophomonas* (21). In contrast to that, in patients with stable lung function a higher abundance of *Bacteriodetes* and *Alloprevotella* was noted. Bacterial products can also affect disease severity. Measurements of short-chain fatty acid (SCFA) in patients with COPD revealed lower levels of total SCFA, acetic, isobutyric and isovaleric acids in patients with COPD with stages 3 and 4 (compared to healthy controls) (22).

Cystic fibrosis is a genetic disorder characterized by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene resulting in viscous epithelial secretion. As this disease affects the function of different organs, including the gut, the association of gut dysbiosis with CF cannot be directly estimated. Data show that CF results in a different pattern of gut bacterial colonization compared to healthy controls (32). Regardless of the disease's impact on gut colonization, a significant association was found between disease exacerbation and gut microbiota composition (23).

Recurrent respiratory tract infections are the most frequent diseases in children under 5 years of age. An analysis of fecal bacteria revealed a decrease in bacterial diversity and distinct community structures in patients compared to healthy controls (24).

A higher abundance of *Enterococcus*, but lower *Eubacterium*, *Bacteroidetes*, and *Faecalibacterium* was noted in children with recurrent respiratory tract infections.

The examination of fecal microbiota in lung cancer patients and healthy controls revealed different bacterial compositions between these groups (25, 26), with higher *Bacteroides*, *Veillonella*, and *Fusobacterium*, and lower *Escherichia-Shigella*, *Kluyvera*, *Fecalibacterium*, *Enterobacter* and *Dialister* in cancer patients (25). Additionally, gut microbiota compositions were shown to correlate with different tumor biomarkers (27), tumor stages, and subtypes (28).

Based on the above data, a clear connection between gut dysbiosis and risk for disease development exists only for asthma, as dysbiosis was documented prior to the disease. Although for other diseases the role of gut microbiota composition in disease development is not so obvious (whether dysbiosis exists before disease symptoms or is a consequence of lung inflammation), gut bacteria might have an impact on disease course and stability (exacerbation, stable disease periods, etc.).

Experimental evidence of a gut-lung axis

Studies examining the impact of gut bacteria on inflammatory reactions in the lungs are based on a comparison of germ-free (GF) animals with conventional or GF animals colonized with specific bacteria, animals with different microbial compositions, or animals treated with antibiotics.

The presence of gut commensal bacteria is important for the control of allergic airway inflammation, as shown in GF mice that develop an exaggerated response to ovalbumin (OVA) administration compared to specific pathogen-free (SPF) mice (33). In the absence of commensal bacteria, a higher goblet cell hyperplasia, increased perivascular and peribronchial infiltration of inflammatory cells were noted, as well as a higher production of IL-4 and IL-5, and augmented IgE response. This exaggerated response can be reversed by the colonization of GF mice with commensal flora of SPF mice (33). Additionally, a comparison of airway inflammatory response to OVA in F1 generation of GF mice colonized with humanized microbiota (fecal microbiota from a patient that developed asthma at the age of 3 years) or with the same microbiota supplemented with *Faecalibacterium* spp., *Lachnospira* spp., *Veillonella* spp. and *Rothia* spp. (FLVR) pointed to beneficial role of FLVR in lung inflammation (14). These genera are decreased in the feces of children with asthma, and enrichment of microbiota with FLVR results in decreased infiltration of total lung cells, neutrophils, and lymphocytes in the lungs in response to OVA (14). The presence of commensal bacteria was shown to impact pulmonary response to bacterial infection also, indicated by higher mortality, higher infection rate in the lungs, and systemic dissemination in GF compared to conventional mice following *Klebsiella pneumoniae* infection (34). The absence of neutrophil infiltration and a lower tumor necrosis factor (TNF) and chemokine CXCL-1 response, but increased IL-10 response, were noted in infected GF mice (34). This aberrant pulmonary response to bacterial infection in GF mice can be reversed by

restoring gut microbiota, pretreating mice with bacterial product lipopolysaccharide, or neutralizing IL-10.

A comparison of animals that differ in the presence of segmented filamentous bacteria (SFB) in the GIT pointed to the role of these bacteria in Th17 cell differentiation, as a higher number of Th17 cells in the lungs was noted in mice colonized with SFB (35-37). The presence of SFB resulted in altered lung antifungal response to opportunistic fungal pathogen *Aspergillus fumigatus* (although the increase in fungal burden was not statistically significant) (35), increased resistance to *Staphylococcus aureus* infection (36), or induction of autoimmunity in prone mice (37). Besides its effect on a number of Th17 cells, SFB stimulates the expression of dual T cell receptors on the Th17 cell surface (for SFB and self-antigens) that contribute to the development of autoimmunity (37). These dual receptor-expressing Th17 cells migrate to the lungs and are responsible for lung pathology noted in rheumatoid arthritis (37). In this regard, it should be noted that many systemic autoimmune diseases have pulmonary manifestations (38). The presence of SFB also results in increased production of antimicrobial proteins (RegIIIγ and IL-22) in the intestine, leading to an increase in serum levels of IL-1α which augments Th17 cell accumulation (35).

A combination of antibiotics such as ampicillin, vancomycin, metronidazole, and neomycin, or gentamycin, in drinking water, is used to deplete gut microbiota. Using this approach, the role of gut microbiota in lung response to viral (39, 40) and bacterial (41-44) infections was investigated. Depletion of gut microbiota results in higher influenza virus titers (39, 40) and bacterial colonization (41-44) in the lungs, higher mortality of infected animals (39, 41-43), and more pronounced lung tissue damage (39, 41, 42). Increased susceptibility of antibiotic-treated animals to pulmonary infections was shown to be a consequence of diminished macrophage function (39, 41-43) and altered cytokine and chemokine production. In general, influenza infection in antibiotic-treated animals results in reduced production of IL-6, TNF, and chemokine MIP-1β (39). In contrast to viral infections, bacterial lung infections result in increased IL-6 and IL-1β, but decreased TNF (41, 42), as well as reduced IL-17A, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand 2 (CXCL2), and CXCL1 (43). Besides innate immunity, adaptive immunity was also affected, as the reduction of pathogen-specific antibody titers (40, 44) and the number and activity of $CD4^+$ T cells (40) and $CD8^+$ cells (39) were noted in the antibiotic-treated group. Microbiota transfer in antibiotic-treated animals was shown to improve lung immunity (42, 43). Additionally, stimulation of receptors that recognize microbial patterns, Toll-like receptors (TLR) (40, 41), and NOD-like receptors (43) can improve immune response in antibiotic-treated animals, suggesting that signals from bacterial products may be sufficient to support immune priming in the lungs. Treatment of mice with a combination of antibiotics prior to exposure to cigarette smoke (CS) was shown to ameliorate lung inflammation (45). An increase in the relative abundance of *Parabacteroides goldsteinii* noted in these animals was shown to correlate with decreased symptoms, and oral treatment with *P. goldsteinii* had a protective effect in CS exposed

mice (45). The previously described models contributed to understanding the impact of gut bacteria on immune reactions in the lungs, but should be carefully interpreted, as a mixture of antibiotics significantly depletes both gut and lung microbiota (43, 44).

Several papers have examined the effect of antibiotics with poor oral absorption, such as neomycin, vancomycin, or colistin. The administration of neomycin solely has a similar effect on anti-viral immunity as an antibiotic mixture (40). The infection of neomycin-treated animals with influenza virus resulted in more pronounced lung tissue damage, which was associated with reduced expression of TLR7 receptor mRNA in the lungs and impaired signal transduction (lower NF-κB expression) (46). Additionally, a lower interferon (IFN)-γ and IL-17 but a higher IL-4 and IL-10 response was noted in the infected neomycin group compared to the infected control group (46). Gut dysbiosis caused by vancomycin application lowered the number of Th17 cells in the lungs, and this effect was associated with a decrease in SFB (35, 37). The application of vancomycin in early life aggravates airway inflammation in adulthood, as a higher number of eosinophils and IL-13 and IL-4 production was noted following OVA application in vancomycin-treated compared to control animals (47). Neomycin and vancomycin affect Gram-positive bacteria, pointing to the role of these bacteria in lung immunity. In one study, both Gram-positive and Gram-negative bacteria, solely in the gut, were depleted following the application of vancomycin (for Gram-positive bacteria) and colistin (for Gram-negative bacteria), which resulted in worse infection outcomes, a higher lung injury, and lower survival of antibiotic-treated animals (compared to controls) following *Pseudomonas aeruginosa* infection as a result of depression of lung cellular immunity (48).

In general, bacteria from the gastrointestinal tract are necessary for adequate lung immunity as the absence of bacteria (germ-free animals) or gut dysbiosis (following antibiotic treatment) results in increased susceptibility to both bacterial and viral infections and an exaggerated allergic response (summarized in Table I). Additionally, in the absence of gut dysbiosis, some bacterial species might also affect the immune response in the lungs, as suggested by more pronounced inflammation in animals containing SFB compared to animals without these bacteria in the gut.

Mitigation of lung inflammation by prebiotics, probiotics or postbiotics

Concurrently with an examination of the mechanisms of the gut-lung axis, there are attempts to modulate immune reactions in the lungs by affecting gastrointestinal microbiota using prebiotics, probiotics or postbiotics (summarized in Table II).

By definition, a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit (49). In other words, prebiotics are compounds (such as fructooligosaccharides, galactooligosaccharides, oligosaccharides present in human milk, some dietary fibers and polyunsaturated fatty acid) metabolized solely by microorganisms in the gut, which modulate the composition and/or activity of gut bacteria resulting in the improvement of host health. Beneficial effects of omega-3 polyunsaturated fatty acids (ω3-PUFA) were noted in a model of lung injury induced by

Table I Overview of data regarding the impact of gut bacteria on immune reactions in the lungs obtained from animal models

Tabela I Pregled podataka o uticaju mikrobiote creva na imunske reakcije u plućima dobijenih u modelima na životinjama

	Model	Effect	Characteristics of response	Ref.
GERM-FREE ANIMALS				
	Allergic airway inflammation	Exaggerated response to allergen	↑Infiltration of inflammatory cells, ↑IL-4, ↑IL-5, ↑IgE	33
	Klebsiella pneumoniae infection	Increased susceptibility to infection	Absence of neutrophil infiltration, ↓TNF, ↓CXCL-1, ↑IL-10	34
ANIMALS CONTAINING SFB IN THE GUT				
	Aspergillus fumigatus infection	No effect on fungal burden in the lungs, altered immune response	↑IL-17, ↑IL-22, ↓IL-4, ↑RegIIIβ and RegIIIγ in intestine	35
	Staphylococcus aureus infection	Increased resistance to infection	↑IL-22, ↑IL-6, ↑Number of neutrophils	36
	Autoimmunity	Triggered lung pathology in susceptible strain	↑Auto-antibody-secreting cells, ↑Th17 cells, Expression of dual T cell receptors on the Th17 cell surface	37
ANIMALS TREATED WITH ANTIBIOTICS				
	Influenza virus infection	Increased susceptibility to infection	↓Number of virus-specific $CD8^+$ T cells, ↓Proinflammatory cytokines (TNF, IFN-γ, IL-2, MIP-1α, IL-1β, IL-17), ↑ IL-4 and IL-10, ↓Titers of specific antibodies (IgM, IgG), Defective innate immune response, ↓TLR7 signaling	39, 40, 46
	Escherichia coli infection	Increased susceptibility to infection	↓Bacterial killing by alveolar macrophages, ↑IL-6, ↑IL-1β, ↑MIP-2	41
	Streptococcus pneumoniae infection	Increased susceptibility to infection	↑IL-6, ↑IL-1β, ↓TNF, ↓IL-10, ↓Phagocytosis in alveolar macrophages, ↓IL-17, ↓Bacterial killing by alveolar macrophages, ↓GM-CSF, ↓CXCL2, ↓CXCL1	42, 43
	Klebsiella pneumoniae infection	Increased susceptibility to infection	↓GM-CSF, ↓CXCL2, ↓CXCL1, ↓IL-17, ↓Bacterial killing by alveolar macrophages	43
	Pseudomonas aeruginosa infection	Increased susceptibility to infection	Depression of lung immunity*, ↓Specific IgA, ↑CXCL2, ↑IL-1α, ↑IL-6	44, 48
	Allergic airway inflammation	Exaggerated response to allergen	↑Infiltration of inflammatory cells, ↑IL-4, ↑IL-13	47

Legend: ↑ - increase; ↓ - decrease; N/A - not available; *Immune response was examined following antibiotic application, but not during infection. Characteristics of the response are presented in comparison to relevant controls, i.e. specific-pathogen free animals for germ-free, animals without SFB, or animals not treated with antibiotics.

Table II Summary of the effects of application of prebiotics, probiotics and postbiotics on immune reactions in the lungs

Tabela II Pregled efekata primene prebiotika, probiotika i postbiotika na imunske reakcije u plućima

	Model	Effect	Mechanism	Ref.
PREBIOTICS				
ω3-PUFA	Lung injury (fine particulate matter)	↓Lung injury	↓TNF, ↓IL-1β, ↓IL-6, ↓IL-17, ↓Oxidative stress	50
Polysaccharides	Lung tumor	↑Antitumor response	↑$CD8^+$ T cells, ↓Treg cells, ↑SCFA, ↓L-kynurenine	51
Dietary fiber	Allergic airway inflammation	↓Lung inflammation	↓Cell infiltration, ↓IL-4, ↓IL-5, ↓IL-13, ↓IL-17A	52
A mixture of galactooligosaccharides and polydextrose	Rhinovirus infection in preterm infants	↓Incidence of viral respiratory tract infection and the incidence of rhinovirus-induced episodes	N/A	53
PROBIOTICS				
B. longum AH1206	Allergy	↓Allergic airway response	↓Number of eosinophils, ↓TNF, ↓IL-6, ↑Number of Treg	55
L. reuteri	Allergic asthma	↓Allergic airway response	↓Number of eosinophils and macrophages, ↓TNF, ↓MCP-1, ↓IL-5, ↓IL-13, stimulation of TLR-9	56
L. acidophilus LA-5, *L. rhamnosus* GG, and *B. animalis*	Allergic asthma	↓Allergic airway response	↓Number of eosinophils, ↓IL-4, ↓IL-5, ↓IL-13, ↓IL-17, ↓IL-25, ↓IL-33	57
L. rhamnosus GG strain	Lung injury (fine particulate matter)	Restored pulmonary function, ↓Pulmonary inflammation	↑Number of Treg, ↓Number of Th17 cells, ↓IL-6, ↓TNF, ↓IL-17A, ↓IL-1β, ↑IL-10, ↑TGF-β1	58
L. rhamnosus GG strain	Respiratory tract infection	↓Risk of respiratory tract infection, ↓Episodes of respiratory tract infection, ↓Severity of infection	N/A	59, 60
L. paracasei subsp. *paracasei*	Influenza infection	↓Duration of upper respiratory symptoms	N/A	61
L. casei	Cigarette smoke	N/A	↑Activity of NK cells, ↑Number of $CD16^+$ cells	62
SYMBIOTICS				
Vegetable and fruit concentrate, fish oil, and *L. salivarius* PM-A0006	Asthma	↓Medication use, ↑Pulmonary function	N/A	63
Galactooligosaccharides, fructooligosaccharides, and *B. breve* M-16V	Asthma	No effect on bronchial inflammation, ↑Peak expiratory flow	↓Th2-cytokines by peripheral blood mononuclear cells	64
Yogurt and high fiber intake	Lung cancer	↓Risk of lung cancer	N/A	65
POSTBIOTICS				
Inactivated non-typeable *Haemophilus influenzae*	COPD	↓Severity of COPD exacerbations	N/A	67
PMBL	COPD	↓Severity of COPD exacerbations	N/A	68
PMBL	Respiratory tract infections	↓Number of infectious episodes	N/A	69
Lantigen B	Respiratory tract infections	↓Number of infectious episodes	N/A	70

Legend: ↑ - increase; ↓ - decrease; N/A - not available.

fine particulate matter (PM2.5) exposure (50). Oral application of ω3-PUFA before induction of lung injury was shown to mitigate inflammation (TNF, IL-1β, IL-6, and IL-17 production) and oxidative stress in the lungs caused by PM2.5. This effect was associated with the attenuation of changes in the relative abundance of bacterial phyla in the gut induced by PM2.5, and with alteration in lung metabolic pathways that positively correlate with *Verrucomicrobiota* (50). In another study, supplementation with polysaccharides isolated from *Panax ginseng* was shown to result in potentiating the antitumor effect of anti-programmed cell death 1/ programmed cell death ligand 1 (anti-PD-1/PD-L1) therapy in a mouse model of lung tumor (51). Combined therapy resulted in higher activation of $CD8^+$ T cells and suppression of regulatory T cells compared with solely anti-PD-1 therapy. The application of ginseng polysaccharides altered microbial composition in the gut, which resulted in an increased concentration of short-chain fatty acids (SCFA) in plasma and a decrease in tryptophan metabolite L-kynurenine (51). The beneficial effect of prebiotics was also shown in a model of allergic airway inflammation (induced by house dust mite extract) in which a diet supplemented with readily fermentable fiber pectin reduced the infiltration of cells into the lungs and decreased IL-4, IL-5, IL-13, and IL-17A (52). The noted effect was mediated by an increased concentration of SCFA. In clinical trials, the application of prebiotic (1:1 mixture of galactooligosaccharides and polydextrose) was shown to lower the incidence of viral respiratory tract infection and the incidence of rhinovirus-induced episodes in preterm infants (53).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host (54). In the context of the beneficial role of probiotics in lung inflammation, *Bifidobacterium* spp. and *Lactobacillus* spp. were examined. Allergic airway response (induced by OVA administration) was attenuated with prior oral administration of *B. longum* AH1206 (55), *L. reuteri* (56), or a combination of probiotic strains (*L. acidophilus* LA-5, *L. rhamnosus* GG, and *B. animalis*) (57). Probiotic strains decreased the number of eosinophils (55-57) and macrophages (56) and reduced the production of TNF (55, 56), IL-6 (55), MCP-1 (56), IL-5 and IL-13 (56, 57), IL-4, IL-17, IL-25 and IL-33 (57). The noted effect is strain-specific and depends on live organisms, as *B. breve* AH1205 (55) and *L. salivarius* (56) or heat-killed *L. reuteri* (56) do not modulate the allergic airway response. The beneficial effect might be mediated by increased numbers of regulatory T cells (in Peyer's patch and spleen) (55) or stimulation of TLR-9 by *L. reuteri* (56). In the model of pulmonary injury, the oral application of *L. rhamnosus* GG strain restored pulmonary function that was decreased in response to PM2.5 exposure and ameliorated pulmonary inflammation (58). Probiotics increased the number of regulatory T cells and decreased the number of Th17 cells in comparison to PM2.5. Additionally, lower levels of proinflammatory (IL-6, TNF, IL-17A, and IL-1β) and higher levels of anti-inflammatory (IL-10 and TGF-β1) cytokines were noted following probiotic administration (58). The beneficial effects of probiotic administration were also examined in humans. In clinical trials, the prevention of respiratory infections with *L. rhamnosus* strain GG (53, 59, 60)

and *L. paracasei* subsp. *paracasei* (61) was investigated. These studies indicated that the application of probiotics reduces the duration of upper respiratory symptoms following influenza infection (61), the risk of respiratory tract infection (59, 60), the severity of infection (60), and episodes of respiratory tract infection that lasted over 3 days in hospitalized children (59). In another clinical study, supplementation with *L. casei* Shirota in smokers for three weeks was shown to increase the activity of NK cells and the number of $CD16^+$ cells (CD16 is a molecule that is expressed on NK cells, but also on other cell types) that are reduced in smokers (62).

Combined administration of prebiotics and probiotics (designated as symbiotics) on asthma (63, 64) and the incidence of lung cancer (65) was estimated. Daily supplementation with vegetable and fruit concentrate, fish oil, and *L. salivarius* PM-A0006 reduced medication use and improved pulmonary function in asthmatic school children (63), while a symbiotic containing galactooligosaccharides, fructooligosaccharides, and *B. breve* M-16V had no effect on bronchial inflammation, but reduced production of Th2-cytokines by peripheral blood mononuclear cells isolated from patients with allergic asthma (64). An analysis of the association between lung cancer risk and dietary fiber and yogurt (containing starter cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, but other *Lactobacilli* spp. and *Bifidobacteria* spp. may also be added) consumption revealed that the risk of lung cancer was reduced by more than 30% in adults with a high yogurt consumption and with the highest quintile of fiber intake, suggesting a protective role of symbiotics against lung carcinogenesis (65).

In the treatment of lung inflammation, postbiotics, which are defined as preparations of inanimate microorganisms and/or their components that confer health benefits to the host (66), might be used as well. Formalin-inactivated non-typeable *Haemophilus influenzae* was shown to reduce the severity of COPD exacerbations, proportions of episodes requiring corticosteroid therapy, and duration of episodes (67). A similar effect in patients with COPD was noted when a polyvalent mechanical bacterial lysate (PMBL) (of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus viridans*, *Klebsiella ozaenae*, *Haemophilus influenzae* type b, *Moraxella catarrhalis* and *Streptococcus pneumoniae*) was added to regular treatment (68). Infection is one of the risk factors in COPD exacerbations, and the use of inactivated bacteria for stimulation of immune response against potential pathogens might be beneficial for these patients. In patients with recurrent respiratory tract infections, a reduced number of infectious episodes was recorded following the application of PMBL (69) and Lantigen B (chemical lysate of suspension containing *Streptococcus pneumoniae* type 3, *S. pyogenes* Group A, *Branhamella catarrhalis*, *Staphylococcus aureus*, *H. influenzae* type b, and *K. pneumoniae*) (70).

Additional perspectives

While this review summarized the effects of bacterial microbiota from the GIT on immune reactions in the lungs, the effect of other microorganisms (viruses, fungi, and protozoa) should not be neglected. For example, the overgrowth of *Candida* spp. in the gut following antibiotic treatment promotes allergic airway inflammation (71, 72) by increasing the level of prostaglandin E_2, which induces M2 macrophage polarization (72).

Another aspect that was neglected is the effect of lung microbiota on the immune homeostasis in this organ. The lungs of healthy individuals have long been considered sterile, but with the development of new technics (sequencing of 16S rRNA gene), it is now established that the lungs harbor a vast range of microorganisms. Bacterial microbiota in the lungs is involved in the regulation of homeostasis in this organ and can be altered during the disease (73). In this context, lung dysbiosis is noted in diseases such as asthma (74), COPD (75), and CF (76), in patients with tuberculosis (77), invasive pulmonary aspergillosis (78), and during influenza A virus infection (79). In recent years, the alteration of lung microbiota in various animal models of lung inflammation/injury has been investigated (80-86). Whether gut microbiota affects bacterial composition in the lungs, thus resulting in altered tissue homeostasis, is still not clear, but data indicate that lung microbiota is enriched in the GIT taxa (gaining access to the lungs through microaspiration) (73).

Communication between the GIT and the lungs is not a one-way interaction (with the GIT microbiota affecting lung immunity), as immune reactions in the lungs might affect the gut microbiome. Dysbiosis in the gut was documented during pulmonary viral (87-91), bacterial (92, 93), and fungal infections (94, 95), as well as in mice exposed to high oxygen levels (80). The effect of lung inflammation on gut dysbiosis is also mediated by the immune system (87, 88).

Conclusion

The bacterial microbiota of the gastrointestinal tract has numerous effects on tissue homeostasis both locally (in the gut) and in extra-intestinal sites such as the lungs. The association of gut bacteria with various pulmonary diseases in humans has been established, and experimental data on animal models confirmed the existence of a gut-lung axis that is mediated by the effect of gut bacteria on immune system activities (Figure 1). The existence of the gut-lung axis provides the basis for modulating pulmonary immune response by affecting gut bacteria with prebiotics, probiotics, or postbiotics.

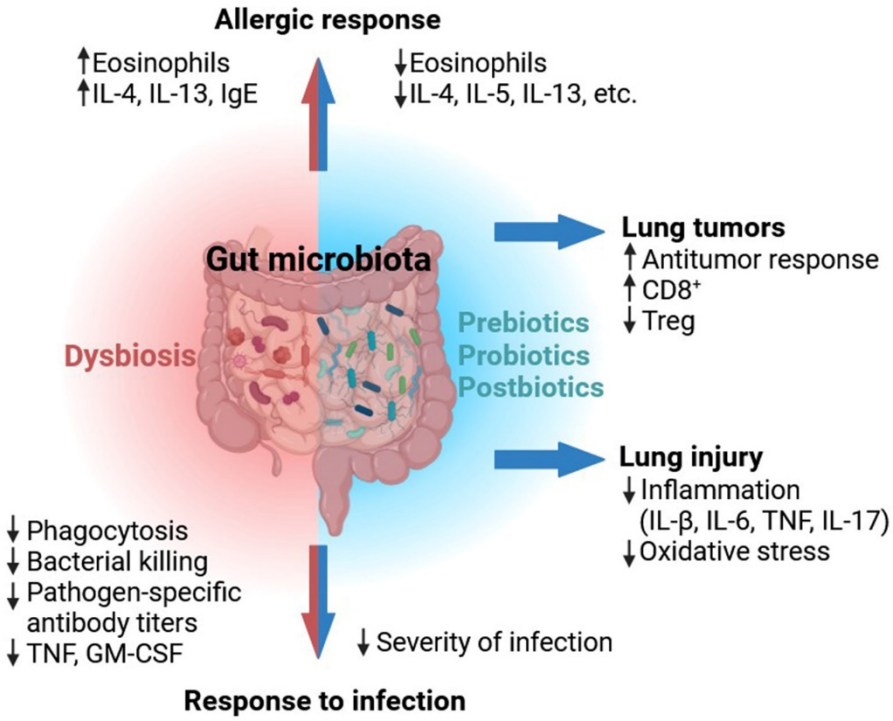

Figure 1. **Impact of gut microbiota on immune reactions in the lungs. While bacterial dysbiosis leads to an exaggerated response to allergen (by increasing the number of eosinophils and IL-4 and IL-13 production) and impaired response to infections (by decreasing activities relevant for pathogen removal), treatment with prebiotics, probiotics or postbiotics was shown to diminish allergies (decreasing the number of eosinophils and IL-4 and IL-13 production), increase antitumor response, decrease lung injury induced by xenobiotics (by lowering inflammation and oxidative stress) and severity of infections.**

Slika 1. **Uticaj mikrobiote creva na imunske reakcije u plućima. Bakterijska disbioza u crevima dovodi do intenzivnijeg odgovora na alergene (povećanje broja eozinofila i produkcije IL-4 i IL-13) i slabijeg odgovora na infektivne agense (smanjenje aktivnosti relevantnih za uklanjanje patogena). Sa druge strane, primena prebiotika, probiotika ili postbiotika smanjuje intenzitet alergijskog odgovora (smanjuje broj eozinofila i produkciju IL-4 i IL-13), potencira antitumorski odgovor, smanjuje stepen oštećenja pluća izazvan ksenobioticima (smanjenje inflamacije i oksidativnog stresa) i doprinosi smanjenju ozbiljnosti infekcija.**

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Uticaj mikrobiote creva na imunske reakcije relevantne za patologiju pluća

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Kratak sadržaj

Poznato je da bakterije prisutne u gastrointestinalnom traktu imaju ulogu u sprečavanju invazije patogenih mikroorganizama, regulaciji propustljivosti creva, varenju hrane, metabolizmu i imunskom odgovoru. Ove bakterije utiču na funkciju, održavanje homeostaze i ishod bolesti kako u gastrointestinalnom traktu, tako i u udaljenim organima kao što su pluća. Ovaj pregledni rad sumira trenutno dostupna znanja o osi creva-pluća. Prikazana je veza između bakterijskog sastava i/ili disbioze u crevima sa različitim bolestima kod ljudi kao što su astma, hronična opstruktivna bolest pluća, cistična fibroza, rekurentne infekcije respiratornog trakta i karcinom pluća, kao i podaci dobijeni u životinjskim modelima inflamacije pluća koji su pokazali da modulacija aktivnosti imunskog sistema leži u osnovi ove interakcije. Potencijalna upotreba prebiotika, probiotika i postbiotika u terapiji inflamacije u plućima je takođe prikazana.

Ključne reči: bakterijska mikrobiota creva, osa creva-pluća, inflamacija u plućima

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Crosstalk between vitamin status and Gut Microbiota: the key to maintaining immune homeostasis in the gut

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Abstract

The human gut microbiota is a diverse ecosystem that harbours a variety of microorganisms, including proteobacteria, bacteria, viruses, fungi, protists, and archaea. These microorganisms are collectively involved in several vital functions, including nutrient metabolism, vitamin synthesis, immune system regulation, neurotransmitter production, drug metabolism, and communication with the central nervous system. Dysbiosis within the gut microbiota has been shown to be a critical factor in the development of chronic disease. Investigating the effects of gut microbiota composition on overall health holds promise for the treatment of inflammatory diseases and the development of new therapeutic interventions. One notable aspect of the functionality of the gut microbiota is its involvement in the production of essential B vitamins. These vitamins exert a significant influence on immune responses and the composition of the gut microbiota. Competition may occur between the host and the gut microbiota for B vitamins, which some bacteria obtain from food or from synthesis by other gut bacteria. Thus, the availability of B vitamins in the diet has the potential to influence the composition of the gut microbiota and thus immune homeostasis. The profile of the gut microbiota varies individually, with diet proving to be an important modulator of both its composition and functional properties. However, further extensive research efforts are needed to understand the complex interplay between the gut microbiota, vitamins, and immune response mechanisms. Such investigations have the potential to develop innovative therapeutic strategies for a spectrum of inflammatory diseases, opening new avenues for improved patient outcomes.

Key words: Gut microbiota, Dysbiosis, Immune system, B Vitamins, Homeostasis

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Gut Microbiota ecosystem

The gastrointestinal system (GIT) is the anatomical site that is regularly exposed to multiple environmental stimuli through food intake, which is why the lumen of GIT is considered the richest source of antigens in the human body. Accordingly, the microbial community inhabiting the human GIT represents a complex and well-structured ecosystem that provides its host with important metabolic (breakdown of indigestible nutrients, synthesis of vitamins) and immunomodulatory functions, and also acts as a dynamic barrier against colonization by pathogenic species (1, 2). The seemingly separate aspects of gut microbiota (GM) function, food digestion and immune modulation are in fact highly intertwined, as byproducts of microbial metabolism act as messengers for epithelial barrier maintenance and drivers of phenotypic changes in local immune cells (Figure 1). Moreover, competition with pathogens for the same ecological niche hinders excessive immune activation and thus also contributes to a more balanced physiological state (3, 4). In addition to dietary components, the GM is also involved in the degradation of xenobiotics, which significantly affects the bioactivity and bioavailability of ingested drugs (5-7). In view of this, experimental attempts aimed at *ex vivo* capturing the drug-metabolism ability of gut microbes at a community-level (8) and machine-learning based approaches for discovering the GM status as a biomarker of medical treatment outcome are starting to emerge (9, 10).

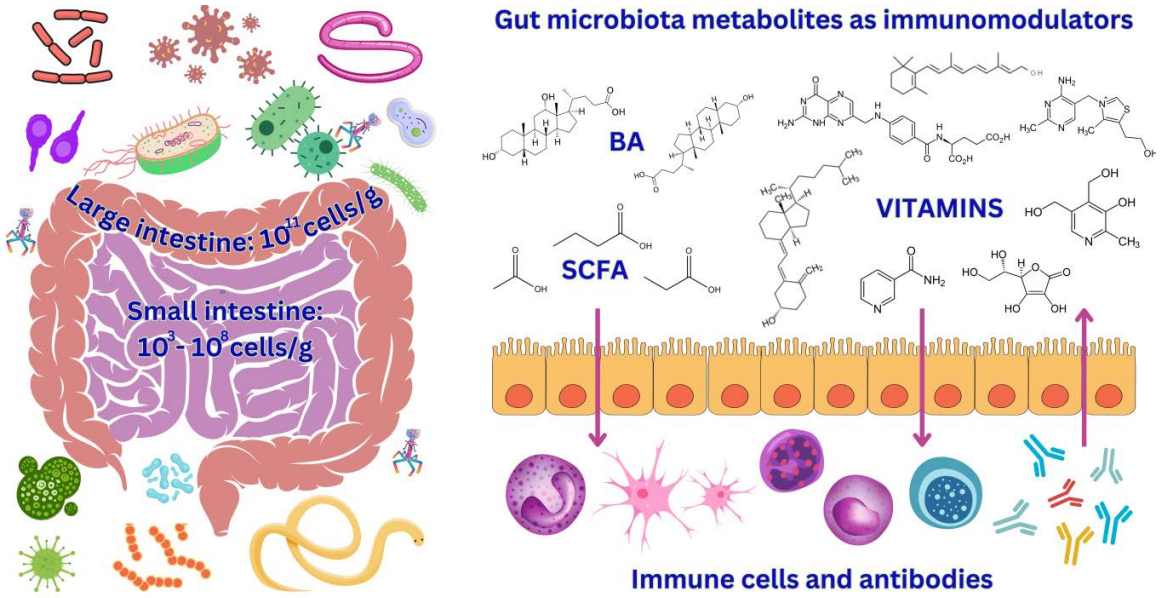

Figure 1. **Gut microbiota composition and cross-talk between microbial-derived metabolites and neighboring immune cell populations**

Slika 1. **Sastav mikrobiote creva i uticaj produkovanih metabolita na populacije rezidentnih imunskih ćelija**

The ratio of symbiotic microorganisms to human host cells is approximately 1:1 (11), mainly due to the high density of microorganism populations along the distal segments of GIT (12). More specifically, the estimated number of bacterial cells per gram of feces is 10^{10}-10^{11}, which is also true for viruses (11, 13). The most abundant gut-associated bacterial phyla are *Firmicutes* (represented by more than 200 genera) and *Bacteroidetes*, which together account for nearly 90% of the richness of the gut community (1, 14, 15). Less common but also functionally very relevant phyla are *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* (1). In addition to the predominant prokaryotic communities (bacteria and viruses), the intestinal microbiota is also composed of microscopic species classified as Eukarya (fungi, helminths and protists) and Archaea (16, 17); however, with lower community richness at the species level. The estimated abundance of archaeal cells per gram of feces is nearly two orders of magnitude lower than that reported for bacteria and viruses (approximately 10^9), while estimates for mycobiome abundance are even lower (10^2-10^6) (13).

The human intestine contains predominantly hydrogentrophic archaea that convert byproducts of bacterial metabolism to methane. Some of these methanogenic species (*Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) are ubiquitous but highly specific representatives of the human GM (18, 19), owing to pronounced adaptability to variable conditions in the habitat. For Western civilization, ubiquitous distribution has also been reported for the fungal genera *Candida*, *Saccharomyces*, and *Malassezia* (16). Interestingly, at the time of initial gut colonization, fungal diversity and abundance correlate negatively with bacterial community richness, and in adulthood this is observed as a fungal bloom in response to antibiotic treatment when a massive depletion of bacterial communities occurs.

The opportunistic drug-induced spread of otherwise harmless commensals implies that both reciprocal and host interactions are highly context-dependent for components of the GM, sometimes complicating the correct positioning of underexplored taxa. For example, the gut-colonizing protist *Blastocystis*, characterized as “the most common eukaryotic organism reported in human fecal samples” (20), has been described as both pathogenic and commensal (21), which may be due in part to a looser definition of commensalism for protists/helminths that implies only a long-lasting tolerance of the immune system under undisturbed conditions (22). Although seemingly of no benefit to the host, *Blastocystis* has also been designated as a block-forming agent essential for the maintenance of the gut ecosystem due to a long history of association with humans (demonstrated in coprolites) and a positive correlation with bacterial community richness and alpha diversity (16, 17). Accordingly, the lower abundance and species richness of intestinal bacteria in industrialized (more sanitized) environments was causally inferred as an indirect effect of the slimming of protozoan and helminths communities in the gut (17). This has also been confirmed by a recent study (23) showing that antibiotic treatment leads to a massive reduction in *Blastocystis* abundance. Remarkably, the dense interaction network of microbial communities in the gut goes beyond the presumed multilevel trophic stratification (24) and also explains the rapid intrahost evolution of its

members due to the high rate of horizontal gene transfer (13). This process plays a prominent role in shaping the gut resistome (collection of genes conferring resistance to antimicrobial drugs), which can be seen as a form of community adaptation to the nowadays widespread use of antibiotics (25).

Apart from antimicrobial drugs, GM also responds to many other conditions/factors in the environment and is probably influenced by the genetic background of the host (11, 26). Accordingly, the human GM is characterized by a high degree of inter- and intra-individual variability. Moreover, primary colonization of the gut is largely stochastically controlled (27), while *sensu stricto* inheritance of the maternal microbiota accounts for only a few species transmitted directly at birth (11). The actual diversification of primary communities occurs in early childhood, most intensely after weaning, when new dietary patterns begin to shape the environmental landscape in the gut. This period correlates with a profound influence of the GM on the education/maturation of the immune system and is often referred to as a "window of opportunity" for major interventions in the composition of the GM. As a proxy of dietary regime, transit time and stool consistency also strongly affect the composition of GM, as stool retention favors the overgrowth of fast-growing microbial taxa, sometimes with pathological consequences in the form of small intestine bacterial overgrowth (SIBO) syndrome (28-30). In addition, drug intake (especially antibiotics and proton pump inhibitors), stress, lack of sleep, high body mass index, and other factors that (negatively) affect host homeostasis and immunity modulate gut microbial communities, but also lifestyle in a modern (highly sanitized) environment characterized by a low parasite burden (16). Consequently, it is unusual to speak of a healthy microbiota, but rather of eubiosis, which refers to the spectrum of different microbial community states capable of balancing their commensal interactions with the host.

Gut Microbiota enterotypes

Despite constant exposure to a highly dynamic environment, the distinctive structural complexity of the gut microbial ecosystem (which correlates with species richness/diversity) provides inherent resilience to perturbation and favors stratified rather than continuous shifts in community composition (31). The stability of the GM in adulthood has been shown to persist over a period of more than 10 years, and the abundance rather than the composition of various microbial species is influenced by environmental stressors (32). Interestingly, the stability of the GM was also confirmed at the level of individual strains (33). Accordingly, pioneering research in this field already attempted to identify characteristic eubiotic states of the GM, leading to the concept of enterotypes – recurrent patterns of GM composition dominated by particular bacterial taxa – that correlate most strongly with long-term dietary habits (31, 34).

In early childhood, *Bifidobacteria* and *Proteobacteria* generally serve as community organizers for primary gut ecosystems, whereas the GM in adulthood is characterized by the prevalence of *Bacteroides*, *Prevotella*, or *Ruminococcus* (and rarely other *Firmicutes* genera) (1). The three major adult stage enterotypes have been widely

validated in the context of different ethnicities, geographies, and lifestyles (rural *vs.* industrial environments), demonstrating diet as the core environmental factor shaping the gut community landscape (14, 35-37). The enterotype dominated by *Prevotella* correlates with a diet rich in complex carbohydrates, due to the high efficiency of the hydrolytic enzymes of *Prevotella* in degrading plant fibers (1). Likewise, the *Bacteroides*-dominated enterotype is a form of GM adaptation to the high-fat animal (Western) diet (14, 38-40), while the *Ruminococcus*-enterotype is best adapted to a diet rich in plants and fermented products (41). The enterotypes dominated by *Bacteroides* and *Ruminococcus* also share the ability to degrade mucin glycoproteins, which affects the turnover rate and stability of the intestinal mucosal barrier(1).

Although rather simple, the concept of enterotyping (40) could have clinical application in diagnostics and prediction of disease susceptibility/medical treatment outcomes, as the dimensionality reduction of the complex GM to a small number of (core) community descriptors facilitates the discovery of medically relevant covariates (38, 42). A year-long cohort study in Sweden (43) has shed light on the long-term enterotype stability (32) and significant intraindividual variation in the GM (44). The study identified three main patterns of variability for GM constituents highlighting stable, bimodal, and variable species. By elucidating ecosystem dynamics, this research supports the concept of core community taxa and offers guidelines for the development of algorithms to predict the evolution of the gut community in response to various (stochastic and tailored) environmental factors.

Gut Microbiota fluctuations along the gastrointestinal system

The concept of enterotypes is a proxy for the composition of GM along the colon only. First, noninvasive methods like fecal DNA sequencing reveal colon microbial communities, while characterizing small intestine microbiota is challenging due to invasive methods prone to artifacts (cross-contamination) and is also unsuitable for healthy subjects (45, 46). Ileostomy samples offer a unique exception to cross-contamination issues, enabling valuable studies of dynamic small intestine microbial communities. However, limitations include a limited study population and the impact of altered ileal anatomy. Secondly, the large intestine hosts microbes 100 times more abundantly than other body compartments, including the small intestine, which lags by 4 times. (31). The density of microbial load along the small intestine (proximal duodenum to terminal ileum) increases from 10^3 to 10^8 cells/g, whereas the microbial load of the large intestine (bacterial only) was estimated to be approximately 10^{11} cells/g of luminal contents (13, 47).

Clearly, GM in the small intestine can only be characterized involving strategies that bypass any type of sampling from the colon, like standard/routine feces collection procedure. Breakthroughs in this area are urgently needed to fully comprehend the effects of various chemical gradients along the GIT – that generate highly specialized microenvironments – on the composition and metabolic variations of the GM, greatly impacting the function of neighboring immune system components. In general, acidity,

concentration of digestive enzymes, bile acids, gasses (pO_2, pCO_2, and pH_2), and dietary antigens decrease from the duodenum to the colon, while the mucus thickness and the total microbial load increase due to the less harsh environment in the distal segments of the GIT (47-49).

In contrast to the dense communities of obligate anaerobes favored in the hypoxic environment of the colon, aerobic bacteria and facultative anaerobes, predominantly from the phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, colonize the small intestine, the most common genera being *Lactobacillus*, *Clostridium*, *Staphylococcus*, *Streptococcus*, *Bacteroides*, *Veillonell*a, *Gemella*, *Actinomyces*, and *Escherichia* (1, 47, 50). Proximally to distally, microbial communities differ more in the lumen, likely due to more uniform conditions in the mucosal environment, which is also protected from rapid digesta flow rates (3-5 h) (1). The proximal luminal communities of the small intestine are particularly dynamic, in response to the rhythmicity of daily food intake and the associated concentration and activity of digestive enzymes. In addition, these communities are severely impacted by proton pump inhibitors, which alter not only gastric, but also the pH of the duodenal compartment. Remarkably, a significant overlap was observed between the GM in the proximal small intestine and the oral microbiota, probably due to the daily influx of more than 1 L of saliva containing about 10^{12} bacteria (51).

How variable transit times alter the biology of bacterial communities from the small intestine is best illustrated by the SIBO syndrome, in which pathological overgrowth of fast-growing taxa occurs in response to slow peristalsis. This has been shown to affect the turnover rate of essential micronutrients functionally related to small intestinal physiology. For example, SIBO is one of the causative factors for cobalamin (vitamin B12) deficiency and malabsorption in the ileum (49), as it is overused by the overgrown bacterial communities for their own metabolic purposes. Similar effects were reported for thiamine (vitamin B1) and nicotinamide (vitamin B3) deficiency, in contrast to the observed increased availability of folate (vitamin B9), probably as a result of biosynthesis mediated by the overgrown microbiota along the small intestine, predominantly the ileal compartment (50). In addition to the synthesis and absorption of B vitamins, the ileum is also characterized by a high turnover rate of vitamins C, D, K, and other micronutrients (Se, Mg, and Mn) (49, 52). Moreover, considerable amounts of bile acids (BAs) and short-chain fatty acids (SCFAs) (53, 54) are produced in the ileum, especially by abundant representatives of various *Clostridiales* genera from the thick mucosal layer. The microbes residing in the ileum, but also duodenum and jejunum, rapidly metabolize simple carbohydrates from the nutrient-rich environment (46), demonstrating once again that the (availability of) dietary components most directly shape(s) highly dynamic microbial communities in the small intestine.

The cross-talk between Gut Microbiota and immunity

In the previous chapter, we saw how specialized microenvironments along the GIT form networks for the establishment of characteristic microbial ecosystems. This regional specialization of the commensal microbiota is primarily reflected in its communication

with neighboring components of the immune system, whose anatomical (and functional) organization is also determined by the anatomy of the associated GIT compartments, including unique organization of the gut-associated lymphoid tissue (GALT) and the presence of a decreasing gradient of pro-inflammatory cytokine concentration along the GIT (48, 55). The structure (maturation) of GALT is also shaped by the composition of GM and this influence extends beyond the local compartment by virtue of soluble immune modulators, targeting extraintestinal lymphoid and non-lymphoid tissues (56). The impact of this extremely close connection between the gut anatomy, resident microbiota, and associated immune system on the host well-being is illustrated by a recent bibliometric analysis of original research articles on the GM for the period between 2010 and 2021 (57). The results of this analysis show that, in addition to microbiology journals, numerous articles were published in clinical medicine journals, with the most frequently cited ones examining the relationship between the GM and human health/disease status.

The defense mechanisms provided by the components of the immune system, located mainly in the lamina propria of the GIT, are largely complemented by a thick mucus layer produced by specialized epithelial cells (goblet cells), which prevents the extraintestinal translocation of microbes and largely isolates the immune cells from the contents of the lumen, enriched with commensals, food components, and transiting microbes (4, 58). The mucosal barrier network is predominantly composed of highly O-glycosylated proteins – mucins, mixed with antimicrobial peptides (cathelicidins and defensins) and secretory immunoglobulin A (IgA), which protect against and opsonize microbes that come into contact with them. This represents the first line of defense in the gut and is also a way to mitigate excessive immune activation, which is achieved mainly by redirecting immune activity to tolerogenic processes (59). The mucosal barrier is further reinforced by tight junctions in the epithelial lining that severely restrict paracellular permeability and the access of microbes and their metabolites to the blood and lymphatic circulation. On the luminal side, the protective role of the intestinal barrier is complemented by the activity of bacteriocins, natural antimicrobial peptides produced by commensals, and the influence that some commensals exert on mucosal renewal by stimulating the production of mucins in goblet cells (58, 60-62).

When microorganisms, or at least their antigens, manage to breach through the mucosal barrier, thanks to the specialized transcytotic activity of epithelial microfold cells (M cells), immune processes are initiated in the GALT, which is one of the largest lymphoid organs of the body, rich in isolated and aggregated lymphoid follicles (48). More specifically, the initiation of immune responses relies on the uniquely organized GALT structures along the ileum – Peyer`s patches, particularly rich in B cell follicles with differentiated germinal centers and antigen-sampling dendritic cells (63). B cell follicles are covered by an epithelium containing M cells specialized for transporting antigens of the intestinal microbiota from the lumen to dendritic cells, which initiate the cascade of adaptive immune responses by presenting antigens to naïve T lymphocytes. GALT, and also the gut-draining lymph nodes, harbor different subsets of dendritic cells biasing the downstream activity of T cells to tolerogenic or pro-inflammatory responses,

which makes them a signaling hub for the maintenance of intestinal homeostasis. In addition to dendritic cells and T lymphocytes (regulatory and effector subsets), immune responses in the gut also depend on the activity of mucosa-associated invariant T lymphocytes (MAIT cells), specialized intraepithelial T lymphocytes (IELs, unconventional γδT cells), innate lymphoid cells (ILCs), natural killer (NK) cells, neutrophils, and macrophages, all of which have distinct surface markers reflecting their functional specialization and gut-homing patterns of recirculation. Under undisturbed conditions, the small intestine is particularly rich in IELs, Th17 subset of $CD4^+$ T cells, ILC2 and ILC3 cells, while the Tregs specific for commensal microbes predominate in the colon (49).

In different compartments of the GIT immune cells are orchestrated to provide either protection from pathogenic invaders or tolerance to recurrent commensals/food components. Tolerance to food antigens is maintained mainly in proximal segments along the GIT, which is the primary site for nutrient absorption. Establishment of food tolerance in the small intestine is controlled in part by diurnal rhythms of major histocompatibility complex (MHC) class II expression on resident epithelial cells that depend on food intake (which in turn causes shifts in local microbial community composition). The function of resident epithelial cells is closely related to the activity of highly mobile IELs, which serve immune surveillance, form the layer along the basolateral side of the epithelium, and also reinforce the integrity of the intestinal barrier (47, 64). The role of IELs in the pathogenesis of celiac disease, which is characterized by abnormal sensitivity to gliadin antigens from cereals, supports the tolerance-promoting effect of IEL to foods (64). Conversely, tolerance to commensal microbes in the colon relies on the regulatory phenotype of $CD4^+$ T cells (65), the induction of which can be influenced by the GM itself (*Akkermansia muciniphila* is a well-characterized trigger of the $ROR\gamma t^+$ regulatory T cell phenotype) or the corresponding microbial metabolites, such as SCFAs, which are abundantly produced in the colon (3).

The choice of inflammation over tolerance and *vice versa* depends on the context of antigen presentation to the immune system, i.e., the balance between pro-inflammatory and anti-inflammatory signals in the surrounding milieu, which is controlled primarily by the GM itself. In this context, the GM functions both as an object and as a modulator of local immune activity. These interactions are especially important in early childhood, when critical processes of immune system education occur and the maturing lymphocytes acquire the ability to discriminate between their own and foreign and between harmless and pathogenic microbial species for proper establishment of regulatory mechanisms (58). Recent evidence shows that the microbiota begins to communicate with the body *in utero*, as metabolites of the maternal microbiota are represented in the fetal metabolome (66, 67) and typical gut microbes, such as representatives of *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, have been detected at the maternal-fetal interface (placenta and umbilical cord) (3). Since the education of the human immune system also begins during intrauterine development (the first trimester) (68), this could be influenced in part by the exposure of maturing T lymphocytes to the colonizing maternal microbiota

or its metabolites. A possible avenue for mediating microbial effects on *in utero* immune system maturation could be epigenetic reprogramming, as a recent study in mice shows that even mild maternal infections cause IL-6 dependent imprinting of the fetal GIT tissue with resulting long-term susceptibility to increased intestinal inflammation in adulthood (69). Consequently, a shift in the balance of immune system activity from a tolerogenic to a pro-inflammatory state in response to altered GM can adversely affect the host health. The persistence of a local pro-inflammatory milieu has systemic effects and, depending on the host's genetic background and lifestyle, can result in a number of different diseases – metabolic, autoimmune, inflammatory, neurological, and even various psychiatric disorders have all been linked to perturbed gut microbial communities (1, 114, 134, 142).

Gut Microbiota Metabolites as Essential Messengers to the Immune System

Communication between gut microbes and the immune system can be mediated either through direct recognition of microbial structures or indirectly *via* metabolites that GM abundantly produce (49). Normally, the recognition of gut microbes is initiated by the cells of innate immunity, since the immune surveillance of the intestinal luminal contents is based on the activity of IECs, whereas the same activity in the lamina propria is predominantly carried out by dendritic cells and macrophages. Common to these cell types is the expression of receptors with specificity for common microbial structures (patterns) – pattern recognition receptors (PRRs), with Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors being two overrepresented classes (70, 71). Upon activation, PRRs activate downstream signaling pathways and ultimately alter gene expression of immune-related (soluble and membrane-bound) mediators, primarily cytokines, chemokines, and several classes of immune receptors. The outcome depends on the specific cascade and target genes being utilized. It can lead to either the promotion of tolerance or the triggering of inflammatory/protective responses. In both cases, adaptive immunity cells, such as effector or regulatory lymphocytes, are also involved. The precise mechanism of differentiation between commensal and pathogenic bacteria remains unclear, as both types of bacteria contain microbial patterns recognized by PRRs; however, there are data supporting the hypothesis of TLR-dependent priming of dendritic cells by intestinal commensal bacteria, in part due to fine-tuning of receptor surface expression (3, 4, 56, 58). Although pattern sharing complicates distinguishing between pathogens and commensals, it is consistent with the dynamic, context-dependent interactions between gut microbes and the host, where a given species may act as both a pathogen and commensal depending on environmental conditions.

The priming of dendritic cells for tolerogenic activity by commensal bacteria and many other immunomodulatory functions of the GM are probably mediated by abundant microbial metabolites, especially in the colon. Some of these metabolites are not produced by the host and are formed exclusively as by-products of microbial metabolism during the breakdown of certain dietary components. Advances in high-throughput omics

technology, particularly metabolomics and metagenomics, have contributed significantly to our understanding of the complex interplay between the immune system and nutrition. The metabolites of the GM act as critical signaling hubs for communication, with the immune system contributing significantly to the maintenance of homeostasis (4). Depending on the starting material, metabolites of the GM can be divided into those derived from ingested materials, host-generated metabolites, or those synthesized *de novo* (72). Based on chemical composition, the major classes of metabolites of the GM include short-chain fatty acids (SCFAs) as byproducts of microbial fermentation, secondary bile acids (BAs), choline metabolites such as trimethylamine N-oxide, tryptophan, and indole derivatives, as well as vitamins, neurotransmitters, and various lipids (73), of which SCFAs and secondary BAs have been repeatedly confirmed as key regulators of intestinal immunity that also affect systemic immunity.

BAs are metabolites derived from cholesterol, primarily synthesized as cholic acid and chenodeoxycholic acid in the liver and conjugated to taurine and glycine before excretion (74). More than 95% of the (conjugated) BAs that reach the intestine are absorbed and recycled to the liver, while the remaining portion undergoes transformation dependent on the intestinal microbiota, mainly by deconjugation but also by oxidation, epimerization, dehydroxylation, and esterification. The deconjugation depends on the activity of specific hydrolases encoded mainly by the genomes of the gram-positive bacterial genera *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus*, and partially by the gram-negative members of the *Bacteroidetes*. Dehydroxylation is predominantly carried out by abundant Clostridia, whereas oxidation and epimerization depend heavily on bacterial representatives of the genera *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, *Ruminococcus*, *Bifidobacterium*, *Egghertella*, *Enterobacter*, and *Escherichia* in addition to Clostridia (75, 76). The abundance of possible enzymatic biotransformations allows for the formation of a highly diverse pool of secondary BAs in the gut that reflects the composition of the microbial community (76). At physiological concentrations, BAs derived from bacteria, such as deoxycholic acid and lithocholic acid, directly modulate intestinal immunity by affecting immune cell differentiation and activity (77, 78). The best-characterized targets of secondary BAs are Tregs and Th17 cells, and these metabolites also largely influence the balance between Th17 cells and Tregs in the GALT. Indeed, a derivative of lithocholic acid attenuates Th17 cell differentiation by interacting with the nuclear receptor RAR-related orphan receptor gamma (RORγt). Conversely, it also stimulates the differentiation of Tregs *via* upregulation of the *Foxp3* gene transcription in conjunction with another nuclear receptor, NR4A1, thereby establishing a tolerance-promoting milieu, which is in contrast to the pro-inflammatory activities of many other secondary BAs and their derivatives (76, 79).

It was found that BAs affect other lymphocyte subsets, in addition to Tregs and Th17 cells, such as cytotoxic T lymphocytes, B cells, Th1 and Th2 subsets of $CD4^+$ T cells, and also innate immunity cells, such as dendritic cells, macrophages, and NK cells (74). The pleiotropic effect depends on the binding of BA to several classes of receptors, of which binding to the farnesoid X receptor (FXR), G protein-coupled

receptor-1 (TGR5/GPBAR1; GPCR), pregnane X receptor (PXR), and vitamin D receptor (VDR) have been most extensively studied to date (76). The FXR receptor is abundantly expressed by macrophages, dendritic cells, and NK cells, so its overall activation has a major impact on gut innate immunity (80, 81). Activated FXR functions as a transcriptional regulator by binding to promoter regions of target genes in complex with the retinoid X receptor. Interestingly, FXR receptor expression is modulated by TLR9, a member of the PRR receptor class that senses microbes by recognizing their genetic material (82). The expression of GPBAR1 on immune cells largely overlaps with the expression pattern of FXR (81), whereas high expression of TGR5 is characteristic of lymph nodes (83). Signaling downstream of TGR5 is cAMP-dependent and interferes with the NF-κB and AKT-mTOR LIP pro-inflammatory signaling pathways, as well as STAT3 signaling in gastric cancer proliferation, which is an inflammation-coupled process. The PXR-dependent anti-inflammatory effect also interferes with NF-κB signaling (84), which is generally known as the most common target of various anti-inflammatory agents/metabolites.

The attenuation of the pro-inflammatory NF-κB pathway is also a prominent role of SCFAs produced by the GM, especially in the proximal segments of the colon (85). The term SCFAs refers to carboxylic acids with aliphatic tails of up to six carbon atoms, with the best-known representatives being acetate, propionate, butyrate, pentanoate, and malonate. These compounds are formed as by-products of microbial fermentation, which usually occurs in the final phase of metabolism of dietary fibers and also proteins (85, 86). The most abundant and biologically active are acetate (C2), propionate (C3), and butyrate (C4), with a 3:1:1 ratio of production in the intestine. Here, the production of acetate and propionate is mainly carried out by the representatives of the bacterial phylum *Bacteroidetes* (especially the genera *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Alistipes*), while the representatives of the phylum *Firmicutes* are devoted to the production of butyrate, the most efficient species being *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Eubacterium hallii*. Butyrate is also abundantly produced by the *Becteroidetes* species *Roseburia intestinalis* and *Anaerostipes butyraticus* (4, 87, 88).

In terms of immunomodulation, SCFAs are probably the most potent and pleiotropic class of metabolites of the GM, having as final targets the key mediators of intestinal homeostasis maintenance (the intestinal barrier, cells of innate and adaptive immunity), due to their distinct ability to influence not only the life cycle (proliferation, growth, differentiation, apoptosis), but also the activity of target cells (secretory phenotype, chemotaxis, metabolism) (85-87, 89, 90). The integrity of the intestinal barrier is modulated by SCFAs both structurally and functionally through several mechanisms. First, the barrier stability is enhanced by SCFAs, particularly butyrate, *via* upregulation of tight junction protein (occludin, zonulin, claudin) expression (72, 91, 92). SCFAs also promote the production of the mucus layer and antimicrobial substances such as defensins, cathelicidins, and C-type lectins by specialized epithelial cells, as well as bacteriocins by representatives of the GM (90). In parallel with stimulation of their

differentiation from monocytes, SCFAs also stimulate the synthesis of antimicrobial peptides in macrophages (87). While this process in intestinal epithelial cells depends on the activation of G protein-coupled receptor 43 (GPR43) and downstream signaling *via* the cell growth pathway involving the mitogen-activated protein kinase cascade, stimulation of defense peptides in macrophages occurs in response to metabolic reprogramming initiated by epigenetic changes in gene expression (93).

In epithelial cells, SCFAs also function as an energy source leading to ATP synthesis by β-oxidation in mitochondria (4, 94). This leads to significant oxygen consumption, which contributes to a hypoxic environment in the colon that favors the growth of obligate anaerobic commensals. Stimulation of hypoxia in response to SCFAs also occurs *via* the stabilization of the transcription factor Hypoxia-inducible factor, with many anti-inflammatory functions in the intestinal tract, particularly at the level of barrier integrity and innate immunity cells (neutrophils, macrophages, dendritic cells) regulation (4, 95-98).

Additionally, SCFAs also act on many other cell populations of innate immunity, such as ILCs, NK cells, eosinophils, and basophils, mainly by activating membrane-embedded GPCRs (88). One such example is the GPCR-dependent activation of the NLPR3 inflammasome leading to the production of IL-18, an important soluble mediator that regulates intestinal barrier turnover and stability (99). Neutrophil recruitment is also influenced by binding of SCFAs to GPCRs. On endothelial cells, this binding event leads to thr production of the chemoattractants CXCL1 and CXCL8, whereas the activation of GPCRs on neutrophils triggers chemotactic movements (100). Further on, in response to SCFA binding to GPCR, ILC3 subset proliferation is upregulated, but also the production of IL-22 *via* downstream AKT/STAT3 signaling cascade (101, 102). Interestingly, the binding of butyrate, but also of vitamin B3, to GPCR109A on antigen-presenting cells promotes the tolerogenic phenotype, leading to an increased proportion of IL-10-producing Tregs in the colon (103). SCFA-binding GPCRs in the gut predominantly include GPR43, GPR41, GPR109A, and OR51E2, which are constitutively (and abundantly) expressed on intestinal epithelial, innate inflammatory (activated macrophages, different ILC subsets, eosinophils) and tolerogenic antigen-presenting cells (88). Depending on the aliphatic side chain length, SCFAs differentially activate these receptors. For example, GPR43 has the largest affinity for acetate (C2) and propionate (C3), while butyrate readily activates GPR41 and GPR109A receptors (104).

Although GPCRs are abundantly expressed on innate immune cells under basal and inflammatory conditions, they are rarely found on lymphocytes. For example, only low expression of GPR43 has been detected on Tregs (88). However, SCFAs undoubtedly exert multiple effects on lymphocyte function, including effector, regulatory, and memory phenotypes (105). For example, in response to butyrate, differentiation of $CD4^+$ T cells into $Foxp3^+$ Tregs occurs, associated with increased expression of anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β). The secretory activity of $CD8^+$ cytotoxic T lymphocytes is also influenced by SCFAs, through the upregulation of CD25, IFNγ, TNF-α, and granzyme B expression (56, 85). In addition,

the differentiation and growth of the $CD4^+$ T cell subsets Th1 and Th17 can be induced by acetate and propionate acting on naïve T cells (72). In this regard, the differentiation of pro-inflammatory $CD4^+$ T cell phenotypes has been widely demonstrated under specific polarizing conditions and in response to elevated SCFA concentrations. Finally, important aspects of B cell functioning, such as differentiation to effector and regulatory phenotypes, IgA antibody production, and control of immunoglobulin class switching and somatic hypermutation, are also responsive to SCFAs, particularly butyrate and propionate, and in a concentration- and context-dependent manner (85). This wide variety of immunomodulatory effects of SCFAs on cells of adaptive immunity have epigenetic reprogramming as a common denominator. Indeed, SCFAs have recently been recognized as a new class of regulators of enzymes that perform acetylation (histone acetyltransferases, HAT) and deacetylation of histones (histone deacetyltransferases, HDAC). At target genes, SCFAs promote histone acetylation and act as potent inhibitors of HDAC, leading to overall chromatin decondensation facilitating transcriptional activation. For example, through HDAC inhibition in T cells, acetate readily promotes T cell growth and differentiation *via* mTOR-S6K kinase pathway (72). In addition to histone modifications, SCFAs were found to affect DNA methylation status, and this was the first pathway ever reported for an effect of SCFAs at the epigenome level (85).

The fact that the catalytic activity of DNA- and chromatin-modifying enzymes (various methyl- and acetyltransferases) crucially depends on the availability of substrates in the milieu is strongly exploited by the intestinal microbiota, which besides SCFAs also produces other classes of metabolites that serve as substrate reservoirs (methyl/acetyl group donors) for epigenetic modification reactions (85, 106). In this sense, B vitamins derived from the GM have been recognized as very potent modulators of epigenome status due to their involvement in folate and one-carbon metabolism (especially B2, B6, and B12), which generates the universal methyl donating intermediate S-adenosylmethionine (SAM) (107, 108). In particular, commensal species belonging to the genera *Lactobacillus* and *Bifidobacterium* are known to produce folate and other B-group vitamins in large quantities (109), which is one of the properties that promotes their use as probiotics. Additionally, vitamins A, D and C are recognized as regulators of host epigenome status.

The role of vitamins and Gut Microbiota in modulating immunity

Vitamins are essential micronutrients that play a key role in many physiological and biochemical reactions. They act as coenzymes and cofactors in numerous processes, including cellular function, energy metabolism, antioxidant protection, neurological function, and immune response. As humans lack the ability to biosynthesize most vitamins, they must be supplied exogenously through a balanced diet (110). However, some vitamins (from B and K group) can be synthesized by the members of GM and consequently absorbed in the colon. Recent studies have revealed a substantial link between vitamins and the GM composition, highlighting the significance of this relationship for immune system functioning and human health (111, 112). Namely, the

interplay between the GM and a host's immune systems is crucial for restraining inflammation and upholding intestinal homeostasis. This interaction not only governs the immune system within the intestines, but also has a significant impact on broader systemic immune mechanisms. The GM role in shaping both innate and adaptive immunity is pivotal in achieving immune homeostasis and maintaining overall health (113). Furthermore, a strong correlation between vitamin deficiency and microbiota dysbiosis has been established, which, in turn, has been associated with a range of pathological conditions (114). Inadequate levels of certain vitamins may prompt alterations in gut composition, fostering the excessive growth of pathogenic strains, ultimately giving rise to persistent inflammatory conditions (115). Although it is not yet fully understood whether the GM is the cause or an outcome of the disease, it has become evident that changes in communities of gut microbes can disrupt immune balance, potentially resulting in the development of various inflammatory conditions, including autoimmune diseases.

Vitamins can be categorized into two groups: fat-soluble vitamins (comprising A, D, E, and K) and water-soluble vitamins (including vitamin C and B vitamins). Vitamin A (retinol, retinal, retinoic acid) is a fat soluble vitamin whose key biological roles encompass vision support, growth promotion, and preservation of epithelial and mucous tissues. Vitamin A plays a pivotal role in the regulation of immunological functions. It influences the differentiation, maturation, and function of both the innate and adaptive immune system. This includes the regulation of macrophage and neutrophil functions, NK T cells, homeostasis of processes in bone marrow, and the proliferation of thymocytes (116). A deficiency of vitamin A may lead to changes in the microbial community, ultimately increasing vulnerability to GIT infections (117). In one study, retinoic acid supplementation hindered Norovirus replication in a murine model. This study showed that retinoic acid treatment increased *Lactobacillus sp.* presence during Norovirus infection (118). Moreover, retinoic acid administration elevated levels of *Allobaculum*, *Aggregatibacter*, *Bifidobacterium*, *Dialister*, and *Enhydrobacter* (112, 115). Additionally, vitamin A supplementation has been linked to reduced mortality and morbidity from infectious gastrointestinal diseases, possibly by altering the GM (117).

Vitamin D (cholecalciferol) has an important role in regulating calcium levels and in promoting bone mineralization. There are numerous studies proving a significant relation of the active form of vitamin D (1,25-dihydroxyvitamin D3) and the immune system (119). Actually, it was demonstrated that the nuclear vitamin D receptor (VDR) is expressed in many immune cells. These findings make vitamin D a potent immunomodulator, having a role in the modulation of pro-inflammatory T cells function and promotion of regulatory T cells. Therefore, vitamin D has been shown to be of crucial significance in the pathophysiology of autoimmune diseases, such as insulin-dependent type 1 diabetes mellitus (T1D), multiple sclerosis (MS), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (120). In addition, roles of vitamin D and the VDR in relation to the GM have also been explored. Several studies have found microbiome-modulatory effects of vitamin D and its potential

in maintaining immune homeostasis through interactions with the GM (112). For instance, in a particular study, the provision of weekly vitamin D supplementation (50,000 IU of ergocalciferol) over a span of 12 months led to elevated levels of SCFA in feces and an increased presence of SCFA-producing genera such as *Ruminococcus*, *Fecalibacterium*, and *Dialister* (115). Moreover, additional research demonstrated that vitamin D supplementation (a single dose of 40,000 IU once weekly for eight weeks) correlated with diminished intestinal inflammation in patients afflicted with active ulcerative colitis (UC) and Crohn's disease (CD). These findings strongly imply that the administration of vitamin D could potentially yield positive effects on a range of autoimmune disorders. This effect could be attributed to the alteration of the composition of intestinal bacteria, thereby increasing the abundance of potentially beneficial bacterial strains (117).

Vitamin E (tocopherol) is a fat-soluble vitamin that is well known for its antioxidant effects. It plays a role in controlling the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), while also influencing signal transduction processes (121). Vitamin E has also shown immunomodulatory effects on the development, functioning, and regulation of dendritic cells, macrophages, NK cells, T, and B cells. Natural antioxidants have been shown to influence the composition of the GM by scavenging excess free radicals and altering the GIT redox potential. Thereby, vitamin E can potentially reshape the GM community towards an anti-inflammatory state, thus helping to alleviate mucosal inflammation. A noteworthy correlation was observed between vitamin E intake and an elevated relative proportion of *Bacteroidetes*, accompanied by a reduction in *Firmicutes* at the phylum level. Additionally, increased vitamin E consumption was linked to a decline in *Proteobacteria*, a phylum housing various pathogens and exhibiting pro-inflammatory characteristics (112, 115, 122). This suggests that vitamin E intake might contribute to cultivating a more favorable GM composition by fostering the proliferation of beneficial bacteria.

Vitamin K (phylloquinone - K1, menaquinone - K2) is a fat-soluble vitamin that has a crucial role in blood coagulation and bone metabolism. Additionally, it can serve as a co-factor for certain plasma proteins, influencing immune responses, especially those modulated by T cells (123). Dietary vitamin K is mainly present in the phylloquinone form, yet it can also be in the form of menaquinones present in fermented foods or synthesized by GM. The menaquinone synthesized *de novo* by GM can serve as a co-factor for specific microbes that need it or its derivatives for growth, benefiting both the host and these microbes (112). This indicates that vitamin K promotes bacterial diversity and acts as a mediator in interactions between diet, GM, and the dynamics of the GM community. Apart from its established functions in blood coagulation and bone health, ongoing research is uncovering the potential of vitamin K to enhance intestinal well-being (124). Vitamin K has been linked to mitigating intestinal inflammation and oxidative stress, promoting the development of intestinal epithelial cells and modulating GM composition and its metabolites. These findings propose a potential utilization of vitamin K as an adjuvant in treating various intestinal disorders, such as IBD.

Vitamin C (ascorbic acid) plays a crucial role as both an antioxidant and a cofactor. Vitamin C acts as free radical scavenger, protecting cellular components from oxidative stress. Moreover, vitamin C promotes the absorption of iron and is essential for the synthesis of collagen, carnitine, and norepinephrine, which are fundamental for various physiological processes in the body. Due to the inability of humans to synthesize vitamin C, it is essential to obtain this vitamin through dietary sources. Furthermore, vitamin C plays a vital role in supporting and regulating the immune system (125, 126). In recent years, there has been significant research on its immunomodulatory properties and its impact on the GM, particularly in the context of oxidative stress. Overall, vitamin C exerts a diverse range of positive effects on cellular functions in both the innate and adaptive immune systems. It influences various components of the immune system, such as epithelial barriers, phagocytes, B and T lymphocytes, and inflammatory mediators (126). Additionally, as an essential vitamin for collagen synthesis, vitamin C plays a crucial role in maintaining the integrity of epithelial barriers. Moreover, it enhances keratinocyte differentiation, fibroblast proliferation, migration, and accelerates the wound healing process. Vitamin C exerts both anti-inflammatory and anti-microbial properties. It aids in promoting neutrophil migration to infection sites, facilitating efficient phagocytosis, and enhancing microbial killing. This multi-faceted role of vitamin C contributes significantly to the adequate immune response and defense against various respiratory and systematic infections. Several studies have shown that vitamin C has the capacity to modulate GM composition (127). In one study, it was demonstrated that vitamin C supplementation increased the alpha diversity (a measure of the diversity or richness of microorganisms within a specific sample or environment) and the SCFA levels (112). As an antioxidant, vitamin C also has a role in preventing oxidative damage within the intestinal tract, increasing the integrity of the epithelial barrier, and thus preventing the infiltration of harmful bacteria and toxins into the bloodstream. It also contributes to the gut homeostasis by promoting the growth and activity of beneficial bacteria, and may play a preventive role in gut-related disorders (127).

B vitamins

Vitamin B1 (thiamin) serves as a cofactor for several enzymes and it is required for the synthesis of nucleic acids, fatty acids, steroids, and aromatic amino acids. Its role is thoroughly described in energy metabolism and is linked to immune cells function. Particularly, it was showed that naïve B cells in Peyer's patches need vitamin B1 for ATP generation and differentiation to IgA-producing B cells, which play the main role in the GALT (128). Several gut bacteria (*Bacteroides fragilis*, *Prevotella copri*, *Clostridium difficile*, some Lactobacillus spp., *Ruminococcus lactaris*, and Bifidobacterium spp.) generate vitamin B1 in both free thiamine and thiamine pyrophosphate form, which play a crucial role in energy metabolism within the colon (128, 129). In addition, these findings indicate that thiamine synthesized by the GM plays a distinct role in shaping the composition or functionality of the GM.

Vitamin B2 (riboflavin), along with its active derivatives flavin adenine dinucleotide and flavin mononucleotide, serves as a cofactor in various enzymatic processes which are integral to the energy-producing reactions involved in metabolizing carbohydrates, fats, and proteins (130). Riboflavin is linked to the regulation of immune cell differentiation and generation of ROS, which represent crucial effector molecules in inflammation and immune responses. In addition, it was shown that riboflavin derivatives are important for the activation of MAIT cells. This population of T cells performs antigen-presentation and production of inflammatory cytokines like IFNγ and IL-17, and therefore takes part in the host defense against pathogens, but is also involved in the development of autoimmune and inflammatory diseases (128). Metagenomic analysis of GM suggests that certain bacteria like *Lactobacillus plantarum, L. fermentum*, *Bacteroides fragilis, Prevotella copri*, *Clostridium difficile*, and *Ruminococcus lactaris* can produce riboflavin, highlighting its potential role in the modulation of GM composition and function of the immune system.

Vitamin B3 (niacin/nicotinic acid/nicotinamide) is a precursor of nicotinamide adenine dinucleotide, a coenzyme which is crucial for a range of metabolic functions, primarily as a redox cofactor (130). Vitamin B3 displays anti-inflammatory characteristics, effectively diminishing the levels of pro-inflammatory cytokines including IL-6, IL-1, and TNF-α. Furthermore, vitamin B3 influences the differentiation of regulatory T cells, thus playing a role in maintaining immunological homeostasis (128). Various intestinal bacteria can synthesize vitamin B3, including *Bacteroides fragilis*, *Prevotella copri*, *Ruminococcus lactaris*, *Clostridium difficile*, *Bifidobacterium infantis*, *Helicobacter pylori*, and *Fusobacterium varium*. Therefore, vitamin B3 can influence the composition of the GM and also contribute to the functioning of colonic epithelial cells, thus aiding in the maintenance of the intestinal epithelial barrier. Through the suppression of inflammatory cytokine production in the colon, vitamin B3 has the potential to effectively mitigate inflammation observed in gastrointestinal disorders like IBD and CD (112).

Vitamin B5 (pantothenic acid) plays a crucial role as a necessary precursor for coenzyme A (CoA), which is a vital component in the synthesis of acetyl-CoA. This compound is pivotal in a range of metabolic pathways, including the citric acid cycle, the synthesis of neurotransmitters, and the oxidation of fatty acids (129, 130). Vitamin B5 contributes to the body's defense mechanisms by enhancing both innate and adaptive immunity. It was demonstrated that vitamin B5 triggers phagocytosis and the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, and thereby induces Th1 and Th17 responses (128). A comprehensive genomic analysis of the human GM has highlighted that the ability for *de novo* synthesis of pantothenic acid is notably restricted within the genomes of *Bacteroidetes* and *Proteobacteria*. Various intestinal bacteria, including *Bacteroides fragilis*, *Prevotella copri*, *Escherichia coli*, *Corynebacterium glutamicum*, *Salmonella typhimurium*, and *Helicobacter pylori* can synthesize vitamin B5, suggesting its important role in the composition of GM.

Vitamin B6 (pyridoxine, pyridoxal, and pyridoxamine) is the precursor of the pyridoxal phosphate and pyridoxamine phosphate, which are important coenzymes that play essential roles in diverse cellular functions. In addition to its role in amino acid synthesis and breakdown, it also participates in the metabolism of fatty acids and carbohydrates (130). Vitamin B6 also plays a significant role in intestinal immune regulation by influencing the metabolism of the sphingosine 1-phosphate, the crucial lipid mediator that controls the gut-homing of lymphocytes (128). Within the mammalian GIT, bacteria engage in the synthesis of vitamin B6 utilizing *de novo* routes or salvage pathways. Comprehensive metagenomic investigations have revealed that certain bacteria possess the capacity for vitamin B6 biosynthesis, such as *Bacteroides fragilis, Prevotella copri*, *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Helicobacter pylori*. Vitamin B6 deficiency is associated with the development of inflammatory diseases such as allergy, RA, and IBD. Research has revealed that a lack of vitamin B6 disrupts the balance between Th1 and Th2 responses, while also causing modifications in microbiota diversity, as well as in gut microbiota metabolites (128, 129).

Vitamin B7 (biotin) is a crucial coenzyme for various biochemical processes, contributing to glucose, amino acid, and fatty acid metabolism. Vitamin B7 also participates in the regulation of epigenetic mechanisms, specifically in the gene expression of the nuclear factor kappa B (NF-κB), a pivotal signaling molecule engaged in the generation of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 (130). In response to a deficiency in vitamin B7, the activation of nuclear transcription of NF-κB is triggered, suggesting that vitamin B7 inhibits the NF-κB activation and restrains the expression of genes linked to pro-inflammatory cytokines. Through metagenomic analysis, it has been revealed that certain bacteria, namely *Bacteroides fragilis, Prevotella copri*, *Fusobacterium varium*, and *Campylobacter coli* possess a biosynthesis pathway for vitamin B7 (128). Studies revealed that insufficient biotin levels result in disruptions to GM balance and the excessive proliferation of *Lactobacillus murinus*, ultimately contributing to the onset of alopecia (129, 131).

Vitamin B9 (folate) functions as a coenzyme in various metabolic reactions, including the synthesis of DNA and amino acids. Vitamin B9 is pivotal in cerebral methylation processes, preserving neuronal and glial membrane lipids, and influencing neurotransmitter metabolism such as serotonin and dopamine (130). Furthermore, vitamin B9 contributes to maintaining immune equilibrium by influencing the function of Treg and MAIT cells, thereby preventing the occurrence of excessive inflammatory responses (128). It has been determined that *Bacteroides fragilis*, *Prevotella copri*, *Clostridium difficile*, *Lactobacillus plantarum*, *L. reuteri*, *L. delbrueckii subsp bulgaricus*, *Streptococcus thermophilus*, *Fusobacterium varium*, and *Salmonella enterica* possess a biosynthesis pathway for folate. Thus, folate deficiency can significantly alter GM diversity.

Vitamin B12 (cobalamine) is a complex, cobalt-containing vitamin involved in various metabolic processes. Its active forms, methylcobalamin and adenosylcobalamin, are important methyl donors crucial for nucleic acid synthesis and protein and lipid

metabolism. It also acts as a cofactor for methionine synthesis by facilitating the recycling of the amino acid homocysteine to methionine. In addition, cobalamin is essential for the proper functioning of the central nervous system, as well as for the formation of erythrocytes (130). Studies have revealed that vitamin B12 operates as an immunomodulator, influencing immune responses via $CD8^+$ T cells and NK T cells (128). In the human gut, only a limited number of bacteria have the capability to synthesize vitamin B12 (around 20% of gut bacteria) (130). Metagenomic analysis has predicted the presence of a vitamin B12 biosynthesis pathway in certain bacteria, including *Bacteroides fragilis*, *Prevotella copri*, *Clostridium difficile*, *Faecalibacterium prausnitzii*, *Ruminococcus lactaris*, *Bifidobacterium animalis*, *B. infantis*, *B. longum*, and *Fusobacterium varium*. An inadequate or excessive intake of dietary vitamin B12 could potentially impact the composition of GM. Notably, vitamin B12 supplementation in humans has led to an increase in the relative abundance of *Prevotella*, while decreasing the abundance of *Bacteroides*.

B vitamins and inflammation-related disorders

The interplay of vitamins, the immune system, and the GM is a complex and dynamic relationship. The overlapping effects in epigenome reprogramming between different gut-microbiota derived metabolites (SCFAs and vitamins), but also the possibility that they bind to the same class of receptors (GPCRs), point to a degree of inherent redundancy in signaling networks at the interface between the GM and immune system in host homeostasis. Any deviation from the delicate functioning of the GM ecosystem disrupts this hub of immune signaling and sets the stage for the initiation of multifaceted pathogenetic pathways. In this context, vitamins from both food intake/supplementation and GM exert a profound immunomodulatory influence on the composition and functionality of the GM (112). This, in turn, positions vitamins as crucial agents in maintaining the gut homeostasis and overall health status. Consequently, deficiencies in vitamins have been linked to various inflammatory disorders (132).

Gastrointestinal disorders encompassing IBD, UC, and CD are characterized by an ongoing and chronic GIT inflammation, reflecting on mucosal immune dysregulation and changes in the composition of the GM (133). A study showcased significant differences in the gene abundance profile related to B vitamins between individuals with IBD and healthy controls (112). Persistent inflammation of the intestinal tract, accompanied by increased levels of pro-inflammatory cytokines, has been demonstrated to induce alterations in the absorptive capabilities of the epithelium (134). As a result, discernible differences in the GM composition seem to be present among IBD patients, who are frequently susceptible to deficiencies in essential vitamins, including B vitamins (135). Disruptions in vitamin absorption have resulted in significantly reduced vitamin B2 levels among individuals with IBD. Additionally, perturbations in vitamin B6 and B12 are frequently observed in IBD patients (132, 135). Given the common occurrence of vitamin insufficiencies among individuals with IBD, it becomes crucial to underscore the particular importance of B vitamins. Furthermore, the assessment of vitamin status in

individuals with CD revealed heightened depletion of vitamins B1, B2, B6, and B9 (135). Additionally, a link was identified between CD and decreased expression of microbial genes responsible for generating vitamins B1, B2, and B9 (112, 136). Another study demonstrated that vitamins B3 and B5 exhibited notable reductions in the feces of individuals with CD, possibly stemming from a diminished population of bacteria that produce these vitamins (136). The diminished levels of this vitamin were directly linked to a decrease in the presence of *Faecalibacterium*, known for its role in preventing mucosal inflammation (137). Inadequacies of B vitamins in GIT inflammatory conditions could stem from hindered absorption due to inflammation, a decrease in the surface area available for absorption, and changes in the composition of gut bacteria responsible for B vitamin production. Consequently, B vitamins might offer a viable adjunctive approach for addressing gut inflammatory disorders, given their potential to display anti-inflammatory properties, foster the growth of beneficial gut bacteria, and re-establish gut-immune homeostasis (138).

Members of the B vitamin group are crucial for the proper functioning of the nervous system, and their deficiency has been linked to various neurodegenerative disorders (139). Studies have shown that the GM influence has systemic effects as well, including the central nervous system (CNS) (140). This bidirectional gut-brain axis enables communication between the GM and the CNS, influencing the blood-brain barrier's permeability and, consequently, homeostasis within the CNS (141). Due to the fact that B vitamins serve as cofactors in various metabolic pathways for the GM, changes in B vitamin levels could potentially result in gut dysbiosis. As a result, this dysbiosis could disrupt immune homeostasis, creating an environment conducive to the colonization of pathogenic strains. Consequently, the modification of GM using B vitamins might offer an additional avenue for therapeutically addressing autoimmune diseases, such as MS (142, 143). This approach could entail enhancing anti-inflammatory responses, thus leading to a subsequent reduction of CNS inflammation. In a recent study (144), authors examined the alterations in microbiota composition within an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). This study involved both animals that did not receive any treatment and animals that were treated with a B vitamin complex (VBC) containing B1, B2, B3, B5, B6, and B12, where a clear change in the gut microbiota (GM) caused by EAE was shown, with the most significant change being a decrease in the abundance of *Prevotellaceae* compared to the healthy control animals. Numerous studies have highlighted a deficiency in *Prevotella* abundance among MS patients when compared to healthy individuals, indicating a potential role of *Prevotella* in promoting anti-inflammatory responses (140). At the peak of the EAE course, *Prevotella* abundance was notably higher in VBC-treated animals when compared to non-treated animals, indicating a potential effect of B vitamins in shaping the GM composition (144). Nevertheless, additional research is required to thoroughly investigate how GM communities interact with B vitamins in influencing the development of MS and EAE disease progression.

One study (145) proposed that an underlying disruption in vitamin B6 metabolism constitutes the fundamental biochemical basis for symptoms associated with Attention-deficit hyperactivity disorder (ADHD). The large intestine's normal microbiota plays a significant role in synthesizing vitamin B6, and key neurotransmitters such as norepinephrine, tryptophan, serotonin, dopamine, and gamma aminobutyric acid are processed or synthesized by enzymes reliant on the coenzyme pyridoxal phosphate, which represents the active form of vitamin B6. These findings may indicate a potential link between ADHD and vitamin B6 production by gut bacteria. Furthermore, reduced levels of serum vitamin B12 are linked to an elevated risk of Alzheimer's disease, Parkinson's disease, and mild cognitive impairment (143, 146). Vitamin B12 acts as a coenzyme for methionine synthase, an enzyme that converts homocysteine into methionine. Elevated plasma homocysteine levels are associated with various clinical manifestations, particularly affecting the CNS (147, 148). The disruption of homocysteine catabolism might stem from deficiencies in vitamin B6, B9, or B12. Additionally, the microbiota composition has the potential to impact circulating homocysteine levels, thereby playing a role in various neurodegenerative disorders. Studies indicate that increasing the consumption of these B vitamins could potentially decrease the risk of such conditions by reducing plasma homocysteine levels (149). B vitamins can potentially affect both the composition and operation of the GM by, among other mechanisms, promoting the metabolism of specific bacteria and inhibiting the colonization of others.

Conclusion

The GM encompasses a variety of microorganisms, including proteobacteria, bacteria, viruses, fungi, protists, and archaea, which are involved in multiple functions, including nutrient metabolism, vitamin production, immune system function, neurotransmitter production, drug metabolism, and brain-gut communication. Consequently, GM dysbiosis may play an essential role in the development of chronic diseases, so that research on the effects of the composition of GM on general well-being could help in the treatment of various inflammatory diseases and in the formulation of new therapeutic strategies. As primary vitamins produced by GM, B vitamins play a role in influencing immune responses and the composition of GM. Because certain bacteria rely on B vitamins from food or vitamins synthesized by other intestinal bacteria, competition for B vitamins may occur between the host and GM. The availability of dietary B vitamins has the potential to influence the composition of the GM and consequently modulate immune homeostasis. Although the profile of GM varies from person to person, diet can alter both its composition and function. However, more in-depth research is needed to fully understand the complex interplay between the GM, vitamins, and mechanisms of immune response to pave the way for innovative therapeutic strategies targeting a range of inflammatory diseases.

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Interakcija vitamina i mikrobiote creva kao ključni faktor u održavanju imunske homeostaze u gastrointestinalnom traktu

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Kratak sadržaj

Mikrobiota creva predstavlja raznovrstan ekosistem mikroorganizama uključujući proteobakterije, bakterije, viruse, gljive, protiste i arheje. Ovi mikroorganizmi učestvuju u sintezi vitamina, regulaciji imunskog sistema, produkciji neurotransmitera, metabolizmu lekova, kao i komunikaciji sa centralnim nervnim sistemom. Nedavna istraživanja pokazala su da dizbioza mikrobiote creva može dovesti do razvoja različitih hroničnih bolesti kod ljudi. Ispitivanje uticaja sastava mikrobiote creva na opšte zdravlje pruža uvid u nove pristupe u lečenju inflamatornih bolesti i razvoj inovativnih terapeutika. Jedna od ključnih uloga mikrobiote creva ogleda se u sintezi vitamina B grupe, za koje je pokazano da ispoljavaju imumodulatorna svojstva. S druge strane, određene bakterije mikrobiote creva metabolišu vitamine B grupe direktno iz hrane, što ih u potrebi za B vitaminima stavlja u konkurentni odnos sa ćelijama domaćina. Zbog toga, dostupnost vitamina B u ishrani može uticati na sastav mikrobiote creva, a samim tim i na održavanje imunske homeostaze. Ishrana predstavlja ključni modulator kako sastava, tako i funkcionalnih svojstava mikrobiote creva čiji se profil značajno razlikuje među individuama. Međutim, neophodna su dodatna istraživanja kako bi se razumela kompleksna interakcija između mikrobiote creva, B vitamina i mehanizama imunskog odgovora. Ovaj tip istraživanja može doprineti razvoju inovativnih terapeutskih strategija za širok spektar inflamatornih bolesti ljudi.

Ključne reči: Mikrobiota creva, Dizbioza, Imunski sistem, B vitamini, Homeostaza

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Next-Generation Probiotics: health-promoting bacteria of the human gut

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Abstract

In recent years, a vast number of human diseases have been correlated with gut microbiota dysbiosis. The development of modern methods in molecular microbiology, such as the culturomics approach, as well as various multi-omics methods like next generation sequencing, transcriptomics and metabolomics analysis, coupled with large data sets correlation analysis, enabled the cultivation and characterization of novel anaerobic hitherto uncultivated Next-Generation Probiotics. In addition, the results of host-microbe interactions studies helped to reveal the mechanisms involved in the beneficial effects of Next-Generation Probiotics. Eventually, the obtained data on Next-Generation Probiotics will help to broaden the scientific knowledge on these bacteria, in terms of both their safety and health-promoting effects, unravel opportunities for the development of novel therapeutic strategies for prevention and treatment of tumors, metabolic, neuropsychiatric and other diseases, with the aim of relieving the symptoms of the diseases and increasing the quality of life for patients and their families. So far, the best characterized probiotics of the new generation are *Akkermansia muciniphila*, *Faecalibacterium prauznitzii* and *Bacteroides fragilis.*

Key words: Next-Generation Probiotics, *Faecalibacterium prauznitzii*, *Akkermansia muciniphila*, *Bacteroides fragilis*

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Introduction

The consumption of artisanal dairy products has been previously correlated with human longevity, and this observation opened the era of probiotics that are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". Probiotics were shown to have beneficial effects on the gut, metabolism, immune system and functioning of the nervous system (1–6). Commercial probiotics are represented predominantly with different species of *Lactobacillus* sp. and *Bifidobacterium* sp., commonly found in the human intestinal tract and dairy products. The probiotic effects of these bacteria are most frequently based on their anti-microbial effects, thus providing help to the host in dealing with different infections, as well as on their contribution in food digestion, thereby helping the host in food processing (7).

The gut microbiota represents a complex microbial community comprising 10^{10}-10^{14} cells of archaea, bacteria, eukarya, viruses, and bacteriophages, which started a new era of research directed towards deciphering host-gut microbiota interactions, providing the opportunity for discovering new commensal bacteria. Gut microbiota evolved together with their host, conveying mutual beneficial effects that are a prerequisite to the host's wellbeing (8). However, infection, inflammation, diet, stress, and similar circumstances could have an impact on gut microbiota diversity and composition, causing dysbiosis and diseases (9, 10). Nowadays, the development of culture-independent high-throughput molecular methods based on 16S rRNA next generation sequencing (NGS), together with developments in bioinformatics, has significantly improved gut microbiota research and enabled the precise identification of distinctive taxa from phylum to the species level (11). Overall, gut microbiota composition is dominated by five phyla with 90% of phylotypes identified in *Bacteroidetes* and *Firmicutes*, and followed by less abundant *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (12). Widespread use of NGS created a firm basis for gut microbiota research where the correlations between specific bacterial taxa and various diseases versus a healthy state are evaluated. Although there are a number of research reports related to the differences in diversity and composition between healthy subjects and various patients' groups**,** studies on metagenomics analysis, host–microbiota cross-talk and the mechanisms behind it, as well as the impact of gut microbiota on intestinal homeostasis, are still sporadic. Functional gut microbiota analysis implies the metagenome studies annotating the relative genes' abundances within the microbial community, while transcriptomic, proteomic and metabolomic analysis enable the interpretation of their interaction with the host (10, 13).

These results provided the idea of using gut commensal bacteria as probiotics to restore a healthy homeostasis of the gastrointestinal tract in a natural way, and facilitated the development of Next-Generation Probiotics (14). The scientific community is increasingly becoming interested in studying and exploitation of Next-Generation Probiotics, especially those with health-promoting properties, whose ability to modulate the host's immune response was confirmed in scientific research. However, substantial work is needed to decipher the molecular mechanisms behind the role of gut microbiota in the amelioration of different diseases. For an in depth studying of these mechanisms,

the cultivation and characterization of predominantly anaerobic gut bacteria in still insufficiently known conditions is crucial and challenging. Hence, while probiotics have been considered safe for human consumption, a regulation on the safety status of Next-Generation Probiotics still does not exist. Due to the unregulated status of Next-Generation Probiotics, the use of postbiotics, referring to functional bioactive compounds, generated in a matrix during bacterial fermentation, which may be used to promote health (15), could be a more than welcome alternative. Some of the interesting candidates for Next-Generation Probiotics are *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, and *Bacteroides fragilis*.

Akkermansia muciniphila

By reviewing the articles dealing with the role of *A. muciniphila* as potential Next-Generation Probiotics, one can conclude this bacterium poses a conundrum, as lower abundances of *A. muciniphila* were correlated with different diseases, but some research has also correlated this bacterium with different pathological conditions. *A. muciniphila* was firstly isolated by Muriel Derrien from human feces as a strictly anaerobic Gram-negative bacterium, and the most abundantly present species in the human fecal microbiota (0.5-5% of total human fecal bacteria) (16). It was characterized as a bacterium using mucin as a carbon, nitrogen, and an energy source. *A. muciniphila*, as well as another species of this genus, *A. glycaniphila*, originating from the feces of python, are found to be the only representatives of *Verrucobacterium*. Karcher et al. found a large phylogenetic diversity of the *A. muciniphila* species in humans, grouping this bacterium into five distinct candidate subspecies (17). This study supported the idea that this species is human-specific, as it was isolated only from animals in captivity. Additionally, according to this study, different subspecies of *A. muciniphila* with functional differences (e.g., the presence of putative exo/lipopolysaccharide operon) could inhabit the human intestine, and this could be the source of somewhat non-uniform effects of this species in different host models. For now, there is little connection between strain-specific differences in genome and functional features.

A. muciniphila is related with obesity in 233,000 Google search results, pointing to the great interest in the role of this bacterium in the overweight state and conditions associated with it, such as diabetes (with 167,000 Google results) and other metabolic disorders. Obesity has been denoted as one of the biggest public-health issues in the 21st century (18). Besides being associated with various chronic diseases (e.g., diabetes type 2, osteoarthritis, tumors, cardiovascular and neuropsychiatric diseases), obesity represents a huge burden on the society, influencing the overall quality of life and often leading to the development of psychosocial disorders (19). In addition to lifestyle, gut microbes have been recognized as important in regulating host metabolism (20). Commonly, the Firmicutes/Bacteroides ratio positively correlates with an increase of body mass index (BMI) (21), but manipulation of specific bacteria abundance is viewed as very promising in preventing or treating obesity. Different studies investigated the potential of using live or pasteurized *A. muciniphila* in the treatment of obesity. By

supplementing C57BL/6 overweight mice fed by a high-fat diet (HFD) with pasteurized *A. muciniphila* (22), Yang et al. showed positive effects of this treatment on obesity parameters. These mice had a decreased caloric intake and consequently decreased the body weight gain. This treatment also improved glucose homeostasis and insulin sensitivity, reduced total fat and major adipose tissues weights, and led to the lowering of intestinal inflammation. Nevertheless, Everard et al. revealed that the effects could only be achieved by supplementation with live, but not with pasteurized bacteria (23). Plovier et al. (24) further challenged the effects achieved with live *A. muciniphila*, showing greater effects of pasteurized bacteria on the weight and fat mass gain in treated animals. This study showed a higher fecal caloric content in the experimental group fed with pasteurized *A. muciniphila*, implying that this treatment reduces caloric absorption. Finally, the treatment with Amuc_1100, an outer membrane protein of *A. muciniphila*, expressed in *E. coli*, resulted in lower fat mass and body weight. Ashrafian et al. also demonstrated that the same and even greater effects on body weight reduction could be achieved by supplementation of HFD-mice with extracellular vesicles produced by *A. muciniphila* in comparison to live bacteria (25). Interestingly, pasteurized *A. muciniphila* was approved by the European Food Safety Authority (EFSA) as a novel food, pursuant to the Regulation (EU) 2015/2283 (26). Plovier et al. further showed that the decrease in body mass in live bacteria treated mice was correlated with normolipemia and a reduction of insulin resistance, while the interaction of Amuc_1100 with Toll-like receptor 2 showed potential to improve gut barrier integrity (24). These effects implicated the potential role of *A. muciniphila* in Type 2 Diabetes (DT2), since impaired glucose tolerance, insulin resistance, obesity, abnormal lipid metabolism, and low-grade systemic inflammation are common manifestations of this disease (27). Indeed, a lot of literature data indicate a lower abundance of *A. muciniphila* in feces of DT2 patients (28–31). An improvement of the metabolism of glucose, lipids, and bile acid by *A. muciniphila* supplementation was proposed as the most important for preventive effects of this bacteria in DT2. The supplementation of mice with *A. muciniphila* increased glucagon-like peptide-1 (GLP-1) secretion and reduced the expression of glucose and fructose transporters in the gut, leading to a reduction in carbohydrate absorption, reduced adipose cell differentiation, and enhanced thermogenesis by upregulation of uncoupling protein 1 (Ucp1) (32), thus influencing body weight and composition (33). Different mechanisms of GLP-1 secretion control by *A. muciniphila* were proposed, such as stimulation by propionate production (34), and stimulation by the interaction of intercellular adhesion molecule 2 (ICAM-2) on immune cells and *A. muciniphila*-derived protein P9, leading to the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway implicated in glucose and lipid metabolism (35, 36). *A. muciniphila* degrades mucin and produces short-chain fatty acids (SCFA), polysaccharides, and indole derivatives, and all these compounds are shown to be involved in lipid metabolism. Lukovac et al. (37), showed that *A. muciniphila* is involved in the control of expression of fasting-induced adipose factor/angiopoietin-like protein (Fiaf/Angptl4), involved in the deposition of triglycerides in adipocytes (38). Furthermore, these authors published that *A. muciniphila* induces a

decrease of G protein-coupled receptor 43 (Gpr43), which activated with SCFAs regulates obesity and inflammatory diseases (39), and peroxisome proliferator-activated receptor gamma (Pparγ), involved in microbiota-induced expression of Fiaf (40). Additionally, *A. muciniphila* stimulated the expression of histone deacetylase 3 (HDAC3) and HDAC5 (41). HDACs have been shown to be correlated with a number of biological processes, such as the stimulation of interleukin-8 (IL-8) and inhibition of monocyte chemoattractant protein 1 (MCP-1) production by intestinal epithelial cells (IEC) (42), thus being important in regulating intestinal inflammation. The reduction of HDAC3 expression was described in tissues of patients suffering from inflammatory bowel disease (IBD) and this enzyme has been proposed to mediate commensal-microbiota interactions (43).

The involvement of *A. muciniphila* in colitis and IBD is controversial. Literature data revealed that supplementation with this species ameliorate IBD (reviewed in (44)). At other hand Ganesh et al., published that treatment with *A. muciniphila* aggravates IBD (45). As it was demonstrated for different microorganisms, several studies have assumed that various *A. muciniphila* strains exhibit strain-specific properties on the gut barrier integrity (46,47) implying the vital role of phenotypic studies in investigation of probiotic potential (45). Liu et al., showed the strain-specific properties of *A. muciniphila* on the regulation of gut epithelial barrier and revealed that this ability depends on the genes implicated in the cellular surface proteins synthesis (47). Strain ATCC BAA-835^T(=CIP 107961^T) has been used in most of the published articles.

Besides metabolic disease, *A. muciniphila* has become a hot topic in cancer research over the last few years (reaching 202,000 Google results). A low level of *A. muciniphila* was shown to be the main microbiota feature in mouse models of colorectal cancer (CRC) associated with colitis and in patients with CRC (48, 49). Furthermore, these studies demonstrated that the supplementation of mice CRC models or patients with Amuc_1100 or pasteurized *A. muciniphila* was able to delay the development of tumor, and that this effect correlates with the direction of the immune response towards the expansion of cytotoxic T lymphocytes in the colon and local lymph nodes, as well as with TLR-2 dependent differentiation of pro-inflammatory (M1) macrophages. Apart from immunomodulatory anti-tumor effects, *A. muciniphila* produces Amuc_1434, a Mucin2-degrading enzyme, highly expressed in mucinous CRC, and could protect p53, thus promoting apoptosis of cancer cells (50, 51). In addition to the effects of solo applied *A. muciniphila* in patients and in cancer mice models, a high number of studies pointed to the possible contribution of *A. muciniphila* application to the anti-cancer effects of different types of anti-cancer therapies, such as cisplatin (52, 53) and abiraterone acetate (54). The main focus on this species in cancer research was caused by results pointing to role in the success of anti-tumor therapy based on Immune Checkpoint Inhibitors (ICIs) that targets the PD-1/PD-L1 axis (55). In this research on patients with renal cell carcinoma (RCC), non–small cell lung cancer (NSCLC), and melanoma, Routy and colleagues found that antibiotics treatment correlated with shorter survival rate of NSCLC patients and even represented a good marker for PD-1 blockade resistance. Moreover,

they showed that *A. muciniphila* abundance in patients' feces at the moment of diagnosis was significantly related to promising clinical outcomes in NSCLC and RCC. In addition, the application of fecal microbiota transplantation (FMT) to avatar mice revealed that the resistance to PD-1 blockade depends on the microbiota, and could be overcome by orally applied *A. muciniphila*. This effect correlated with higher enrollment of CCR9+CXCR3+CD4+T lymphocytes into mouse tumor and was dependent on IL-12 production. Even though these results sound convincing, taking into consideration overall results dealing with the implication of the human gut microbiota in ICI response, no consensus on the exclusive role of *A. muciniphila* was observed, while other species like *Faecalibacterium prausnitzii* (56), *Bifidobacterium longum* (57), *Bacteroides caccae* (58) were shown to be important for ICI response. Furthermore, the investigation on the correlation of microbiota composition with the efficacy of dendritic cells-based anti-cancer vaccine *in vitro* pointed to a strong negative correlation of *A. muciniphila* abundance with the pro-inflammatory properties of differentiated dendritic cells and with their potential to induce differentiation of Th1 cells *in vitro*. Thus, the positive role of *A. muciniphila* should not be taken for granted and should be investigated in every type of tumor and in every type of anti-cancer therapy.

Although the exact role and the mechanism of action of *A. muciniphila* in the gut-brain axis has not been completely clarified, a vast amount of evidence points to its potential as a therapeutic target for brain function and disease. While only a few studies have evaluated the therapeutic effects of *A. muciniphila* applications in these disorders, Xu et al. gave a comprehensive review of the mechanisms of *A. muciniphila* in the gut-brain axis, including its protective effect on the intestinal epithelial barrier, immunomodulation and production of metabolites, like SCFAs, amino acids, and their derivatives (59). The results of Xu et al. suggested that *A. muciniphila* is involved in the production of SCFAs, primarily butyrate, considered to be histone deacetylases regulators (41), and recognized as molecules important for brain development and correlated with depression (60), schizophrenia (61), and Alzheimer's disease (62), pointing to the possible role of *A. muciniphila*-derived SCFAs in the microbiota-gut-brain axis. Dooling et al. showed that *A. muciniphila* reduced the gamma-glutamylation of amino acids and increased the ratio of hippocampal gamma-aminobutyric acid (GABA)/glutamate, preventing seizures (63). Oral supplementation of *A. muciniphila* in chronic restraint stress mice led to the restoration of corticosterone, dopamine, serotonin, and brain-derived neurotrophic factor levels, indicating its role in the regulation of hormones, neurotransmitters, and neurotrophic factors (64). Additionally, some evidence of the therapeutic potential of *A. muciniphila* in different neuropsychiatric disorders, such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis, was also reviewed (59).

Faecalibacterium prausnitzii

Faecalibacterium prausnitzii (*F. prausnitzii*) is a Gram-negative, non-sporeforming bacterium, belonging to the *Ruminococcaceae* family, phylum Firmicutes.

Based on a phylogenetic evaluation using 16S rRNA sequencing, the species *F. prausnitzii* can be divided into two phylogroups and three clusters (65). This obligatory anaerobic bacterium thrives in oxygen-free conditions. Despite being extremely oxygen sensitive, *F. prausnitzii* may tolerate the presence of oxygen if the media contain glutathione, cysteine, or flavins (66). It has been discovered that in healthy humans, *F. prausnitzii* represents more than 5% of fecal bacteria. Hence, it is thought to be one of the most abundant anaerobic bacteria in the human gut (67–69).

F. prausnitzii represents an important part of the gut microbiota with a huge impact on the host's health (70). Literature data showed that alterations in the abundance of *F. prausnitzii* are often correlated with dysbiosis, leading to various human disorders including ulcerative colitis, chronic idiopathic diarrhea, acute appendicitis, colorectal cancer, type 2 diabetes, obesity, atopic diseases, neuroendocrine tumors of the mid gut, liver transplantation, etc. (68, 71, 72). Its low incidence in many intestinal diseases, especially in IBD, Crohn's disease, coeliac disease, and irritable bowel syndrome (IBS), raises the possibility that it might serve as a marker for intestinal health (68). Additionally, the association between frailty in the elderly, which is linked to increased incidence of depressive disorder, and the diversity of the fecal microbiome was investigated (73).

Due to its impressive metabolic abilities, *F. prausnitzii* is found to be very important for the production of various metabolites with health benefits. Its capacity to metabolize a variety of carbohydrates, such as apple pectin, inulin, and various carbon sources from the host, is well documented (74–76). It generates SCFAs, particularly butyrate, during the fermentation process (77). Butyrate was shown to modulate immune responses and exert anti-inflammatory effects in the gut through its inhibition of NF-κB (78). Hence, it points to the immunomodulatory effects of *F. prausnitzii*, promoting anti-inflammatory responses and contributing to immune homeostasis in the gut.

According to the obtained scientific data, anti-inflammatory compounds originating from *F. prausnitzii* could be involved in protecting gut barrier integrity and reestablishing zona occludens 1 (ZO-1) expression under diabetic conditions, presumably via the tight junction pathway (79). Due to its ability to elicit high levels of IL-10 and low levels of IL-12 and IFN-γ production, an anti-inflammatory profile of *F. prausnitzii* was revealed (70). Furthermore, through excreted metabolites, *F. prausnitzii* is able to inhibit the activation of NF-κB and production of IL-8, factors involved in the inflammation process (80). In addition, the microbial anti-inflammatory molecule (MAM) originating from *F. prausnitzii* may inhibit the NF-κB pathway in IEC, preventing the occurrence of colitis in an animal model (81).

According to studies, *F. prausnitzii* can be engaged in cross feeding interactions with other gut microbiota members, e.g., *F. prausnitzii* is often balanced with the other main commensal bacterium *Bacteroides thetaiotaomicron*, since they are metabolically complementary in the sense that *B. thetaiotaomicron*-produced acetate is consumed by *F.* prausnitzii, which in turn produces butyrate, allowing colonic epithelial homeostasis to be maintained by modifying goblet cells and mucin glycosylation (82).

Furthermore, *F. prausnitzii* has been linked to improved glucose metabolism, insulin sensitivity, and lipid metabolism. A negative correlation has been revealed between insulin resistance and *F. prausnitzii* (28). The obtained results revealed that *F. prausnitzii* may be an excellent candidate for a new treatment approach for type 2 diabetes, since it improves insulin resistance index (IR) and lipid metabolism, while lowering inflammation (83).

Additionally, research findings indicate that *F. prausnitzii* may be used as a psychobiotic due to its mental health benefit linked to the alleviation of anxiety and depression symptoms in rats (84). Based on the results of the microbiome analysis of the feces from patients with Alzheimer's disease and mild cognitive impairment (MCI), it can be concluded that *F. prausnitzii* has a positive correlation with cognitive scores in the MCI group in comparison to healthy subjects (85). Further experiments confirmed that two *F. prausnitzii* strains, live Fp360 and pasteurized Fp14, isolated from the healthy group, improved cognitive impairment in an Alzheimer's disease mouse model, indicating the possible application of *F. prausnitzii* in gut microbiome modulation in people suffering from Alzheimer's-type dementia.

A comprehensive study of *F. prausnitzii* and its metabolites in human feces may be useful in developing treatment options and personalized therapy. Available knowledge points to the potential significance of *F. prausnitzii* in maintaining gut function and host wellbeing. Although *F. prausnitzii* shows promise as a potential probiotic, more work is needed to completely reveal its mode of action, optimal dose, and safety issues. The challenges associated with its anaerobic nature and the need for further clinical studies limit its current availability as a stand-alone probiotic. Nonetheless, ongoing research in the field of gut microbiota and its potential therapeutic applications may shed more light on the benefits of *F. prausnitzii* as a candidate for Next-Generation Probiotics.

Bacteroides fragilis

Bacteroides fragilis (*B. fragilis*) is an obligate anaerobe usually found in the human gut, but it has also been detected in the upper respiratory and female genital tract. It is a Gram-negative bacteria, one of the most common members of the genus *Bacteroides* (Phylum Bacteroidetes, Class Bacteroidia, Order Bacteroidales, Family *Bacteroidaceae*) (86). The first discovery of *B. fragilis* was in infected patients, and it was thus isolated as a pathogen strain (87). *B. fragilis* was frequently found in individuals with a variety inflammatory disorders, such as: IBD, endocarditis, bacteremia, septicemia, as well as infections of the abdomen, skin, bone and joint, female reproductive tract, central nervous system and lower respiratory tract (88–94). Besides humans, this bacteria colonizes mucosal surfaces in the lower gastrointestinal tracts of various mammals, mainly infant sheep, beef, rabbits and pigs (95–98). *B. fragilis* is able to metabolize polysaccharides as sources of carbon and energy, and even though it is an obligate anaerobe, it could be tolerant of oxygen exposure (99). There have been reports stating that a number of variables, including physical health, drug use, lifestyle choices, but most importantly food, might influence the amount of *B. fragilis* in the gut. A diet

high in carbohydrates significantly affected the amount of this bacteria in several studies (100, 101). Other studies suggest that vitamin D has a positive correlation with the quantity of *B. fragilis* (102), while the intake of probiotic drinks containing *Lactobacillus casei* Shirota (103) or heat-killed *L. kunkeei* YB38 (104) reduces the amount of this bacteria in the gut.

Previous studies indicate that there are two kinds of *B. fragilis* strains: nontoxigenic *B. fragilis* (NTBF) that do not harbor or secrete *B. fragilis* toxin (BFT), and enterotoxigenic *B. fragilis* (ETBF) strains that have *bft* genes, coding *B. fragilis* toxin in their pathogenicity islands (BfPAI) (105, 106). NTBF strains are frequently thought of as advantageous commensal inhabitants that could compete with ETBF. By releasing specific favorable chemicals, one of which has been identified as polysaccharide A (PSA), these beneficial strains support intestinal health (107). PSA possesses zwitterionic properties (having both positive and negative charges on the sugar molecule), which is a rare feature among a few known bacterial polysaccharides (108, 109). Further experiments need to determine the mechanism of the delivery of PSA to host cells. One possible mechanism is secretion via outer membrane vesicles, which have components that form the outer membrane, such as phospholipids, proteins and polysaccharides (110).

It is interesting that a large portion of the genome of *B. fragilis* gives information for capsular polysaccharide synthesis, enabling this bacteria to produce at least eight distinct capsular polysaccharides (111). An essential basis for *B. fragilis* colonization and function in the human colon may be provided by the extensive and variable expression of surface polysaccharide combinations, since *B. fragilis* mutants lacking surface polysaccharide expression have problems colonizing the intestine. It is important to note that these mutants use an alternative way of restoring the expression of multiple capsular polysaccharides, leading to stable commensalism. (112). Besides, in their capsular surface *Bacteroides* incorporate polysaccharides and glycoproteins with L-fucose, which is a plentiful surface molecule of intestinal epithelial cells, leading to the coordinated expression of this surface molecule by the host and symbiont. Therefore, a *Bacteroides* mutant which cannot cover its surface with L-fucose is incapable of successfully colonizing the mammalian intestine in a competitive environment. (113).

Despite previous reports that *B. fragilis* was frequently detected in various diseases, it is reported as a commensal, with potential as a probiotic (114), due to its immunoregulatory and health-promoting effects. In experimental colitis-related mouse models, it can direct an anti-inflammatory response and provide protection (115, 116), showing similar effects in autoimmune encephalomyelitis (117), colorectal cancer (118), pulmonary inflammation (119) and asthma (120). Additionally, *B. fragilis* has been shown to have positive effects on graft-versus-host disease (GVHD), a serious complication following allogeneic hematopoietic cell transplantation (allo-HCT) and proinflammatory syndrome brought on by donor T cells. It was shown that the consumption of a *B. fragilis* isolate improved gut health by enhancing gut diversity and helpful commensal bacteria, while reducing proinflammatory bacteria. PSA-deficient *B. fragilis* failed to protect recipients from GVHD, even though the administration of live or

heat-killed *B. fragilis* significantly reduced GVHD. Additionally, the administration of *B. fragilis* increased intestinal tight junction integrity and SCFAs, particularly butyric acid and acetic acid, in gut tissues (121). Due to its immunomodulatory effects on the gut-brain axis, *B. fragilis* PSA could be used as promising agent for potential new treatments of multiple sclerosis (MS) (122). It appears that PSA is a potent modulator of neuroinflammation, since PSA given orally was able to protect against a lethal viral neuroinflammatory disease, like herpes simplex encephalitis (HSE). A possible mechanism could be that PSA binds and stimulates intestinal $TLR2^{+}$ plasmacytoid dendritic cells and B cells to secrete IL-10, followed by the induction of regulatory T cells producing IL-10 and IFN-γ, which together suppress pathogenic inflammatory monocytes and neutrophils to prevent encephalitis (123). It is interesting that changes in PSA expression in the gut lumen can cause an imbalance that may lead to peripheral systemic autoimmune disorders like human MS or EAE, and changing the gut microbiota's composition may enable to control the ratio of disease prevention to disease induction (124).

Taking into account their controversial status, like any other potential probiotic strains, different *B. fragilis* strains need to be evaluated systematically to determine whether they comply with the probiotic safety requirements. For example, *B. fragilis* strain ZY-312 was evaluated systematically, and it was shown that its metabolite profile closely resembles descriptions of *B. fragilis* in Bergey's manual and that it lacks BFT toxin, and both healthy and immune-deficient mice were used to demonstrate *in vivo* safety. This strain has 11 antibiotic resistance genes, but they are located on the chromosome, eliminating the risk of plasmid-mediated transfer of antibiotic resistance (125).

Finally, it is important to have in mind that research in this field is ongoing, and more studies are needed to fully understand the therapeutic potential and mechanisms of action of *B. fragilis* and its potential for Next-Generation Probiotics.

Conclusion

Although studies investigating the role of microbiota-gut-brain axis in neurodegenerative and psychiatric disorders have shown promise, their exact mechanisms of action are still not clear, and strong evidence-based treatments have not yet been developed. Hence, the isolation of novel gut microbiota members, their detailed safety and health-promoting characterization, as well as the identification of the metabolites produced by gut microbiota members involved in host-microbe interactions, are part of a fascinating journey leading to a full understanding of their role in health and diseases and shedding a new light on the field. Finally, the new knowledge acquired in this pioneering field will enable the development of novel Next-Generation Probiotics for prevention and treatment of various diseases, along with the development of effective diagnostic/prevention strategies based on detecting the presence/absence of key gut microbiota players/metabolites in the human gut microbiome before the symptoms of the disease occur.

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Probiotici sledeće generacije: crevne bakterije koje unapređuju zdravlje

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Kratak sadržaj

Poslednjih godina se veliki broj patoloških stanja i bolesti dovodi u vezu sa disbiozom crevne mikrobiote i promenama u njenom funkcionisanju. Razvoj savremenih metoda molekularne mikrobiologije, uključujući kulturomiku i integrativne pristupe kao što su sekvenciranje sledeće generacije, transkriptomska analiza dualne RNK sekvence i analiza metabolomike, omogućio je identifikaciju, kultivaciju i karakterizaciju novih anaerobnih, do sada nekultivisanih probiotika, nazvanih probiotici sledeće generacije. Pored toga, rezultati *in vitro* i *in vivo* studija proučavanja interakcija domaćina sa mikrobiotom pomogli su u rasvetljavanju mehanizama delovanja probiotika sledeće generacije. Na kraju, dobijeni podaci o probioticima sledeće generacije pomoći će da se prošire naučna saznanja o ovim bakterijama, kako u pogledu njihove bezbednosti, tako i u pogledu njihovog uticaja na zdravlje, otvarajući mogućnost za nove terapijske pristupe u prevenciji i terapiji metaboličkih bolesti, tumora, neurodegenerativnih i psihijatrijskih bolesti i drugih bolesti, u cilju ublažavanja simptoma bolesti i poboljšanja kvaliteta života pacijenata i njihovih porodica. Do sada najbolje opisani probiotici sledeće generacije su *Akkermansia muciniphila*, *Fecalibacterium prauznitzii* i *Bacteroides fragilis.*

Ključne reči: probiotici sledeće generacije, *Akkermansia muciniphila, Fecalibacterium prauznitzii, Bacteroides fragilis*

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Polyphenols as a new class of prebiotics for gut microbiota manipulation

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Abstract

A thriving community of microorganisms resides in our intestines, forming complex interactions and producing signaling molecules that can affect human physiological processes. Intrinsic and environmental factors modulate the composition of the microbial ecosystem, with diet representing a key factor affecting the formation of gut microbiota. The epidemic of non-communicable chronic diseases has been associated with the Western diet, which may modulate the gut microbiota, resulting in a detrimental derangement in the microbial community, termed dysbiosis.

Dysbiosis may be reverted through dietary interventions and the application of probiotics and prebiotics. Phenolic compounds represent plant-based nutraceuticals, which can be separated into phenolic acids and polyphenols, that exert prebiotic-like effects and may prevent the development of diseases. Besides direct health-promoting activities, beneficial effects of phenolic compounds may be exerted through their interaction with the gut microbiota. A bidirectional relationship between the gut microbiota and phenolic compounds has been observed, as microorganisms are involved in the metabolism of phenolic compounds, whereas phenolic compounds may affect the composition of the microbiota, with selective stimulatory or inhibitory activity towards the growth of microbial members. In this review, insight into the gut microbiota-polyphenols relationship will be given, with a focus on the application of polyphenols for modifying microbiota and promoting health.

Key words: polyphenols, microbiota, prebiotic, quorum-sensing

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Introduction

More than 2000 years ago, Hippocrates postulated that “all disease begins in the gut”, indicating that even in ancient ages people knew about the importance of healthy intestines. However, only in recent years did it become apparent that the community of microbes residing in the gastrointestinal tract and their function are important for maintaining health and promoting disease. Microorganisms reside in and on our bodies, and the most diverse microbial ecosystem, termed gut microbiota, is present in the gastrointestinal tract. Human intestines are colonized by more than 100 trillion microorganisms, with distal parts of the colon characterized by the highest diversity and density of microorganisms (1, 2).

The recent advantages in microbiota analysis methods, along with the improvements in culture-based methods, provided an important insight into the complex composition and activity of the human microbiota (3-6). Human gut microbiota contains over 1000 bacterial species, several hundreds of fungi species, and several thousands of viruses and other microorganisms. Today, with the application of advanced sequencing methods combined with various bioinformatics tools, it is possible to study this ecosystem with species or even strain-level resolution (3, 7-9).

Microorganisms residing in the intestines are involved in various functions, including catabolism of nutrients and xenobiotics, synthesis of vitamins, as well as the maintenance of intestinal barrier integrity and resistance to pathogen colonization. Moreover, microbial metabolites may enter the circulation and exert effects on distal organs. Through their activity, gut microbiota affects human metabolic pathways responsible for energy balance, appetite control, immune function, cardiometabolic and neurobehavioral processes (1, 10, 11).

Gut microbiota is a dynamic ecosystem, which is affected by intrinsic and environmental factors during its formation. Throughout life, gut microbes are under the influence of acute or long-term perturbations – such as diet changes, treatment with antibiotics or other medications, the existence of gastrointestinal pathologies, level of physical activity, pregnancy, type of delivery and breastfeeding, and different environmental exposures, among others (2, 12). Among all these factors, diet represents a major factor in the modification of the microbial ecosystem. Diet provides necessary nutrients for humans, but it also provides substrates for microbial growth. In addition, many microbial species are ingested through diet. Differences in dietary patterns significantly shape the microbiota of each individual (13-16). Unfortunately, the industrialization and mass production of ultra-processed food contributed to the development of the dietary style prevalent in most of the world. This Western diet is characterized by a high intake of saturated fats, simple sugars, sodium, and a deficient intake of essential micronutrients, phytochemicals, and complex plant carbohydrates, as shown in Figure 1 (17, 18). The Western diet, combined with the irrational use of antibiotics, pesticides, and other chemicals, has led to the great extinction of microbial species that used to colonize the human gastrointestinal tract for centuries. Differences in

gut microbiota composition between urban and rural populations are remarkable, and urbanized populations have significantly lower microbial diversity, with a higher abundance of pathogenic species (19-21).

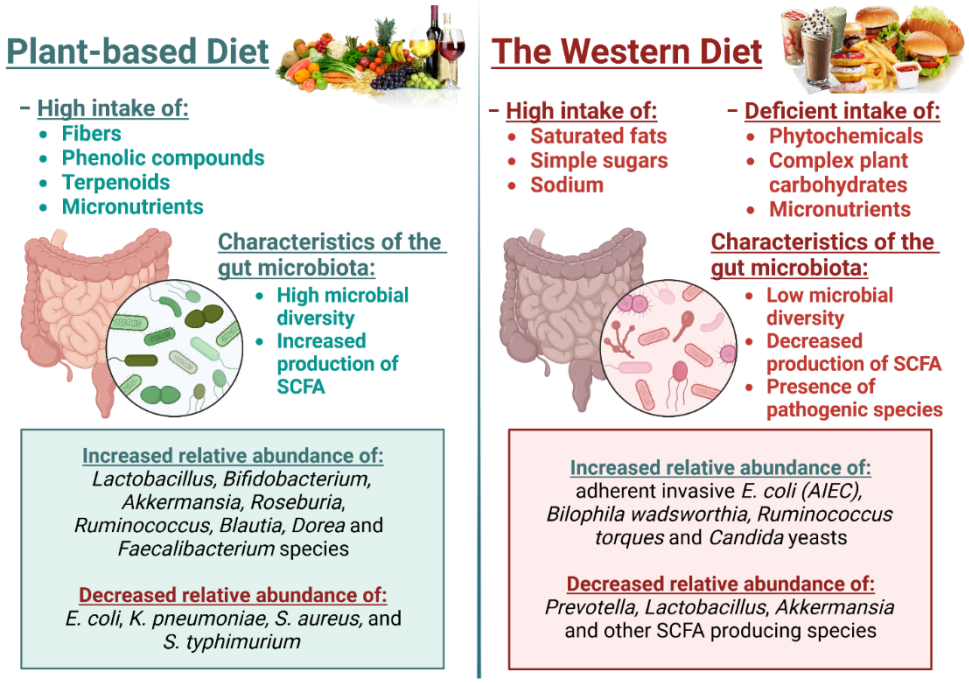

Figure 1. Comparison of plant-based and Western diet in nutrients and phytochemicals intake, and the composition of the gut microbiota

Slika 1. Poređenje ishrane zasnovane na namirnicama biljnog porekla i zapadnjačkog načina ishrane u unosu nutrijenata, fitohemikalija i sastavu mikrobiote

Under the persistent and/or intense influence of stressors, a detrimental derangement in the structure and function of microbial communities, termed dysbiosis, may occur. Dysbiosis, characterized by low diversity, loss of beneficial microbial species, and colonization of potential pathogenic microorganisms, is associated with various proinflammatory diseases, from local gastrointestinal pathologies, to cardiometabolic, neurologic, respiratory, and immune-related disorders, indicating the importance of maintaining the integrity of the intestinal microbial ecosystem (22-24). The epidemic of non-communicable chronic diseases is probably linked to microbiota dysbiosis, while the low effectiveness of traditional therapies most likely reflects the fact that we are underusing the ability to modify microbiota in a desired direction. Microbiota modification is possible through dietary and functional medicine interventions, including dietary changes, and the use of probiotics, prebiotics, or synbiotics (25-28).

Since its introduction in 1995, the term prebiotic has changed its definitions with new evidence regarding the prebiotic activity of various food compounds (22, 29, 30). Previously, prebiotic potential was associated with non-digestible carbohydrates, and to

be defined as prebiotics, substances had to meet three criteria, including resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, being fermented by intestinal microbiota, and selectively stimulating the growth and/or activity of intestinal bacteria associated with health (30). According to the most recent definition of the International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics are defined as substrates selectively utilized by host microorganisms and conferring a health benefit. Besides focusing not only on specific bacterial species, but on the entire microbiota, the latest definition encompasses the beneficial activity of prebiotics not only in the gastrointestinal tract, but also in other parts of the body (22, 31).

In addition to the well-established prebiotics from the group of non-digestible polysaccharides and oligosaccharides such as β-glucans, inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS), recent studies have shown that non-carbohydrate molecules may also exert prebiotic activity. These molecules include polypeptide polymers, poly-unsaturated fatty acids, polyphenols, and other phytochemicals (1, 32). As of 2016, ISAPP has included polyphenols as a potential class of prebiotics, as the health-promoting activities of polyphenols have been shown to be mediated through their interaction with the gut microbiota (22). In this review, we discuss the possibilities of using polyphenols as a tool for modifying microbiota composition and function to promote health.

Phenolic acids and polyphenols

Phenolic compounds represent a chemically diverse group of phytochemicals, widely distributed in fruits, vegetables, herbs, spices, and beverages, such as wine, tea, and cocoa. More than 8,000 phenolic compounds have been identified. These secondary plant metabolites are involved in the regulation of plant development and protection against reactive oxygen species (ROS), ultraviolet radiation, and pathogens (1, 23, 33).

The classification of phenolic compounds varies according to different criteria, with several existing classifications based on the chemical structure (34). Phenolic compounds may be broadly divided into simple phenols, phenolic acids and their derivatives, and more complex polyphenols (35-37). The main constituent of phenolic compounds is an aromatic ring with at least one hydroxyl group. Phenolic acids possess one carboxylic acid group and can be separated, based on the length of the chain containing the carboxylic group, into hydroxybenzoic, hydroxycinnamic acids, and other hydroxyphenyl acids, including acetic, propanoic, and pentaenoic (35). Polyphenols possess one or more aromatic rings that have more than one hydroxyl group and can be divided into flavonoids and non-flavonoid compounds (38, 39). Flavonoids are a diverse class of low-molecular-weight polyphenols consisting of two phenyl rings connected through a heterocyclic pyrane ring and forming a phenyl benzopyran skeleton. Based on the differences in the pyran ring, flavonoids can be further separated into six groups, including flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanidins. Among different flavonoid classes, compounds differ in hydroxylation and methylation patterns of phenyl rings, which are generally mono, di, or trihydroxylated (1, 35, 36, 40).

Non-flavonoid phenolic compounds are characterized by more complex and heterogeneous structures, and they include lignans, stilbenes and tannins, among others (39, 41). Tannins can be subdivided into hydrolysable (gallotannins and ellagitannins), condensed and complex tannins. Gallotannins and ellagitannins contain gallic acid units, whereas condensed tannins consist of catechin units and are also called proanthocyanidins. Stilbenes and lignans possess a similar structure, as stilbenes contain two phenol units linked by two double bonded carbons, whereas in lignans the phenol units are connected by four carbons (36).

Polyphenols and phenolic acids are naturally found predominantly in glycosylated forms, bound to sugar constituents as O-glycosides, and C-glycosides, and except flavan-3-ols and proanthocyanidins, they only exceptionally appear in deconjugated form of aglycones. Some flavonoids form complex oligomeric and polymeric structures, such as proanthocyanidins or condensed tannins, including procyanidins, prodelphinidins, or propelargonidins (41, 42).

The consumption of phenolic compounds varies significantly among different populations and age groups, as it is highly correlated with dietary patterns. The estimated daily intake of polyphenols in Europe varies from 500 mg to 2 gr/day, with coffee, tea, and fruit representing the main sources of phenolic compounds. Other major dietary sources include vegetables, wholegrains and nuts, cocoa, herbs and spices, and the different sources of phenolic compounds are shown in Figure 2 (43, 44). Among fruits, berries, cherries, plums, and apples have the highest amounts of polyphenols, whereas artichokes, onions, olives, and spinach represent vegetables that are an excellent source of polyphenols (45, 46).

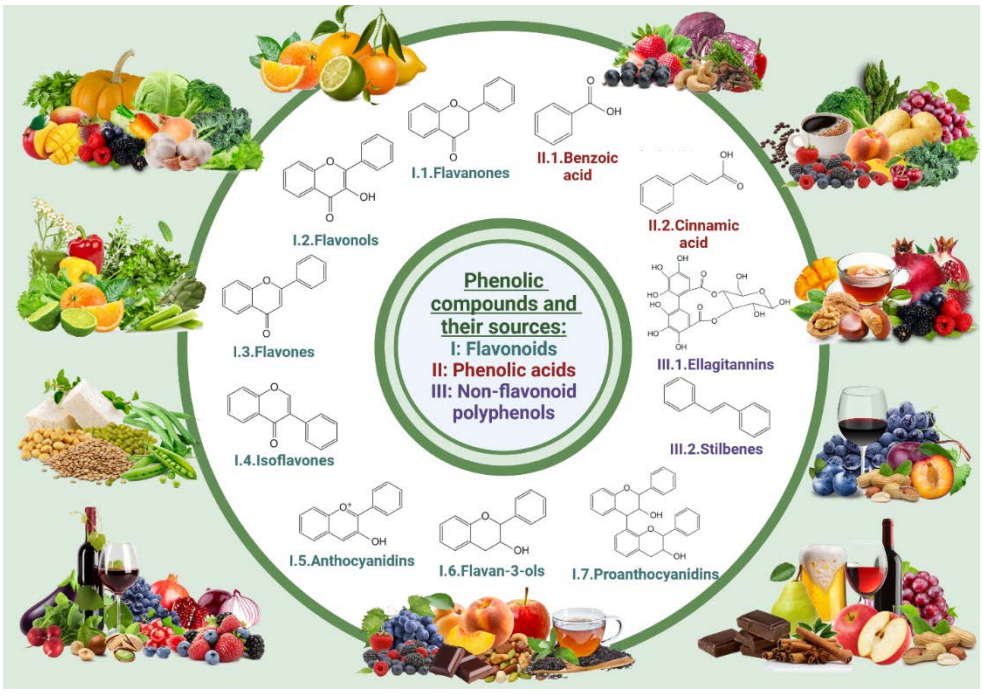

Figure 2. Classification of phenolic compounds and their sources in nature
Slika 2. Klasifikacija fenolnih jedinjenja i njihovi prirodni izvori

Medicinal plants and spices, traditionally used in culinary recipes and/or for prevention and therapy of diverse pathologies, have been identified as a rich source of phenolic compounds, with many yet unresearched phytochemicals that may possess significant biological activity. Frequently isolated phenolic compounds in culinary spices and medicinal herbs include chlorogenic, caffeic, isovanilic, and cichoric acids, kaempferol, luteolin, apigenin, and their derivatives and glycosides (47-50).

Resveratrol, curcumin, quercetin, rutin, genistein, daidzein, naringin, hesperidin, ellagitannins, and proanthocyanidins represent the most studied polyphenols with many suggested biological activities (51). Quercetin and its glycosides are among the most widely consumed polyphenols, mainly found in onions, pepper, tea, red wine, berries, pomegranates, and apples. Due to its abundance and health-promoting effects, quercetin has been thoroughly researched, resulting in many quercetin supplements currently available on the market (42). Likewise, resveratrol, a stilbenoid found in the skin of grapes, berries, peanuts, wine, and chocolate, has attracted significant attention regarding its beneficial effects on cardiometabolic parameters and cancer prevention (52). Another frequently consumed flavonoid is epigallocatechin gallate (EGCG), flavan-3-ol esterified with gallic acid, which is the main phenolic compound in green tea (~ 50% of total green tea polyphenols). Studies have revealed the high antioxidant activity of EGCG, and the ability to modulate the molecular signaling pathways through which it exerts anti-inflammatory, anti-proliferative, and other beneficial effects (53-55).

Metabolism of polyphenols

The majority of ingested polyphenols are not absorbed in the small intestine. It has been estimated that about 90-95% of consumed polyphenols reach the colon intact. In the colon, polyphenols undergo extensive biotransformation by intestinal microorganisms. Although phenolic compounds have low bioavailability, through gut microbiota-mediated metabolism, an array of metabolites is produced, which may have greater bioaccessibility and significant biological activity, and which may be responsible for the positive effects of polyphenols on health (23, 51, 56). As most phenolic compounds in plants are conjugated with sugars and organic acids and need to be hydrolyzed before absorption, the bioavailability and bioactivity of polyphenols largely depend on the metabolic capability of intestinal microbes. Gut microbiota possesses glycosidases and esterases responsible for the deconjugation of glycosides, glucuronides, and organic acids, which leads to the hydrolysis of glycosides. The released aglycones are further transformed into various derivatives through the activity of microbial enzymes involved in dehydroxylation, reduction, decarboxylation, demethylation, and isomerization reactions, among others (23, 41, 42, 57).

The catabolic pathways of phenolic compounds are complex and highly dependable on the composition and metabolic potential of the gut microbiota. As a result of polyphenol catabolism, various end products can be produced by different microbial species. In addition, the metabolism of specific phenolic compounds also depends on other factors, including the number, type, and position of specific functional groups,

polymerization degree, and stereoisomerism (41, 56). Therefore, both the nature of polyphenols and the metabolic potential of the microbiota define the type of polyphenol transformation in the colon.

Only a few of the metabolic pathways involved in the microbiota-mediated biotransformation of polyphenols have been described. One of the most thoroughly investigated is the biotransformation of soy isoflavones daidzein and genistein into equol or O-desmethylangolensin. Equol possesses more potent bioactivity compared to its precursors. Several bacterial species, including *Slackia isoflavoniconvertens, S. equolifaciens,* and *Adlercreutzia equolifaciens,* can produce equol from daidzein. It has been shown that the presence of these microorganisms is essential for the beneficial effects of soy isoflavones (41, 58). Likewise, the metabolism of ellagic acid and ellagitannins into urolithins (3,4-benzocoumarin derivatives) was found to be dependent on the gut microbiota composition, with several *Gordonibacter* and *Ellagibacter* species possessing tannin acyl-hydrolase enzymes necessary for the metabolism of ellagic acid (59).

Despite the presence of a few illustrative examples, due to the great structural diversity, the catabolism of polyphenols is still under-researched, and more studies are necessary to identify produced metabolic intermediates and end-products (41).

Activities of polyphenols

Similar to the diverse functions that polyphenols have in plants, results from *in vitro* and *in vivo* studies suggest that phenolic compounds possess a wide range of biological activities, which appear to be associated with the maintenance of human health and the prevention of chronic diseases (60). As mentioned earlier, medicinal plants, in addition to spices, are the richest sources of polyphenols. Medicinal plants have been traditionally used for the treatment of various gastrointestinal diseases, including gastritis, inflammatory bowel disease, and irritable bowel syndrome, among others. The positive effects of medicinal plants can largely be attributed to the presence of polyphenols. Likewise, the consumption of food rich in polyphenols has been inversely associated with the risk of the development of various non-gastrointestinal diseases, including cardiometabolic (type 2 diabetes mellitus, atherosclerosis, coronary heart disease, non-alcoholic fatty liver disease, metabolic syndrome, and others) and neurodegenerative diseases (Alzheimer's disease and multiple sclerosis), several types of cancer, other inflammatory and immune-related disorders (37, 43, 61).

The beneficial effects of phenolic compounds have usually been explained by their potent antioxidative properties; however, a plethora of other health-promoting activities have been associated with the consumption of polyphenols and phenolic acids, including anti-inflammatory, antimicrobial, anticarcinogenic, neuroprotective, antiadipogenic and immunomodulatory activities, among others (62-64). Although *in vitro* studies have proven a remarkable free-radicals scavenging activity, the *in vivo* antioxidant activity of phenolic compounds is not solely based on the free radicals elimination, as it involves the regulation of the activity of endogenous antioxidant enzymes and more complex

molecular mechanisms associated with the modulation of gene transcription and cellular signaling pathways (43).

The ability to reduce tissue inflammation has been documented for many phenolic compounds, and the effects of polyphenols on the maintenance of the gut barrier integrity have been observed in studies (1, 61). Moreover, studies have shown that polyphenols can bind to specific proteins, such as kinases associated with the regulation of pro-inflammatory cytokines (transcription factor NF-κB, interleukin-6 (IL-6), and IL-1), and mitogen-activated protein kinase 2 (MAP kinase 2), subsequently affecting the expression of genes associated with inflammation, cell adhesion, antioxidant defense, and cell signaling (43, 62, 65-67). Results also indicate that polyphenols may alleviate chronic oxidative cellular and DNA damage, in addition to recent findings suggesting the involvement of polyphenols in the regulation of cell cycle processes and the ability to induce apoptosis by modulating mitochondrial functions and bioenergetic control (53, 68).

In addition to the direct effects on human health, polyphenols and their metabolites exert modulatory effects on the gut microbiota, affecting the composition and activity of present microorganisms. More than a decade ago, the existence of a bidirectional relationship between polyphenols and the gut microbiota was indicated, and the importance of a highly diverse microbiota for adequate polyphenol metabolism was emphasized (41, 64, 69).

Polyphenol - gut microbiota - host axis

The bidirectional interaction between polyphenols and gut microbiota has been researched using *in vitro* and *in vivo* approaches, including batch culture fermentations and gastrointestinal simulators, as well as animal model and human intervention studies (13). Polyphenols may alter the composition and activity of intestinal microbes by directly modulating the metabolic activity of microorganisms, or by exerting growth-stimulating or growth-inhibiting effects on the gut microbiota members. Reciprocally, the microbiota is involved in the biotransformation of phenolic compounds into metabolites, which may be responsible for the health-promoting properties of polyphenols and phenolic acids (58).

As the effects of consumed polyphenols are highly dependent on the gut microbiota, a great inter-individual variability in response to polyphenols is explained by the differences in microbial activity and composition. Moreover, some microorganisms require polyphenols as a nutrient source and prefer carbohydrates attached to the phenolic compounds, explaining the role of microorganisms in the biotransformation of glycosylated polyphenols. In addition to glucose, other sugar moieties, including rutinoside and neohesperidoside, may be attached to phenolic compounds and act as a preferred energy source for certain microbial species (41, 43).

Considering the high interindividual variability in gut microbiota composition and activity, the beneficial effects of ingested phenolic compounds have been inconsistent across studies, resulting in the need for tailored prebiotic interventions based on an

individual's microbiota profile. A personalized microbiota-focused approach has been investigated, with some authors investigating the possibility of categorizing individuals into metabotypes, based on variations in the microbial metabolism of phenolic compounds. By classifying individuals into producers and non-producers of certain phenolic metabolites, a personalized polyphenol-rich diet may be tailored to support a unique gut microbiota, with the goal of promoting health and treating specific diseases (41, 70, 71).

Prebiotic effects of phenolic compounds

Due to a great diversity of phenolic compounds, the effects of ingested polyphenols and phenolic acids on the growth of microorganisms vary significantly. However, several commensal species have been frequently observed to be stimulated by polyphenols. Interestingly, the utilization of polyphenols has been paralleled with the increase in the production of health-promoting short-chain fatty acids (SCFAs). Polyphenols exert prebiotic effects by modulating the gut microbiota through complex interactions with microorganisms, and they have a unique ability to promote the growth of commensal microorganisms, in parallel with the inhibition of pathogen growth (41, 64).

Studies have shown that polyphenols exert prebiotic effects through the selective growth stimulation of beneficial microorganisms, including species belonging to the genera *Lactobacillus, Bifidobacterium, Akkermansia, Roseburia, Ruminococcus, Blautia*, *Dorea* and *Faecalibacterium* (13, 63, 72). For example, stimulatory effects on the growth of *Lactobacillus*, *Akkermansia*, and *Ruminococcus* species were observed after the intake of orange juice, rich in hesperidin and naringenin (73). Likewise, the intake of anthocyanin-rich fruits (red and purple berries, red grape, pomegranate, and plums) promoted the growth of *Akkermansia muciniphila*, a mucin-degrading bacteria associated with beneficial effects on the integrity of the intestinal barrier, anti-inflammatory activity and protection against obesity and cardiometabolic diseases (74). In addition to anthocyanins, other phenolic compounds, including phenolic acids, flavan-3-ols, flavonols, flavanones, stilbenes, and hydrolyzable tannins, were associated with the increased abundance of *A. muciniphila* (58, 75). Supplementation with pomegranate extract, rich in ellagitannins, was found to stimulate the growth of *A. muciniphila* in urolithin A producers. Additionally, pomegranate consumption increased the abundance of several bacterial genera including *Butyrivibrio, Enterobacter, Escherichia, Lactobacillus, Prevotella, Serratia*, and *Veillonella*, whereas the abundance of *Collinsella* significantly decreased following dietary intervention (76).

In addition to selectively stimulating the growth of probiotic and commensal species, polyphenols have been found to have potent inhibitory activity against the growth of many pathogens and opportunistic pathogens. Phenolic compounds exert antimicrobial activity through several mechanisms, including metal-chelating ability (flavan-3-ol) and the disruption of cell membrane function, or by altering the membrane permeability (anthocyanins), antiadhesive activity (resveratrol), inhibition of biofilm formation and quorum sensing (23, 41, 42).

For instance, naringenin, quercetin, and rutin were found to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*, whereas quercetin also exerted inhibitory activity towards the growth of *Klebsiella pneumoniae* and *Proteus vulgaris* (1).

The anti-adhesion effect of cranberry, rich in proanthocyanidin-A – procyanidin A2 and cinnamtannin B1, as well as other flavonoids and phenolic acids, on the growth of *E. coli*, represents a well-known antimicrobial effect of polyphenols, and many cranberry supplements were formulated targeting the *E. coli* urinary tract infection (UTI). In addition to suppressing the growth of *E. coli*, cranberry extracts were found to exert antimicrobial activity towards the growth of other microorganisms responsible for UTIs, including *K. pneumoniae*, and some Gram-positive staphylococci and enterococci, indicating the great potential of polyphenols in restoring microbiota balance (77, 78). Likewise, green and black tea polyphenols were found to inhibit the growth of pathogen bacteria, including *S. aureus*, *E. coli*, *Helicobacter pylori*, *S. typhimurium*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*, as well as several viruses such as hepatitis C virus, influenza, and HIV, but also *Candida* yeasts (79).

Resveratrol intake has been associated with an increase in the relative abundance of *Bacteroides*, *Lactobacillus*, *Christensenella*, *Bifidobacterium*, and *Akkermansia* (80-82). In addition to the growth-stimulating effects, resveratrol also possesses antibacterial activity against several clinically important microbial species, including *E. coli*, *Enterococcus faecalis*, and *Salmonella enterica* (81).

Similarly, the consumption of mango, rich in gallotannins and gallic acid, has been associated with selective antimicrobial activity against gram-positive bacteria, such as *Bacillus subtilis* and *S. aureus*, with beneficial effects on the growth of probiotic and/or *SCFA*-producing microbial species, including bifidobacterial and lactobacillus, *A. muciniphila* and butyrate-producing bacteria *Faecalibacterium* spp. and *Clostridium butyrium* (83).

Investigation of the prebiotic effects of several medicinal herbs, including willow gentian (lat. *Gentiana asclepiadea*), St John's wort (lat. *Hypericum perforatum*), winter savory (lat. *Satureja montana*), and yarrow (lat. *Achillea millefolium*), showed the selective growth stimulation of probiotic lactobacilli and probiotic yeast *Saccharomyces boulardii*, in parallel with the antimicrobial activity towards the growth of *E. coli*, *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, and *Candida* yeasts (84). Moreover, the prebiotic effect of baicalin, a flavone glycoside found in root extracts from *Scutellaria baicalensis*, a traditionally used plant in Chinese medicine, was found to be exerted through the selective stimulation of growth of *Streptococcus* and *Bifidobacterium* species, with parallel inhibition of pathogenic bacteria growth, such as *H. pylori*, *E. coli* and *S. aureus* (23, 66, 85).

In addition to compositional changes, polyphenols also modulate the metabolic activity of the microbiota. Phenolic compounds influence the production of organic acids, including lactate and SCFAs. The fact that polyphenol intake leads to increased levels of

SCFAs is of particular importance, since SCFAs have several important functions in the gut, including maintenance of epithelial barrier integrity, provision of necessary nutrients, and immunomodulation (72, 86). Moreover, some polyphenols, such as quercetin, galancin, and fisetin, were found to increase the production of anti-inflammatory molecules, while the consumption of anthocyanins and ellagic acid was associated with the reduction in plasma lipopolysaccharide levels. The health-promoting effects of polyphenols can be summarized by their stimulation of SCFAs and anti-inflammatory molecules production, with the parallel inhibition of the synthesis of proinflammatory molecules (1, 72).

Quorum sensing and phenolic compounds

Phenolic compounds impact microbial growth by various mechanisms. In addition to the unique property of these compounds to stimulate beneficial and inhibit pathogenic microorganisms, their ability to interfere with quorum sensing signaling might be of major importance.

Quorum sensing is an important mechanism of communication between unicellular microorganisms, which impacts their metabolism. Although each cell is independent, microorganisms are capable of conducting coordinated activity. The possibility of group functioning has many advantages, and it allows microorganisms to, e.g., migrate towards more favorable environmental conditions, or to adapt to new growth modes such as sporulation or biofilm formation (87). Microorganisms can respond to changes by modifying their phenotype, while mutual interactions are based on the expression of quorum sensing (QS) dependent genes (88). Quorum sensing involves the production, detection, and response to extracellular molecules called autoinducers. Autoinducers accumulate in the environment when microbial population density increases, and microorganisms monitor this information to detect changes in cell numbers and collectively alter gene expression (89). When cell density increases, accumulation of autoinducers inside and outside the cell occurs, leading to the specific activation of transcriptional regulatory proteins by binding to them (90). Many important functions relevant to pathogenic phenotypes, such as toxin production or biofilm formation, are QS-dependent. Thereby, interference with QS signaling can impact the activity of particular members of an ecosystem and the ecosystem as a whole.

Phenolic compounds can impact microbial growth in various manners. In addition to growth inhibition or stimulation, phenolics can block quorum sensing, which consequently changes microbial mobility, adhesion properties, and the ability to express factors relevant to microbial virulence (91). It has been shown that grape and apple extracts, as well as various phenolic compounds, including syringic, gallic and vanillic acid, (+)-catechin, and resveratrol, can inhibit quorum sensing in a laboratory test on model organism *Chromobacterium violaceum* CV026, as well as inhibit the formation of biofilms of pathogens *L. monocytogenes, S. aureus, E. coli, S. enterica, P. putida* and *P. aeruginosa in vitro*, at concentrations of 100 μg/ml (92). Anti-QS effects have been proven for various polyphenols, including naringenin, quercetin, taxifolin, and

apigenin (93). Interestingly, a significant effect on QS-dependent functions can be achieved even at concentrations lower than the minimum inhibitory concentration (94). Although it is not completely clear how polyphenols interfere with QS signaling, it seems that polyphenols do not affect the production or degradation of autoinducers, but they rather make complexes with signaling molecules or interfere with their receptors (95, 96). These results indicate that polyphenols can induce modifications of ecosystem function even without the elimination or stimulation of the ecosystem members.

Conclusion

Studies have shown that polyphenols may exert beneficial effects by directly affecting human physiological functions and indirectly, by modulating the gut microbiota composition and activity and preserving the balance of the gut microbial ecosystem. Although the results indicate a positive effect of polyphenol intake on the gut microbiota, it is still necessary to clarify the exact mechanisms through which beneficial effects are exerted, as they are not seen in all individuals. In addition to the impact of the selected study design, genetic, physiological, and lifestyle differences between participants, the great discrepancy in results can also be attributed to a high inter-individual variability in microbiota structure and activity, as a complex bidirectional interaction between phenolic compounds and gut microbiota influences the effects of polyphenol intake. However, polyphenols represent a unique group of prebiotics that can interfere with molecular signaling and thereby impact the phenotype of members of the microbiota, boost the growth of beneficial and suppress the growth of pathogenic species. These properties make polyphenols an extremely interesting group of health-affecting phytochemicals, especially in relation to microbiota-mediated health effects. Given the complexity of both microbiota and polyphenols, their interaction needs to be thoroughly researched, and exciting findings could be expected to emerge from this research.

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Polifenoli kao nova klasa prebiotika za manipulaciju crevne mikrobiote

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Kratak sadržaj

Raznovrsna zajednica mikroorganizama kolonizuje naš intestinalni trakt, u kom formira složene interakcije i proizvodi signalne molekule koji mogu uticati na ljudske fiziološke procese. Unutrašnji faktori i faktori životne sredine utiču na obrazovanje strukture mikrobnog ekosistema. Mešu njima, ishrana predstavlja ključni faktor koji utiče na formiranje crevne mikrobiote. Epidemija nezaraznih hroničnih bolesti povezana je sa zapadnjačkim načinom ishrane koji može negativno uticati na crevnu mikrobiotu i uzrokovati stanje disbioze.

Dijetarnim intervencijama i primenom probiotika i prebiotika moguće je ponovo uspostaviti ravnotežu u strukturi mikrobiote. Fenolna jedinjenja, koja predstavljaju biljne nutraceutike i mogu se podeliti na fenolne kiseline i polifenole, ispoljavaju prebiotski efekat i mogu sprečiti nastanak brojnih patologija. Pored direktnih pozitivnih dejstava na stanje čoveka, efekti fenolnih jedinjenja mogu se ostvariti i kroz njihovu interakciju sa crevnom mikrobiotom, Nedavno je uočen dvosmerni odnos između crevne mikrobiote i fenolnih jedinjenja, u kom su mikroorganizmi uključeni u metabolizam fenolnih jedinjenja, dok fenolna jedinjenja mogu uticati na strukturu mikrobiote selektivnom stimulativnom ili inhibitornom aktivnošću prema rastu mikrobnih članova. U ovom preglednom radu biće dat uvid u povezanosti crevne mikrobiote i fenolnih jedinjenja, sa fokusom na primenu fenolnih jedinjenja u cilju modifikacije crevne mikrobiote i održavanja zdravlja.

Ključne reči: polifenoli, mikrobiota, prebiotici, *quorum sensing*

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Probiotic Potential of Dairy Western Balkan Countries *Enterococcus faecium* strains

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Abstract

One of the major genera of the lactic acid bacteria family, *Enterococcus* sp., has a controversial status, reflected in the fact that enterococci are utilized as starter cultures and probiotics, in addition to being known to cause nosocomial infections. The qualified presumption of the safety list and the widely acknowledged safe status for *Enterococcus* species are absent. Rich sources of *Enterococcus faecium* species with possible probiotic characteristics can be found in artisanal dairy products, typically made from raw milk. To further understand the probiotic potential and health-promoting effects, this study looked at the presence of virulence factors and adhesion properties of *En. faecium* isolated from artisanal dairy products from Western Balkan countries.

Key words: enterococci, virulence factors, probiotics, extracellular matrix, adhesion, survival

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Introduction

Enterococci, a group of lactic acid bacteria (LAB), are highly debated due to their controversial nature (1). They are facultative anaerobes highly tolerant to diverse environmental conditions such as extreme temperatures, pH, and salt concentrations. This tolerance contributes to their colonization of diverse host niches and persistence in the environment (2, 3). *Enterococcus faecium* is one of the most common types of bacteria that cause infections in humans and is ranked third among multidrug-resistant nosocomial pathogens that cause bacteremia (4). To persist in nosocomial infections, enterococci exhibit a variety of virulence factors, such as gelatinase activity (GelE), enterococcal surface protein (Esp), aggregating substances (Agg), hyaluronidase (Hyl), and cytolysin (Cyl, -hemolysin) (5, 6). Biofilms are intricate communities of microorganisms that are widespread in the natural environment. Biofilm-forming enterococci are a significant cause of various infections, exhibiting increased virulence and antimicrobial resistance (7). Conversely, enterococci are also used as probiotics and starter cultures for various types of cheese. Probiotic enterococci are live microorganisms identified at the strain level that, when given in an appropriate amount, have a beneficial effect on the host's health (8). The first probiotic *En. faecium* SF68® strain is frequently used in veterinary applications to prevent and treat diarrhea in cats and dogs, as well as for treating human digestive system diseases (9, 10). Enterococci are frequently found in Mediterranean-style cheese curds that contribute to the taste and flavor development during cheese ripening, most likely through proteolysis, lipolysis, and citrate breakdown (11). Additionally, enterococci are capable of producing bacteriocins that are effective against pathogenic and spoilage-causing microorganisms in food, as well as suitable probiotic qualities, which are compelling grounds for their use in the fermentation of food (12, 13). Despite their controversial reputation, awareness of enterococci's probiotic potential has recently increased. Because they can survive in harsh digestive conditions, stick to intestinal epithelial cells, and actively keep pathogens out, which are all important qualities of probiotics, they have gained a lot of attention (14). Probiotic enterococci express cell-surface adhesins, facilitating adhesion to host tissue components like mucin, fibronectin, collagen, laminin, or fibrinogen (15, 16). Conversely, pathogenic bacteria also employ specific adhesiveness to collagenous proteins, a crucial factor in early-phase infections and pathogen virulence (17). This interaction enables pathogens to interact with extracellular matrix proteins, ensuring colonization and tissue infection (18).

This study aimed to investigate the probiotic potential of artisanal dairy strains of *En. faecium* isolated from milk and cheese from various locations in the Western Balkans, including survival in simulated GIT conditions, adhesion to the components of the extracellular matrix (ECM) and human intestinal cell line, and the ability to counteract the harmful effects of pathogens.

Material and Methods

Media, Bacterial strains, and Growth factors

Ten dairy isolates of the enterococci species *En. faecium* that had previously been identified were used (Table I). M17 broth (Merck, GmbH, Darmstadt, Germany) supplemented with glucose (0.5% w/v) (GM17) was used to grow enterococci, *Listeria monocytogenes* ATCC19111, and *Lactococcus lactis* subsp. *lactis* at 37 °C and 30 °C, respectively. *Escherichia coli* ATCC25922 and *Salmonella* Enteritidis 654/7E were grown at 37 °C in Luria-Bertani broth (LB), which contained 0.5% NaCl, 0.5% yeast extract (Torlak, Belgrade, Serbia), and 1% tryptone (Torlak). Each broth was mixed with agar (1.7% w/v, Torlak) to create corresponding agar plates.

Table I List of Enterococcus faecium strains used in this study and their origin

Tabela I Spisak sojeva Enterococcus faecium i njihovo poreklo korišćenih u ovoj studiji

Strain	Origin	Region
BGPAS1-3	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-4	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-10	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-20	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-58	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-71	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGZLM1-5	Milk	Serbia, Zlatar mountain
BGGO9-28	Cheese	Serbia, Golija mountain
BGGO11-27	Cheese	Serbia, Golija mountain
BGGO11-29	Cheese	Serbia, Golija mountain

PCR Detection of Virulence Determinants

According to Parish, the total DNA extracted from ten *En. faecium* species was used in PCR tests to determine whether virulence-related genes were present or absent (19). Table II lists the target gene primer sequences, anticipated amplicon sizes, and annealing temperatures.

Antimicrobial Activity Assay

The deferred antagonism approach was employed for evaluating antimicrobial substances synthesized by enterococci using different indicator strains (14). Table III contains a list of the indicator strains utilized in this test.

Table II List of primers used in this study

Tabela II Spisak prajmera korišćenih u ovoj studiji

Genes	Primers	Product size	T°C[a]	Reference
Virulence factors				
gelE	5′-CGGAAGGCGTTACTGTTGAT-3′ 5′-GAGCCATGGTTTCTGGTTGT-3′	957 bp	46°C	(14)
sprE	5′- TTGAGCTCCGTTCCTGCCGAAAGTCATTC-3′ 5′-TTGGTACCGATTGGGGAACCAGATTGACC-3′	591 bp	58°C	(36)
ace	5′-AAAGTAGAATTAGATCCACAC-3′ 5′-TCTATCACATTCGGTTGCG-3′	320 bp	56°C	(37)
hylN	5′-ACAGAAGAGCTGCAGGAAATG-3′ 5′-GACTGACGTCCAAGTTTCCAA-3′	276 bp	56°C	(38)
agg	5′-AAGAAAAAGAAGTAGACCAAC-3′ 5′-AAACGGCAAGACAAGTAAATA-3′	1553 bp	54°C	(39)
cylA	5′-TGGATGATAGTGATAGGAAGT-3′ 5′-TCTACAGTAAATCTTTCGTCA-3′	517 bp	58°C	(39)
esp	5′-TTGCTAATGCTAGTCCCAGACC-3′ 5′-GCGTCAACACTTGCATTGCCGAA-3′	933 bp	58°C	(39)
efaA^{fs}	5′-GACAGACCCTCACGAATA-3′ 5′-ATGTCATCATGCTGTAGTA-3′	705 bp	56°C	(39)
efaA^{fm}	5′-AACAGATCCGCATGAATA-3′ 5′-CATTTCATCATCTGATAGTA-3′	735 bp	56°C	(39)

Note: [a] - annealing temperature for a given primer pair

Table III The list of indicator strains used in this study

Tabela III Spisak indikatorskih sojeva korišćenih u ovoj studiji

Bacterial strains	Source
Lactococcus lactis subsp. *lactis* BGMN1-596	Laboratory collection
Enterococcus faecalis BG221	Laboratory collection
Listeria monocytogenes ATCC19111	ATCC [a]
Escherichia coli ATCC25922	ATCC
Salmonella Enteritidis 654/7E	Scientific Veterinary Institute ‘Novi Sad’, Serbia

Note: [a] ATCC-American Type Culture Collection

MTT Assay

A microculture tetrazolium [MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test was used to assess the cytotoxicity of enterococci on the HT29-MTX cell line, which was kindly provided by Dr. T. Lesuffleur (INSERM UMR S938, Paris, France; (20)). After 24 hours of seeding (40–60% confluency), filtered supernatants (using 0.22 µm Nalgene syringe filter units, Sarstedt, Nümbrecht, Germany) and UV-irradiated non-viable bacterial cells were added to the eukaryotic cells in various concentrations (ratios 1:1 and 10:1 bacteria: eukaryotic cell). Live cells exposed to MTT produced formazan crystals that were dissolved in 10% sodium dodecylsulfate (SDS) in 0.01% HCl. After that, the wells were incubated overnight at 37 °C. Plate Reader Infinite 200 Pro was used to measure the adsorption of the dissolved formazan crystals at 570 nm. The percentage of optical density (metabolic activity) relative to the control (cultures of untreated cells), which was utilized to express the results, was calculated as follows: Metabolic activity (%) = 100 divided by (OD of treated cells minus the OD of untreated cells).

Survival in Simulated Gastrointestinal Tract

Survival in a chemically imitated gastrointestinal tract was tested in vitro, as previously described (22). The viable cells recovered after each chemically simulated GIT stage about the initial counts were used to calculate survival. The results were expressed as log colony forming units (CFU) per mL.

Assay for Extracellular Matrix Adhesion

Mucin, collagen, and fibronectin adhesion experiments were done in 96-well polystyrene microtiter plates (Sarsted, Newton, USA) using Valeriano and colleagues' (23) method with slight modifications. At 4 °C for 24 hours, the wells of microtiter plates were coated with 200 L of 100 g/mL porcine stomach mucin-type II (Sigma, Germany), collagen type I (Sigma), and human fibronectin (Serva, Heidelberg, Germany). Wells were rinsed twice with 200 µL PBS before being incubated for 2 hours at 4 °C with 100 µL (20 mg/mL) bovine serum albumin (BSA) (Sigma, Germany). To eliminate detached BSA, the wells were washed twice with 200 µL of PBS. A bacterial culture containing approximately 10^8 CFU/mL (100 µL) was washed, suspended in PBS (pH 7.0), and applied to the wells. Plates were incubated for 2 hours before being rinsed twice with 200 µL of PBS to wash out unattached bacteria. To isolate the adhering bacteria, another 200 µL of 0.5% (v/v) Triton X-100 (Sigma) was added at 37 °C. Plating on GM17 determined the viable cell count expressed as CFU/mL in all cases. The percentage adhesion was obtained by multiplying the viable counts adhered to the mucin, collagen, and fibronectin by the initial count (%) = (CFU/mL recovered bacteria/CFU/mL initial bacteria) ×100.

HT29-MTX Cell Line Adhesion

According to Živković et al. (24), 13 ± 1 day-old cellular monolayers were used for adhesion to HT29-MTX cell line studies. Cellular monolayers were thoroughly cleaned before bacterial suspensions were introduced at a 10:1 (bacteria: eukaryotic cell) ratio. Adhesion tests were performed for 1 hour at 37 °C and 5% CO_2. To calculate the percentage of adhesion we used the formula: (%) = (CFU/mL adhering bacteria/CFU/mL presented bacteria) x 100.

Assay for Pathogen Exclusion

Salmonella Enteritidis 654/7E and *E. coli* ATCC25922 were examined for their capacity to attach to the intestinal epithelium in the presence and absence of enterococci (24). Bacterial suspensions (1 x 10^8 CFU/mL) containing *E. coli*, or a combination of *E. coli* and enterococci (ratio 1:1) and 1 x 10^8 CFU/mL *Salmonella* Enteritidis, or a combination of *Salmonella* Enteritidis and enterococci (ratio 1:1) were added to the HT29-MTX monolayers at a 10:1 (bacteria:eukaryotic cell) ratio and incubated for 1 hour at 37 °C with 5% CO_2. The adhesion percentage was calculated as follows: bacteria attached to HT29-MTX monolayers at 100 CFU/mL / total CFU/mL bacteria introduced (corrected for dilution). Viable cell count expressed as CFU/mL measured by plating on LA was used to determine the count of bacteria. To test the ability of the enterococci to inhibit *E. coli* and *Salmonella* Enteritidis adhesion to HT29-MTX monolayers, the data were compared to that obtained with *E. coli* and *Salmonella* Enteritidis alone (i.e., 100% adhesion).

Statistical Analysis

Each of the experiments was carried out in duplicate and independently performed at least twice. All separate experiments' data are presented as mean values with a standard deviation. For multiple group comparisons, a one-way ANOVA with Tukey's post hoc test was utilized. $p < 0.05$ was considered statistically significant. GraphPad Prism 9 software (California, San Diego, USA) was used to perform statistical analysis and create graphics.

Results and Discussion

Probiotic usage is widely acknowledged as a viable strategy for enhancing or stabilizing the digestive system. The typical microbial community of the human GIT includes enterococci. They can be found naturally in many food products, as well as frequently being linked to various types of traditional fermentations or purposefully added as starting cultures (13). Ten strains of *En. faecium* used in this study were isolated from different dairy products from the Western Balkans (Table I). Previous research has demonstrated that autochthonous dairy products from the Western Balkan counties can be used as a source of novel enterococci probiotic strains (25). Our previous results based on the safety assessment analysis showed that ten strains of *En. faecium* showed sensitivity to nine relevant clinical antibiotics according to the Clinical and Laboratory Standards

Institute (CLSI) standards, and they did not express gelatinase and hemolytic activity (14). The ten strains employed in this research were examined for the presence or absence of the genes associated with virulence encoding aggregation factor (*agg*), collagen adhesin (*ace*), cytolysin (*cylA*), enterococcal surface protein (*esp*), cell wall adhesins ($efaA^{fs}$ and $efaA^{fm}$), gelatinase (*gelE*), hyaluronidase (*hyl*), and serine protease (*sprE*) (Table IV). Six strains (60%) and eight strains (80%), respectively, tested positive for the *esp* gene and the *agg* gene. The $efaA^{fm}$ gene was found to be present in five strains (50%) and the $efaA^{fs}$ gene in three strains (30%). The *ace* gene was not found to be present. It is noteworthy that neither the *cylA* gene, which codes for CylA serine protease, nor the *hyl* gene, which codes for hyaluronidase, a degradative enzyme linked to tissue damage (26), were found. CylA serine protease is involved in processing and activating cytolysin, also known as hemolysin, a bacterial toxin with beta-hemolytic properties in humans. Additionally, neither the *gelE* gene encoding gelatinase nor the *sprE* encoding serine protease were detected in any of the strains. Both gelatinase and serine protease play a part in the pathogenesis of enterococci, supplying the bacteria with nutrients by destroying host tissue, but they also play a part in the development of biofilms (27). Additional crucial probiotic characteristics include persistence in the intestine, competitive exclusion of pathogens, and adhesion to intestinal epithelial cells (IEC), which are essential to colonizing the intestinal mucosa (28, 29). Considering these results, we hypothesize that the presence of genes coding for adhesins, but not for pathogenesis-associated enzymes, enables these strains to adhere to the host surfaces, which can exert health-promoting effects.

Table IV Presence of the virulence genes and genes for biofilm formation

Tabela IV Prisustvo gena virulencije i gena za formiranje biofilma

Strains	Enzymes				Adhesins				
	gelE	*sprE*	*Hyl*	*clyA*	*agg*	*esp*	*ace*	$efaA^{fs}$	$efaA^{fm}$
BGPAS1-3					■				■
BGPAS1-4					■				
BGPAS1-10					■	■			
BGPAS1-20					■	■			■
BGPAS1-58						■			
BGPAS1-71					■	■		■	
BGZLM1-5					■			■	■
BGGO9-28								■	■
BGGO11-27					■	■			
BGGO11-29					■	■			

Note: The shaded areas reflect the presence of the respective gene.

To achieve these effects, probiotic enterococci must survive the unfavorable conditions of the GIT. To examine the survival of enterococci in GIT conditions, ten strains of *En. faecium* were exposed to conditions simulating the GIT (Figure 1). All of the bacteria survived well in highly acidic stomach conditions (from initial 8.79 to 8.23 log CFU/mL), demonstrating the isolates' adaptability to such conditions. After prolonged exposure to lower bile concentrations (0.3%) (8.37–7.40 CFU/mL) and pancreatic enzymes (7.83–7.00 log CFU/mL), the survival rate was either maintained or significantly lowered. This is because earlier research has shown that the GIT is resistant and can survive challenging circumstances like those seen there (14).

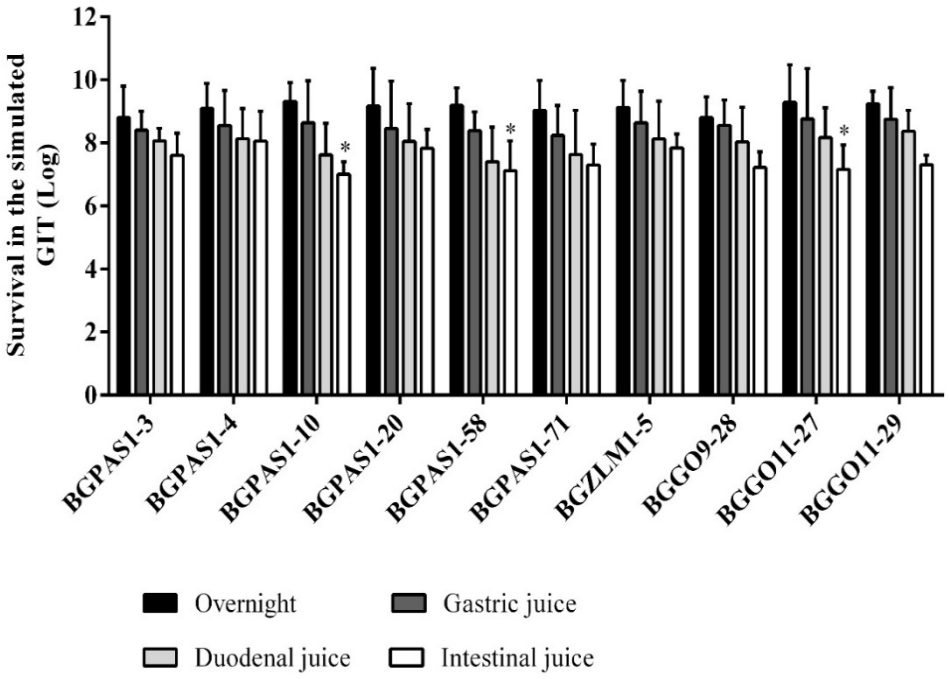

Figure 1. Survival of the enterococci in *in vitro* simulated gastrointestinal conditions. The statistical differences between treatments are annotated with asterisks (*$p < 0.05$).

Slika 1. Preživljavanje enterokoka u *in vitro* simuliranim gastrointestinalnim uslovima. Statističke razlike između tretmana su označene zvezdicama (*$p < 0,05$).

The integrity of the intestinal epithelial barrier is essential for the intestinal epithelium's delicate sensitivity to modulations by commensal and pathogenic microorganisms (30). In addition to the absence of pathogenesis-associated genes, excluding strains with cytotoxicity effects is a very important step. To investigate any potential negative effects, ten *En. faecium* strains were each exposed to the supernatants of overnight bacterial cultures (soluble bacterial products) and UV-irradiated non-viable bacterial cells (surface bacterial cell molecules). The findings of the MTT test showed that none of the

investigated bacteria, their soluble products, or surface chemicals had any significant effects on the metabolic activity of HT29-MTX cells (Figure 2). The data demonstrate the safety of all tested strains because none of the dairy isolates investigated have a cytotoxic effect on the intestinal epithelial barrier. In addition to analyzing the presence of adhesion genes, we further investigated the potential of these strains to adhere to the main components of the ECM, such as mucin (Figure 3A), collagen (Figure 3B), and structural glycoprotein fibronectin (Figure 3C), to assess its probiotic potential (31, 32). Each strain that was evaluated showed a strong affinity for certain ECM elements. Our findings showed that 10 enterococci dairy isolates had a strong ability to bind to mucin, with an average value of 72.9% + 2.77, to collagen, with an average value of 75.9% ± 1.83, and to fibronectin, with an average value of 74% ± 1.74. Interestingly, most strains showed the ability to bind collagen and fibronectin with greater affinity than pathogenic species *Salmonella* Enteritidis 654/7E and *E. coli* ATCC25922, while only BGZLM1-5 and BGGO9-28 bound to mucin were stronger than pathogenic bacteria with significant affinity. To estimate the percentage of attachment of the prospective probiotic strains, the ability of tested enterococci to adhere to the epithelial intestinal cell line HT29-MTX was also assessed. According to the study findings, the tested strains had strong adhesion capabilities, adhering to the HT29-MTX cell line at a rate of 89.7% ± 1.2 (Figure 4). These results are by the properties of enterococcal strains selected in other studies to adhere to components of the ECM and intestinal epithelial cells (14, 33, 34).

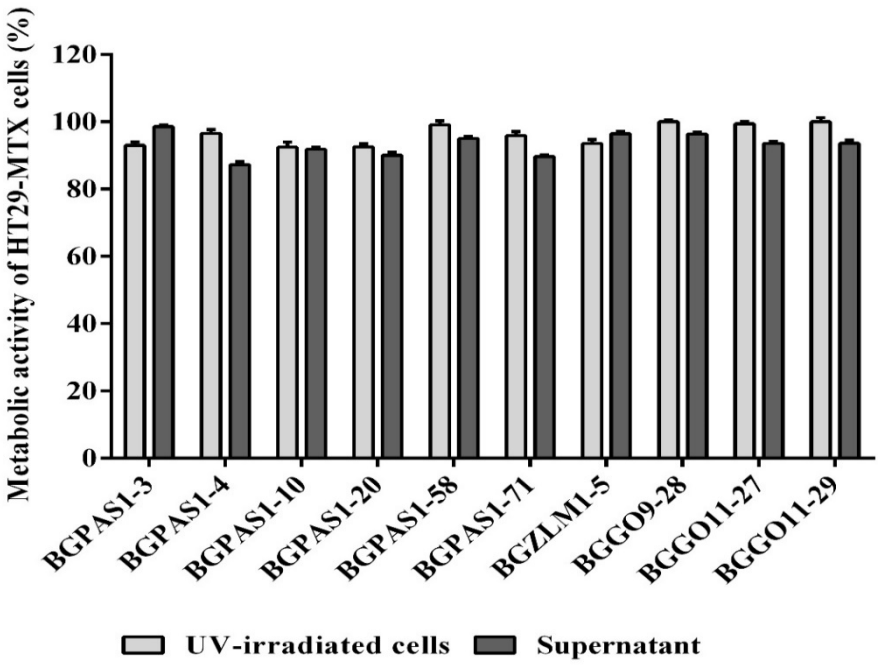

Figure 2. The cytotoxicity of enterococci on HT29-MTX cell line
Slika 2. Citotoksičnost enterokoka na ćelijskoj liniji HT29-MTX

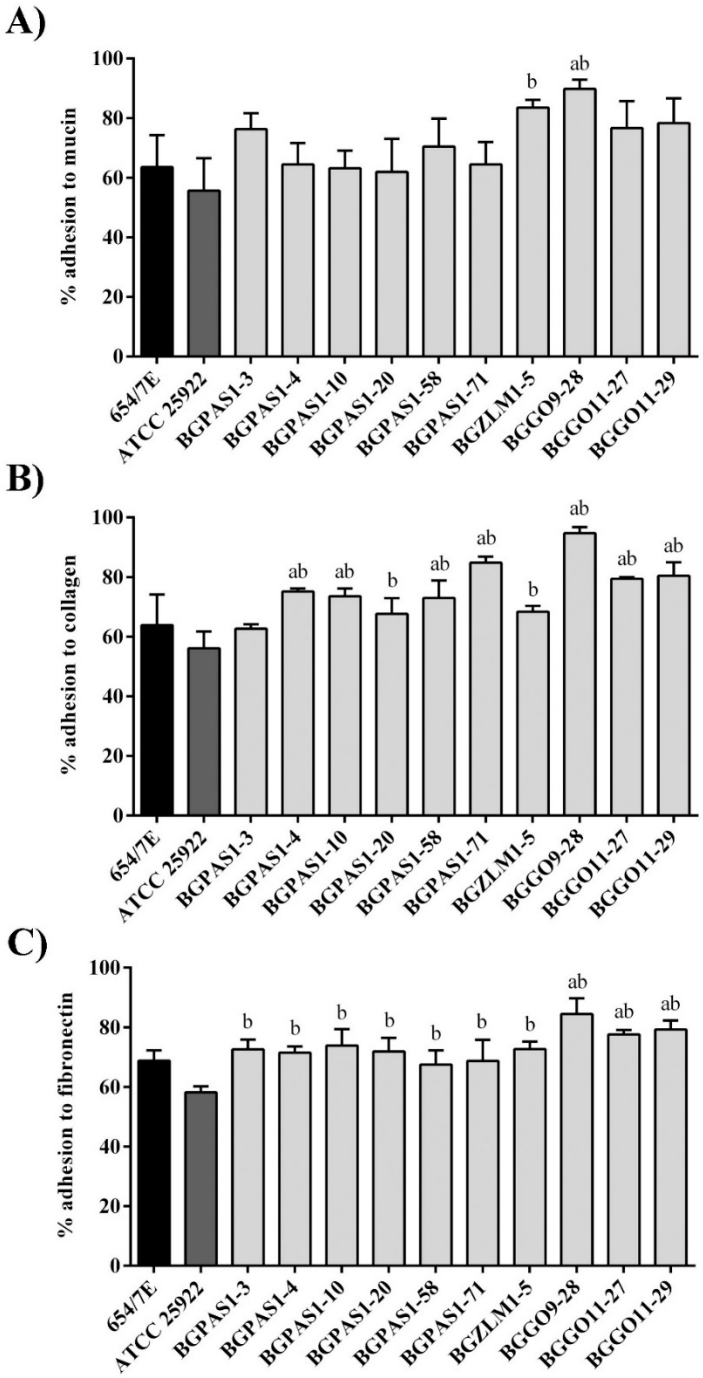

Figure 3. **Adhesion of the enterococci strains to mucin (A), collagen (B), and fibronectin (C). Statistical differences ($p < 0.05$) associated with *Salmonella* Enteritidis 654/7E are marked with the letter a, and the association with *E. coli* ATCC25922 with the letter b.**

Slika 3. **Adhezija sojeva enterokoka za mucin (A), kolagen (B) i fibronektin (C). Statističke razlike ($p < 0,05$) povezane sa *Salmonella* Enteritidis 654/7E označene su slovom a, a povezanost sa *E. coli* ATCC25922 slovom b.**

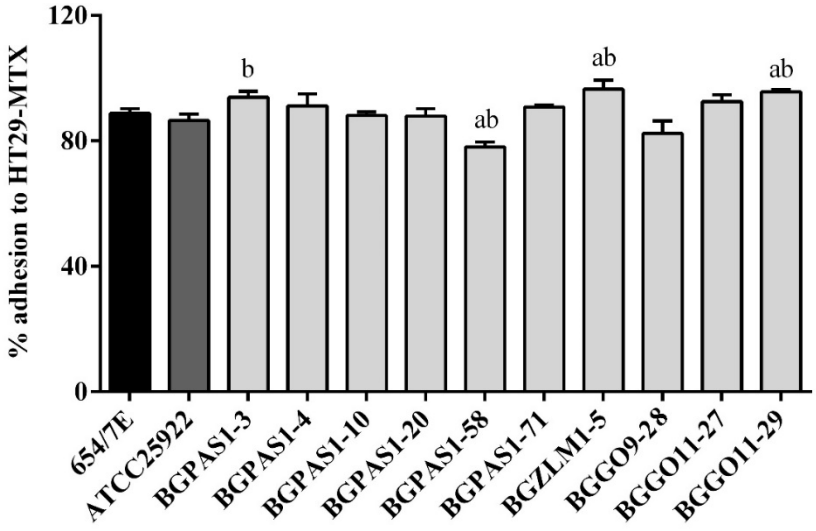

Figure 4. Adhesion of the enterococci strains to the human intestinal epithelial cell line HT29-MTX. Statistical differences ($p < 0.05$) associated with *Salmonella* Enteritidis 654/7E are marked with the letter a, and the association with *E. coli* ATCC25922 with the letter b.

Slika 4. Adhezija sojeva enterokoka za ćelijsku liniju humanog crevnog epitela HT29-MTX. Statističke razlike ($p < 0,05$) povezane sa *Salmonella* Enteritidis 654/7E označene su slovom a, a povezanost sa *E. coli* ATCC25922 slovom b.

One of the first and most common health-promoting properties of probiotic strains is their ability to counteract the harmful effects of pathogens (35). One of the ways they achieve this is through antimicrobial activity, involving the production of bacteriocins, as well as processes like colonization competition and pathogen exclusion (3). We thus showed that only two strains (BGPAS1-3 and BGZLM1-5) have antimicrobial activity (Table V) against specific pathogens; however, none of the tested strains showed activity against *Salmonella* Enteritidis 654/7E and *E. coli* ATCC25922, so we analyzed other potential anti-pathogenic mechanisms. It is proposed that certain cell surface components, such as S-layer macromolecules or auto-aggregation factors, could be essential (3, 11). These components may contribute to the probiotic strains' ability to compete with pathogens for colonization and maintain a healthy microbial balance within the host. The study findings showed that the presence of the two enterococci strains under test decreased the adhesion of *E. coli* ATCC25922 and *Salmonella* Enteritidis 654/7E to HT29-MTX (Figure 5). The adhesion of *Salmonella* Enteritidis 654/7E in the presence of *En. faecium* BGPAS1-3 during co-incubation assay is 94.6% compared to control, whereas the adhesion of *E. coli* ATCC25922 in the presence of *En. faecium* BGPAS1-4 is 96.1% compared to the control.

Table V Antimicrobial activity of *Enterococcus faecium*

Tabela V Antimikrobna aktivnost *Enterococcus faecium*

	BGMN1-596	BG221	ATCC19111	ATCC25922	654/7E
BGPAS1-3					
BGPAS1-4					
BGPAS1-10					
BGPAS1-20					
BGPAS1-58					
BGPAS1-71					
BGZLM1-5					
BGGO9-28					
BGGO11-27					
BGGO11-29					

Note: The shaded areas reflect the presence of antimicrobial activity.

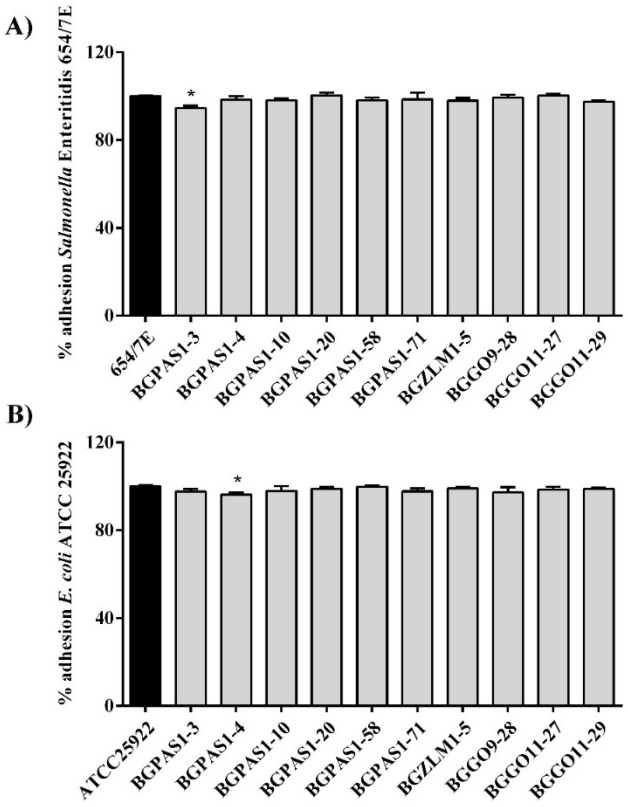

Figure 5. Association of *Salmonella* Enteritidis 654/7E to HT29-MTX cells in the presence of enterococci (A) and association of *E. coli* ATCC25922 to HT29-MTX cells in the presence of enterococci (B). The statistical differences concerning the control strains are annotated with asterisks (*$p < 0.05$).

Slika 5. Adhezija *Salmonella* Enteritidis 654/7E za HT29-MTX ćelijsku liniju u prisustvu enterokoka (A) i adhezija *E. coli* ATCC25922 za HT29-MTX ćelijsku liniju u prisustvu enterokoka (B). Statističke razlike u odnosu na netretirane kontrole su označene zvezdicama (*$p < 0,05$).

Conclusion

As far as we know, this is the first study that combines data on virulence genes and probiotic features of dairy *En. faecium* isolates from the Western Balkan, which expands our understanding of virulence factors implicated in dairy enterococci's probiotic properties. While virulence genes may be sporadically present in enterococci, they could potentially serve as advantageous features, aiding in their successful colonization of the gut.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Probiotički potencijal sojeva *Enterococcus faecium* izolovanih iz mlečnih proizvoda sa područja Zapadnog Balkana

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Kratak sadržaj

Rod *Enterococcus* je jedan od glavnih rodova koji pripada bakterijama mlečne kiseline i ima kontroverzni status, koji se ogleda u činjenici da su enterokoke prepoznate kao uzročnici bolničkih infekcija, dok se istovremeno koriste i kao probiotici i kao starter kulture. Pripadnici vrste *Enterococcus* nemaju opštepriznat bezbedni status, niti su uvršteni na liste bezbednih sojeva Evropske agencije za bezbednost hrane. Autohtoni mlečni proizvodi, posebno oni proizvedeni od sirovog mleka, predstavljaju bogate izvore vrsta *Enterococcus faecium* sa potencijalnim probiotičkim svojstvima. U ovoj studiji je istraživano prisustvo faktora virulencije i sposobnost adhezije vrsta *En. faecium* izolovanih iz mlečnih, autohtonih proizvoda sa područja Zapadnog Balkana sa ciljem boljeg razumevanja njihovog probiotičkog potencijala, kao i efekata koji doprinose unapređenju zdravlja korisnika.

Ključne reči: enterokoke, faktori virulencije, probiotici, ekstracelularni matriks, adhezija, preživljavanje

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Probiotic characterization of *Limosilactobacillus fermentum* BGHV110 strain and its influence on innate immune response in *Caenorhabditis elegans*

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Abstract

Probiotic lactobacilli exhibit the potential to promote health benefits for the host. Thanks to its numerous beneficial effects on human health, *Limosilactobacillus fermentum* stood out as an excellent candidate for the development of commercial probiotic preparations aiming to prevent community-acquired infections. In this study, several *in vitro* tests, including biofilm formation assay, assessment of antibiotic susceptibility, survival in simulated gastrointestinal tract conditions and attachment to intestinal Caco-2 cells, were used to estimate the safety and probiotic potential of *L. fermentum* BGHV110 strain. Additionally, *Caenorhabditis elegans* was used as an *in vivo* model system for the evaluation of *L. fermentum* BGHV110 influence on the host's innate immune response. The results revealed that *L. fermentum* BGHV110 strain showed an excellent capability to survive harsh conditions of the gut, to attach to intestinal Caco-2 cells and to stimulate conserved p38 MAPK immunity pathway and expression of the *clc-1* claudin-like gene and antimicrobial peptides in *C. elegans* in order to enhance the immune response against pathogens. Finally, *L. fermentum* BGHV110 showed no virulence traits and susceptibility to tested antibiotics, confirming its safety status which enables it to be applied as a future probiotic.

Key words: *Limosilactobacillus fermentum*, probiotic, innate immune response, gastrointestinal tract, *Caenorhabditis elegans*

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Introduction

Interaction between the complex microbial community in the gut which forms intestinal microbiota and the host is essential for the regulation of homeostasis of the gastrointestinal tract (GIT) (1). Gut bacteria are responsible for multiple physiological processes such as nutrient digestion, protection against pathogens, proper epithelial barrier function, and immunomodulation, and therefore manipulation of the intestinal microbiota represents a potential strategy for the prevention and treatment of different diseases (2, 3, 4). These mutually beneficial interactions essential for the host's wellbeing could be further potentiated by the application of probiotics, commonly used microbiota-based therapeutics. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (5). The most common feature of probiotics is the ability to enhance the immune response and prevent community-acquired gastrointestinal infections (6). Innate immune pathways in the gut are kept in the primed state to instantly respond to microbe-associated molecular patterns (MAMPs), such as peptidoglycans, teichoic acids, lipopolysaccharides, dsRNA, flagellin, and microbial polysaccharides (7). Because of their simplicity and nonspecific detection of MAMPs, these pathways are highly conserved through evolution and their orthologs could be found from invertebrates (e.g., nematodes, fruit flies) to complex organisms (8). In mammals, the activation of mucosal intestinal immunity usually induces the expression of different genes responsible for the recruitment of immune cells (dendritic cells and macrophages), stimulation of IgA production by plasma cells from lamina propria, but also activation of Paneth secretory cells specialized in the production of antimicrobial peptides (AMP) including defensins, lysozymes, or C-type lectins similar to those involved in the *Caenorhabditis elegans* response to pathogens (9).

C. elegans is a bacterivore nematode which has been used as a model organism to study response to infection by several bacterial pathogens. Conserved signalling pathways identified to be essential for a worm's defence against intestinal pathogens include the PMK-1 pathway corresponding to mammalian p38 mitogen-activated protein kinase (MAPK) pathway and the DBL-1 pathway similar to transforming growth factor β (TGF-β) signalling (10). Due to the high similarity of the *C. elegans* digestive system to mammalian intestines, in terms of expression of epithelial tight junction proteins (TJP) like claudins (e.g., claudin-like in Caenorhabditis, CLC-1) and above-mentioned immunity pathways that control the expression of AMP, *C. elegans* became an excellent model to study probiotic-host interaction in general (11, 12). Recent evidence from experiments conducted on the *C. elegans* model implies that several stress-response mechanisms, including the HLH-30/TFEB dependent autophagy (13), canonical PMK-1/p38 MAPK immunity (8), SKN-1/NRF2 mediated antioxidative response (14), and serotonin signalling (15) could be triggered by different strains of commensal lactobacilli resulting in increased longevity of worms.

Limosilactobacillus fermentum is a heterofermentative gram-positive bacterium from the lactic acid bacteria (LAB) group of the Firmicutes phylum. Reported beneficial

roles of *L. fermentum* are mainly related to the prevention or treatment of gastrointestinal disorders including intestinal infection, immunomodulation in colitis and Crohn's disease, reduction of colorectal cancer risk, and hepatoprotective effect against drug-induced toxicity and alcoholic liver disease (6). Based on the described effects, *L. fermentum* were used for the development of various probiotic preparations commercially available on the USA market (6); however, probiotic preparations in Serbia are predominantly composed of *Lactobacillus acidophilus* and *Lactiplantibacillus plantarum* strains. Our previous results described the promising probiotic potential of *L. fermentum* BGHV110 strain, highlighting its ability to activate protective cellular autophagy, a clearance process for the recycling of damaged organelles and misfolded proteins, resulting in a beneficial effect for the host which can be used to design novel probiotic preparations (13, 16).

Here, we demonstrated that *L. fermentum* BGHV110 strain possesses excellent probiotic features for further health promoting applications, and that it showed the capability to stimulate conserved p38 MAPK immunity pathway and expression of TJP and AMP in *C. elegans* necessary for host defence against intestinal infection and invading microbes.

Materials and Methods

Bacterial Strain and Culture Condition

Limosilactobacillus fermentum BGHV110 was used in this study (16). The strain was cultivated overnight at 37° C in deMan-Rogosa-Sharpe (MRS) broth (Sigma-Aldrich) under anaerobic conditions. The *Escherichia coli* OP50 strain for worms' maintenance was cultivated in Luria Bertani (LB) medium overnight at 37° C aerobically. *Enterococcus faecium* BGZLM1-5 was cultivated in GM17 medium overnight at 37° C.

Antibiotic Susceptibility Testing

Minimal inhibitory concentrations (MICs) were determined by microdilution assay (17). Antibiotics used in the assay were: ampicillin (2 mg/L), gentamicin (16 mg/L), kanamycin (64 mg/L), streptomycin (64 mg/L), erythromycin (1 mg/L), clindamycin (4 mg/L), tetracycline (8 mg/L), and chloramphenicol (4 mg/L) proposed by EFSA for obligate heterofermentative lactobacilli, including *L. fermentum* (17). Microdilutions were made in the Hi-Sensitivity Test Broth (HiMedia, India). The final CFU per well was 5×10^6. Cell density was monitored after 24 h incubation at 37 °C with 5% CO_2 at 600 nm using a spectrophotometer Plate Reader Infinite 200 pro (MTX Lab Systems, Vienna, Austria).

Biofilm Formation

Biofilm formation assay was done according to Macovei et al. (18), with minor modifications. The microtiter plates (Sarstedt, Germany) were filled with 180 µl Hi-Sensitivity Test Broth (HiMedia) medium and 20 µL of overnight grown culture of *L.*

fermentum BGHV110 (adjusted to 0.5 McFarland units). After 24 h incubation at 37 °C with 5% CO_2, microtiter plates were washed with phosphate-buffered saline (PBS) and incubated for 30 min at 65 °C for the drying process. Formed biofilm was dissolved by using 0.1% crystal violet (HIMedia, India), and absorbance was measured at 595 nm using a Plate Reader Infinite 200 pro (MTX Lab Systems). The strains were categorized as no biofilm producer (OD 595 ≤ 0.2), weak biofilm producer (OD 595 0.2 - 0.7), strong biofilm producer (OD 595 0.7 - 1.4), and very strong biofilm producer (OD 595 ≥ 1.4). *En. faecium* BGZLM1-5 was used as a positive control.

Survival in Simulated Gastrointestinal Tract Conditions

The survival of *L. fermentum* BGHV110 strain during the passage through GIT was performed in a model of simulated GIT conditions described by Sánchez et al. (19). *L. fermentum* BGHV110 strain, which was grown overnight in MRS medium, was centrifugated at 5000 × g for 10 min, washed in 0.9% NaCl and resuspended in gastric juice (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, 0.3% pepsin (Sigma), pH 2). After a 90 min challenge, bacterial cells were pelleted and resuspended in duodenal juice (1% bile salt (Sigma), pH 8). After 10 min of incubation, bacteria were collected with centrifugation and resuspended in intestinal juice (0.3% bile salt, 0.1% pancreatin ("Pancreas acetone powder porcine Type I", Sigma), pH 8) for a 120 min challenge. Serial 10× dilutions in 0.9 % NaCl were made after 0, 90, 110 and 180 min of bacterial incubations and plated on MRS agar plates, which were incubated at 37 °C for 24 h. The results were expressed as colony forming units (CFU)/mL of survived bacterial cells after every challenge to GIT juices.

Adherence to intestinal cells

The intestinal Caco-2 cells were used to estimate the adhesion ability of *L. fermentum* BGHV110 strain. The Caco-2 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, all purchased from Thermo Fisher Scientific. The cells were seeded in 24-well plates (1×10^5 cells/well) and cultivated until a monolayer was formed. Overnight bacterial culture was washed with PBS and resuspended in the DMEM media without antibiotics. The bacterial suspension was added to the cells in a 1:10 ratio. Following co-incubation for 2 h at 37 °C and 5% CO_2, the cells were gently washed with PBS and detached with 0.25% Trypsin–EDTA solution (Sigma). The bacteria before adhesion and the bacteria collected after adhesion were diluted in PBS and plated on MRS agar plates. The results were expressed as a % of adhesion, calculated as CFU/mL of adhered bacteria/CFU/mL of added bacteria × 100.

***Caenorhabditis elegans* maintenance and treatment**

C. elegans wild-type N2 (Bristol) strain were maintained on nematode growth medium (NGM) plates seeded with *E. coli* OP50 strain at 20 °C by using standard protocols (8). Worms' synchronization was done from a population of egg-bearing

worms by using 0.5 M NaOH and 1% Na-hypochlorite solution, followed by washing with M9 buffer. Eggs were plated to OP50 seeded NGM plates in order to obtain synchronized L1 animals. For the treatments, the overnight culture of *L. fermentum* BGHV110 strain was pelleted by centrifugation at 5000 × g for 10 min at room temperature, washed twice with PBS, resuspended in LB medium and seeded on NGM plates. The control treatment was prepared by seeding an overnight culture of *E. coli* OP50, grown in LB, on NGM plates. Age-synchronized worms in the L4 larval stage were transferred on *E. coli* OP50 NGM plates or *L. fermentum* BGHV110 containing NGM plates, and incubated overnight.

RNA isolation and quantitative real-time PCR (qRT-PCR)

After overnight treatment, worms were collected with M9 buffer and washed three times to remove the remaining bacteria. Total RNA was isolated using a Trizol reagent by following the manufacturer's protocol (Thermo Fisher Scientific). Genomic DNA contamination was removed by using RapidOut DNA Removal Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Reverse transcription was performed with RevertAid Reverse Transcriptase (Thermo Fisher Scientific) with 0.5 µg of isolated RNA, random hexamers (Thermo Fisher Scientific), and RiboLock RNase inhibitor (Thermo Fisher Scientific) used in the reactions. Quantitative real-time PCR was performed with an IC Green qPCR Universal Kit (NIPPON Genetics, Düren, Germany) under the following conditions: 2 min at 95 °C activation, 40 cycles of 5 s at 95 °C and 30 s at 60 °C in Line-Gene 9600 Plus Real-Time PCR (Hangzhou Bioer Technology). The results were normalized by using the housekeeping *act-1* gene. All used primers are listed in Table I and were purchased from Thermo Fisher Scientific. For each condition, three independent replicates were used.

Statistical Analysis

All values are presented as mean ± standard deviation (SD). Student's t-test was used to compare the differences between the control and treatment groups, while one-way ANOVA followed by the Tukey post hoc test was used for multiple comparisons. P value lower than 0.05 was considered statistically significant. The statistical analysis and graphs were done in GraphPad Prism version 8.0.0 for Mac, GraphPad Software, San Diego, California USA.

Results and Discussion

The safety of *Limosilactobacillus fermentum* BGHV110 strain as a potential probiotic

Traditionally long application in human nutrition listed *Lactobacillus* species on the QPS (qualified presumption of safety) list. However, based on the new evidence which pointed out the increased prevalence of antibiotic resistance among different dairy lactobacilli (20), we first tested the susceptibility of *L. fermentum* BGHV110 to recommended concentrations of clinically relevant antibiotics according to guidelines

Table I Primers used for the analysis of the gene expression in *Caenorhabditis elegans*
Tabela I Prajmeri korišćeni za analizu ekspresije gena kod *Caenorhabditis elegans*

Primer name	Primer sequence 5′–3′	Reference
tir-1 forward	CCGACCACCAAAGAAATGCC	8
tir-1 reverse	CTTGGTCCACCGATGCTTCT	
pmk-1 forward	ACTTCATCCGACTCCACGAG	8
pmk-1 reverse	CAGCAGCACAAACAGTTCCA	
lys-1 forward	GGATCTGGAGCATTCGACACA	This work
lys-1 reverse	GCTGGGGAGGTAACCTGAATC	
lys-8 forward	TTGTCCGTGCATACAACCCA	This work
lys-8 reverse	TCCTTGCTTGCTTGAAGCCG	
dbl-1 forward	TTTTGCGGCGAACAAATCGT	8
dbl-1 reverse	TTCGCTGTTGCCTGTTTGTG	
clc-1 forward	CCACTCACCCTCTTTGCAGT	8
clc-1 reverse	CGAGTATCCAAGCTGCGAGT	
act-1 forward	TGCAGAAGGAAATCACCGCT	13
act-1 reverse	TGCAACGAGAGCAACTGAAC	

provided by EFSA. We demonstrated that *L. fermentum* BGHV110 is susceptible to ampicillin, gentamicin, erythromycin, clindamycin, tetracycline, and chloramphenicol, while resistant to streptomycin and kanamycin (Table II). According to the previous study, most lactobacilli are intrinsically resistant to aminoglycoside antibiotics, which could explain the obtained results (21). However, the genome of *L. fermentum* BGHV110 should be sequenced for the final confirmation of the absence of acquired or transferable antibiotic resistance genes (21). On the other hand, the potential risk of transfer of antibiotic resistance genes could be reduced by following the novel postbiotic trend in probiotic supplementation, which proposes the use of unviable inactivated bacteria or bacterial metabolites as active ingredients which mimic the beneficial effect of probiotics (22).

Additionally, we investigated the potential of *L. fermentum* BGHV110 to produce biofilm as a common virulent characteristic of different pathogenic bacteria. Biofilm is a structured membrane composed of a polysaccharide matrix, proteins, and other elements, and containing microorganisms, with a complex internal arrangement and channels that facilitate the transport of nutrients within the network which enables pathogen survival in harsh conditions (23). Our results showed that the investigated strain does not have the ability to produce a biofilm on a plastic substrate, suggesting the absence of virulence traits of *L. fermentum* BGHV110 (Figure 1). Overall, these results demonstrated that *L. fermentum* BGHV110 does not possess any of the tested virulent traits. However, genome sequencing is needed for final confirmation of its safety status.

Table II Susceptibility of *Limosilactobacillus fermentum* BGHV110 strain to selected antibiotics

Tabela II Osetljivost *Limosilactobacillus fermentum* BGHV110 soja na odabrane antibiotike

Antibiotics (mg/L)	Amp	Gen	Kan	Str	Ery	Cli	Tet	Chl
MIC value	2	16	64	64	1	4	8	4
Result	< 2	< 16	≥ 64	≥ 64	< 0.5	< 2	< 4	< 2

Note: MIC - Minimal inhibitory concentration, Amp - ampicillin, Gen - gentamicin, Kan - kanamycin, Str - streptomycin, Ery - erythromycin, Cli - clindamycin, Tet - tetracycline, and Chl - chloramphenicol

Napomena: MIC - minimalna inhibitorna koncentracija, Amp - ampicilin, Gen - gentamicin, Kan - kanamicin, Str - streptomicin, Ery - eritromicin, Cli - klindamicin, Tet - tetraciklin i Chl - hloramfenikol

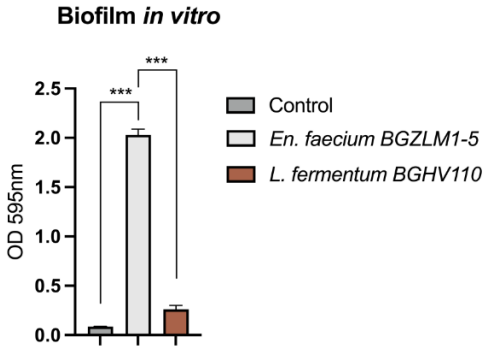

Figure 1. ***Limosilactobacillus fermentum*** **BGHV110 does not have potential to form biofilm *in vitro*. Cristal violet assay showing biofilm forming potential of *L. fermentum* BGHV110 strain in comparison with *Enterococcus faecium* BGZLM1-5 used as a positive control. Data are presented as the mean ± SD from results obtained from three independent experiments. One-way ANOVA followed by the Tukey *post hoc* test for multiple comparisons was used (***p<0.001).**

Slika 1. ***Limosilactobacillus fermentum*** **BGHV110 nema potencijal za formiranje biofilma *in vitro*. Kristal violet test pokazuje potencijal formiranja biofilma soja *L. fermentum* BGHV110 u poređenju sa *Enterococcus faecium* BGZLM1-5 izolatom koji je korišćen kao pozitivna kontrola. Rezultati su predstavljeni kao srednja vrednost ± SD iz rezultata dobijenih iz tri nezavisna eksperimenta. Jednofaktorska ANOVA i Tukey *post hoc* test su korišćeni za poređenje tretmana (***p<0,001).**

Limosilactobacillus fermentum BGHV110 tolerates intestinal juices during simulated gastrointestinal passage

The first step for establishing host-microbe interaction in the gut is the survival of indigenous bacteria in the gastrointestinal environment and potential gut colonization (5).

Therefore, based on the FAO/WHO guidelines, we further evaluated the capability of *L. fermentum* BGHV110 strain to survive simulated transit through the gastrointestinal tract. Results revealed that *L. fermentum* BGHV110 tolerated passage through all gut compartments, including the stomach, duodenum and small intestines (Figure 2). Overnight culture of *L. fermentum* BGHV110 contained 1.8×10^8 CFU/mL of bacteria. After 90 min of exposure of the overnight grown culture to gastric juice, bacterial counts slightly decreased to 6.5×10^7 CFU/mL. As expected, this trend of decreased viability continued after 10 min of incubation in duodenal juice with 1.3×10^7 CFU/mL of viable bacteria. Finally, after exposure to intestinal juice for 120 min, the bacteria count returned to its initial level, with 1.5×10^8 CFU/mL bacteria. As reported, the presence of digestive enzymes and bile acids negatively influences the survival of bacteria during transit through the intestine (24). *L. fermentum* BGHV110 exhibited increased sensitivity to pepsin, low pH and high bile acid concertation (1%), resulting in a 1 log decrease of bacterial counts compared to the initial concentration. However, with a dilution of digestive enzymes and bile salts concentrations which occurs in small intestines, *L. fermentum* BGHV110 recovered to its initial count. A similar result was obtained for *L. fermentum* TCUESC01, showing a slight decrease in viable bacteria after exposure to pepsin and bile acids, even with the addition of 10% of milk as a protecting agent (25). This result suggests that *L. fermentum* BGHV110 survives unfavorable conditions of the GIT, making it a desirable candidate for the development of probiotic preparation, without the need to use the gastro-resistant coated pharmaceutical formulations to enable the survival of probiotic bacteria.

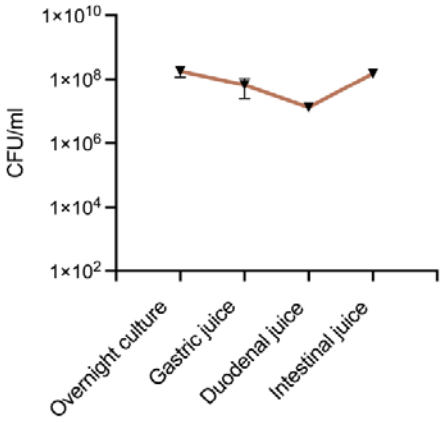

Figure 2. ***Limosilactobacillus fermentum*** **BGHV110 survives simulated gastrointestinal tract conditions. Colony forming units (CFU)/mL of *L. fermentum* BGHV110 strain after exposure to gastric, duodenal and intestinal juices presented as the mean ± SD from results obtained from three independent experiments.**

Slika 2. ***Limosilactobacillus fermentum*** **BGHV110 preživljava simulirane uslove gastrointestinalnog trakta. Jedinica formiranja kolonija bakterija po ml (CFU)/ml *L. fermentum* BGHV110 soja nakon izlaganja želudačnom, duodenalnom i crevnom soku predstavljeno kao srednja vrednost ± SD iz rezultata dobijenih iz tri nezavisna eksperimenta.**

Limosilactobacillus fermentum BGHV110 shows good adhesion properties to Caco-2 cells and strengthens the epithelial barrier in *Caenorhabditis elegans* model

The ability of probiotic strains to adhere to gut mucosa is an important feature for the selection of future probiotics (5). Therefore, we used Caco-2 cells as an *in vitro* model for the intestinal epithelium, to estimate the percentage of bacterial binding. *L. fermentum* BGHV110 showed good adhesion properties of 7.7 % ± 2.02 % of binding, which was almost comparable with the standard probiotic strain *Lacticaseibacillus rhamnosus* GG (ATCC 53103), often used in commercially available probiotics with binding ability of 9.7 % ± 3.3 % (26). Additionally, as binding ability is species-dependent, the obtained result for *L. fermentum* BGHV110 is similar to that reported for *L. fermentum* BIF-19 strain, estimated to be around 8.78 % ± 0.74 % (27). This result implies that a sufficient number of administrated probiotics will come into close contact with host receptors with the potential to activate different cellular pathways in the gut mucosa.

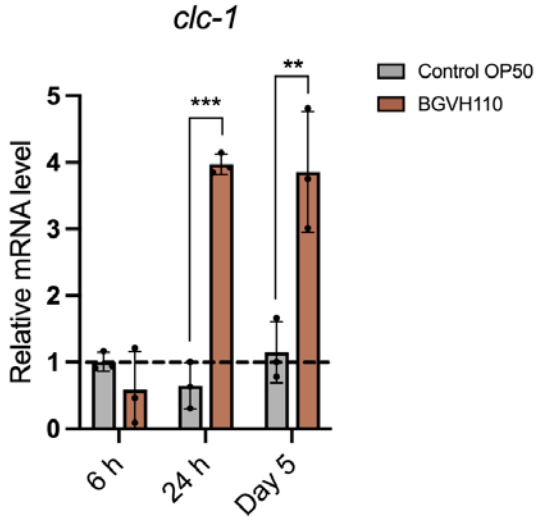

Figure 3. ***Limosilactobacillus fermentum* BGHV110 stimulates expression of tight junction protein in *Caenorhabditis elegans*. The expression of the claudin-like *clc-1* gene was measured by qRT-PCR in *C. elegans* after 6 h, 24 h and 5 days of treatment with *L. fermentum* BGHV110 relative to *E. coli* OP50 control. All data are presented as mean ± SD and Student's t-test was used to compare the treated group relative to control (***p<0.001).**

Slika 3. ***Limosilactobacillus fermentum* BGHV110 stimuliše ekspresiju proteina tesnih međućelijskih veza u *C. elegans*. Ekspresija gena sličnog klaudinu *clc-1* merena kvantitativnom PCR metodom kod *C. elegans* nakon 6 h, 24 h i 5 dana tretmana sa *L. fermentum* BGHV110 u odnosu na *E. coli* OP50 kontrolu. Svi rezultati su predstavljeni kao srednja vrednost ± SD i Studentov t-test je korišćen za poređenje tretirane grupe u odnosu na kontrolu (***p<0,001).**

As the interaction between bacterial macromolecules and host receptors is essential for the beneficial effect of probiotics (4), we next evaluated the effect of *L. fermentum* BGHV110 on the *clc-1* gene expression, a human ortholog of claudin TJP in the *C. elegans* model (11). Worms fed with *L. fermentum* BGHV110 for 6 h showed no changes in transcription levels of the *clc-1* gene, in comparison with *E. coli* OP50 used as a standard laboratory food. However, prolonged treatment for 24 h and 5 days showed significant upregulation of *clc-1* transcripts, suggesting that *L. fermentum* BGHV110 has the potential to straighten the epithelial barrier (Figure 3). This observation is in line with the reported results for *Latilactobacillus curvatus* BGMK2-41 strain, which showed PMK-1 dependent induction of the *clc-1* genes in *C. elegans*, thus providing higher resistance of treated worms to pathogens (8). Additionally, literature data showed that another strain of *L. fermentum*, labelled as KBL375, exhibits the potential to upregulate TJPs like E-cadherin or Claudin 3 in mice with dextran sulfate sodium-induced colitis, suggesting that probiotic lactobacilli could maintain epithelial barrier integrity not only in infection, but also in inflammation (28).

Limosilactobacillus fermentum BGHV110 triggers conserved p38 MAPK immunity pathway essential for *Caenorhabditis elegans* defence against pathogens

Next, we focused on the evaluation of conserved immunity pathways and AMP production in the *C. elegans* model. We examined the expression of immune-related genes at three time points, starting with 6 h treatment. After 6 h of feeding, no changes in the mRNA transcription of tested genes were observed **(**Figure 4A**)**. Furthermore, since we had identified an elevation of *clc-1* expression after 24 h, we fed worms for 24 h and the expression results revealed elevated transcript levels of PMK-1/p38 MAPK immunity pathway genes, including the *tir-1* and *pmk-1*, as well as the effector *lys-1* gene which encodes a human ortholog of AMP **(**Figure 4B**).** Finally, worms treated for 5 days with *L. fermentum* BGHV110 maintained a high level of transcription of *tir-1* and *lys-1* immunity genes, with additional activation of the DBL-1 pathway, and another worm AMP effector *lys-8* **(**Figure 4C**)**. These results are consistent with our previous finding that *L. curvatus* BGMK2-41 probiotic strain can trigger PMK-1/p38 MAPK immunity to survive lethal *Staphylococcus aureus* and *Pseudomonas aeruginosa* intestinal infection in *C. elegans* (8). Moreover, this study brings new findings about probiotic-mediated activation of lysozyme-like genes *lys-1* and *lys-8* expressed mainly in the intestines, implying potential indication of *L. fermentum* BGHV110 strain in gastrointestinal infections caused by both gram-positive and gram-negative bacteria. It has been shown that some probiotic lactobacilli have the potential to tune the innate immune response and transiently boost proinflammatory cytokines production (29). In mammals, p38 MAPK controls the synthesis of proinflammatory cytokines (IL-1β, TNF-α and IL-6), induction of enzymes important for the innate immune defence such as COX-2 and iNOS, and induction of endothelial adherent proteins along with other inflammatory related molecules necessary for combating incoming infections (30). For example,

Lacticaseibacillus casei ATCC27139 was shown to significantly upregulate proinflammatory cytokines by mice splenocytes via p38 MAPK signalling pathways (31). On the other hand, the activation of DBL-1 pathways pointed out that this strain could increase the production of TGF-β cytokine, an important inflammation regulator in mammals, highlighting the dual role of *L. fermentum* BGHV110 in fine-tuning between pro- and anti-inflammatory responses. For some lactobacilli there is evidence that they can trigger the TGF-β signalling pathway in *C. elegans* to mediate *S. aureus* resistance (32).

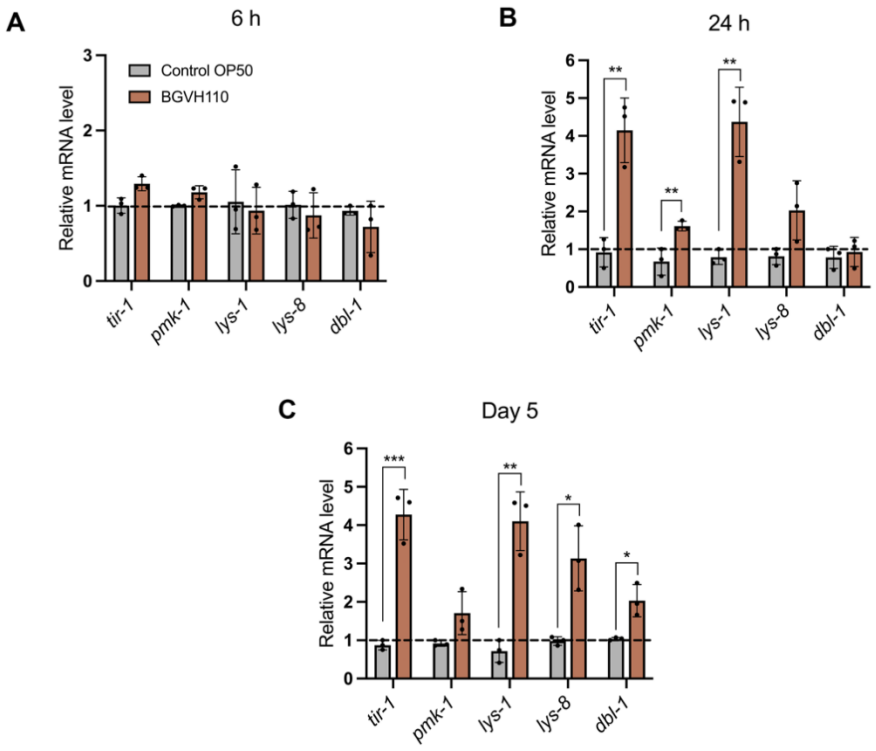

Figure 4. ***Limosilactobacillus fermentum*** **BGHV110 activates immunity pathways and AMPs in** ***Caenorhabditis elegans*****. The expression of the immune-related genes (*tir-1, pmk-1, dbl-1*) and AMPs (*lys-1, lys-8*) measured by qRT-PCR in *C. elegans* after 6 h, 24 h and 5 days of treatment with *L. fermentum* BGHV110 relative to *E. coli* OP50 control. All data are presented as mean ± SD and the Student's t-test was used to compare the treated group relative to control (*p<0.05, **p<0.01, ***p < 0.001).**

Slika 4. ***Limosilactobacillus fermentum*** **BGHV110 aktivira imunske puteve i antimikrobne peptide u** ***C. elegans*****. Ekspresija gena povezanih sa imunskim odgovorom (*tir-1, pmk-1, dbl-1*) i gena koji kodiraju antimikrobne peptide (*lys-1, lys-8*) merena kvantitativnom PCR metodom kod *C. elegans* nakon 6 h, 24 h i 5 dana tretmana sa *L. fermentum* BGHV110 u odnosu na *E. coli* OP50 kontrolu. Svi rezultati su predstavljeni kao srednja vrednost ± SD i Studentov t-test je korišćen za poređenje tretirane grupe u odnosu na kontrolnu (*p<0,05, **p<0,01, ***p < 0,001).**

Overall, having in mind all the collected results of beneficial effects of *L. fermentum* BGHV110 strain from this and previous studies, we highlight its potential to be used for the prevention or treatment of gastrointestinal infections, with the additional possibility for it to be tested for the treatment of other diseases, especially those related to liver damage and lipid metabolism.

Acknowledgements

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Probiotička karakterizacija soja *Limosilactobacillus fermentum* BGHV110 i njegov uticaj na urođeni imunski odgovor kod *Caenorhabditis elegans*

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Kratak sadržaj

Probiotički laktobacili pokazuju potencijal da pozitivno deluju na zdravlje domaćina. *Limosilactobacillus fermentum* se zahvaljujući brojnim korisnim efektima na zdravlje ljudi izdvojio kao odličan kandidat za razvoj komercijalnih probiotičkih preparata koji imaju za cilj prevenciju širenja infektivnih bolesti. U ovoj studiji, korišćeno je nekoliko *in vitro* testova, uključujući test formiranja biofilma, test procene osetljivosti na antibiotike, test preživljavanje u simuliranim uslovima gastrointestinalnog trakta i test adhezije na intestinalne Caco-2 ćelije, za procenu bezbednosti i probiotičkog potencijala soja *L. fermentum* BGHV110. Dodatno, *Caenorhabditis elegans* je korišćen kao *in vivo* model sistem za procenu uticaja *L. fermentum* BGHV110 na urođeni imunski odgovor domaćina. Rezultati su pokazali da soj *L. fermentum* BGHV110 poseduje odličnu sposobnost da preživi nepovoljne uslove u crevima, da se veže za intestinalne Caco-2 ćelije i da stimuliše evolutivno konzervisani p38 MAPK imunski put i ekspresiju gena sličnog klaudinu *clc-1* i antimikrobnih peptida u *C. elegans* u cilju jačanja imunskog odgovora na infekciju. Dodatno, *L. fermentum* BGHV110 je pokazao odsustvo faktora virulencije i osetljivost na testirane antibiotike, što je potvrdilo njegov bezbednosni status u skladu sa kojim se može primeniti kao budući probiotik.

Ključne reči: *Limosilactobacillus fermentum*, probiotik, urođeni imunski odgovor, gastrointestinalni trakt, *Caenorhabditis elegans*

Arh. farm. 2023; 73: 586 – 601 *Original scientific paper/Originalni naučni rad*

Effect of immunostimulating *Limosilactobacillus* strain in rats with trinitrobenzenesulfonate (TNBS)-induced colitis

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Abstract

The aim of the study was to test the potential of immunostimulating *Limosilactobacillus fermentum* BGHI14 strain to reduce the damage of colon tissue in rats with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. Wistar rats were treated with *L. fermentum* BGHI14 in the regime of preventive, therapeutic and continuous treatment 22 days prior to and/or 7 days after the administration of TNBS. After sacrifice, the colon tissue samples were taken for RNA isolation, gene expression analysis, histopathological analysis, and malondialdehyde measurement. Judging from the body weights, histopathological scores, malondialdehyde levels and transcription of IL-1β and Tight junction protein 1 (Tjp-1) coding genes, preventive and therapeutic treatment proved to be the most protective in the applied conditions. On the other hand, continuous treatment did not affect the intensity of tissue damage. Considering these results, we discussed the possible mechanisms which might stand behind the protective action of immunostimulating probiotic bacteria in the case of mucosal barrier damage.

Key words: colitis, *Limosilactobacillus fermentum*, probiotic, pro-inflammatory cytokines, lipid peroxidation

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Introduction

Gut barrier dysfunction is a major cause of digestive tract inflammations designated as inflammatory bowel diseases (IBD). Crohn's disease (CD) is a highly prevailing inflammatory disease and commonly involves the ileal part of the gastrointestinal (GI) tract (1,2). At the moment there is enough evidence showing that CD weakens acute immune response, leading to defective clearance of luminal bacteria that invade the intestinal tissue and cause chronic inflammation (3). One of the simplest experimental models of CD is 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis that develops after intracolonic TNBS administration. Although the TNBS model results only in tissue damage in the colon, due to the nature of inflammatory lesions that include the entire intestinal wall, it corresponds to CD in humans (4, 5). Gut wall necrosis induced by TNBS leads to the entrance of luminal microorganisms in the colonic tissue and mounting of acute inflammatory response. Accordingly, extermination of intestinal microbiota has been able to prevent the progression of TNBS colitis (5, 6).

Strategies developed until now have been aimed at modulating the immune response in order to maintain remission in Crohn's disease (7). Lactobacilli have been tested in IBD in the context of the prolongation of remission after surgery (8). However, in some CD patients, the incidence of relapse was higher after treatment with probiotic lactobacilli (9). Ambiguous results obtained with lactobacilli in IBD treatment impose the need for a comprehensive examination of mechanisms of action of probiotic lactobacilli (10).

Limosilactobacillus fermentum is the heterofermentative lactic acid bacterium present in foods, milk of mammals, vaginal tract and GI tract of new-borns and children (11, 12, 13, 14, 15). Although it does not belong to the indigenous intestinal microbiota (16), different *L. fermentum* strains exhibit various probiotic effects on the host (17, 18). In our previous study, we followed the reaction of healthy colon tissue of rats after ingestion of *L. fermentum* BGHI14 (19). In this study, we tested the effect of the same strain in the late phase of acute TNBS-induced colitis. In addition, due to the immune response of colon tissue to BGHI14, as reported in our previous work, we have chosen to apply TNBS three weeks after the start of BGHI14 treatment, because we expect that at this point transient immune reaction has already withdrawn.

Materials and methods

Bacterial preparation

The strain *Limosilactobacillus fermentum* BGHI14 isolated from breast-fed neonate faeces was used in the study. The bacteria were cultivated in MRS medium (Oxoid Limited, Hampshire, United Kingdom) at 37 °C anaerobically (19). For animal treatment, 10 ml of fresh overnight bacterial culture (with approximately 10^{10} colony forming unites (CFU)/ml) was pelleted, washed in saline and resuspended in 1 ml of sterile 11 % skimmed milk (Mlekara Subotica, Serbia).

Experimental animals

Female Wistar rats, 5-6 weeks of age, weighing 140 ± 10 g, were purchased from the Farm of Military Medical Academy, Belgrade, and for experimental purposes were housed in the animal facility of the Faculty of Pharmacy, University of Belgrade. The research was approved by the Ethical Committee of the Faculty of Pharmacy, University of Belgrade, and experimental procedures were performed in accordance with the institutional guidelines on care for experimental animals No. 2/09. A veterinarian inspected the animals once a week, checking for distress or existence of physical injuries.

Study design

The experimental design is shown in Figure 1. Animals were grouped in five treatment regimens (nine per group) and treated daily by oral gavage using a stainless steel feeding tube (18 G, Instech Solomon, Plymouth Meeting, PA, USA). Two groups of animals were treated with BGHI14 suspension in skimmed milk for 21 days, while the remaining three groups received skimmed milk. On day 22 of treatment onset, rats from two BGHI14-treated and two milk fed groups were administered TNBS through the anus, as described in our previous paper (19). The remaining group was administered phosphate buffered saline (PBS). On the day of colitis induction, two colitic groups, one BGHI14-treated and one milk fed group, had the treatment changed so that for BGHI14-treated rats' bacterial treatment was stopped and feeding was continued with milk (preventively treated colitic rats), and for milk receiving rats BGHI14 treatment was started (therapeutically treated colitic rats). For rats from the remaining groups, the same treatment was continued (colitic continuously BGHI14-treated, colitic controls, and healthy controls). Animals were fed for the following seven days and were sacrificed

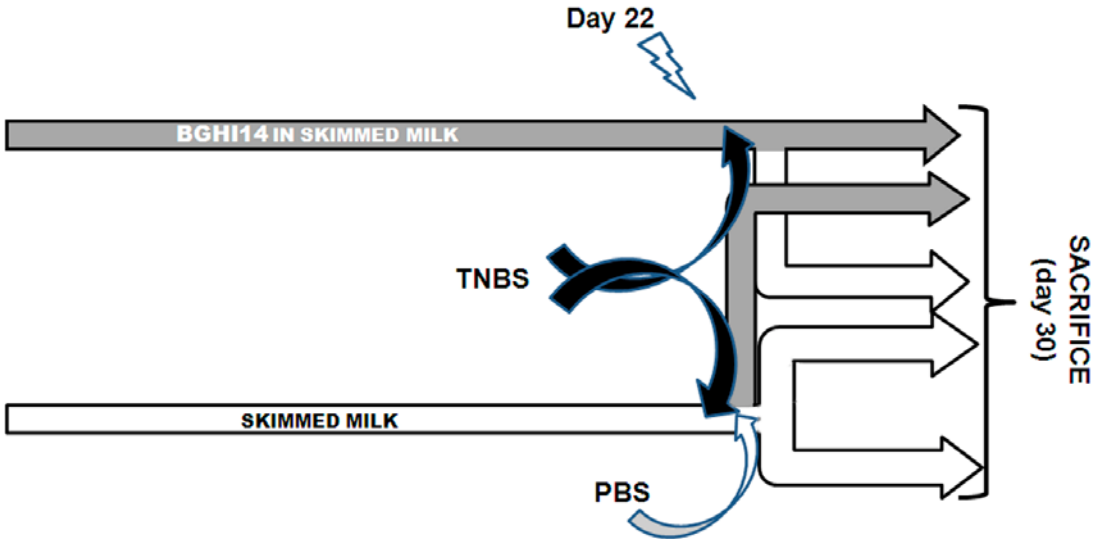


Figure 1. Study design
Slika 1. Dizajn studije

using increasing CO_2 concentration on day 30 from the beginning of the experiment. Colons were sampled for histological analysis, RNA isolation and biochemical assay. Additionally, colonic content was sampled for total DNA isolation.

Histological processing of tissue

Colon tissue was processed for histopathological analysis as described in our previous paper (19). Paraffin blocks were prepared using a rotary microtome (RM2125RT, Leica Microsystems, Wetzlar, Germany). Damage and inflammation in the colon tissue were estimated semi-quantitatively using the following criteria: crypt and submucosal muscle layer hypertrophy, presence of immune cells in underlying fat tissue, presence and type of immune cell infiltrate in submucosal loosely organized tissue and in submucosal muscles, crypt damage, submucosal widening, absorptive surface decrease and necrotic tissue presence. Histological sections were photographed using NIS-Elements Microscope Imaging Software 2.3 (Nikon Instruments Inc., Tokyo, Japan).

RNA isolation

The isolation of RNA was performed according to the protocol by Chomzynski & Sacchi (20), with modifications described in our previous paper (19). Shortly, tissue was pulverized in liquid nitrogen and resuspended in denaturing solution containing guanidine thiocyanate as denaturing agent. Repeated acid phenol, pH 4, extractions were performed for protein and DNA contamination removal. Centrifugation steps at 15 000 × *g*, + 4 °C were performed in an Eppendorf 5417R centrifuge (Eppendorf). RNA concentrations were measured spectrophotometrically using a Nanovue Plus Spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

Quantitative real-time PCR (qRT-PCR)

Reverse transcription (RT) reaction was set according to the instructions of the enzyme manufacturer (Thermo Scientific). Random hexamers, RNase inhibitor and dNTP set were purchased from Thermo Scientific. All reaction steps were performed in the Gene AmpR System 2700 apparatus (Applied Biosystem). Controls without reverse transcriptase were included for a DNA contamination check. Complementary cDNA obtained in reverse transcription was used as a template in a qPCR reaction performed in the 7500 Real Time System apparatus (Applied Biosystems). All primers used were from Invitrogen (Paisley, United Kingdom) and described in our previous paper (19). KAPA SYBR FAST Universal Master Mix (KAPA Biosystems) was used, and two-step reaction conditions were as follows: 3 min at 95 °C, 40 cycles with 15 s at 95 °C and 60 s at 60 °C. For relative quantification, tenfold cDNA dilutions were used.

Lipid peroxidation assay

The level of oxidative stress in rats' colon tissue was determined as the malondialdehyde (MDA) level in tissue homogenates. The test was done according to

McCluskey *et al*. (21), with modifications. Colon tissue was pulverized in liquid nitrogen and next homogenized as a 10 % suspension in 1.15 % KCl in glass homogenizers (Sigma-Aldrich, St. Louis, Missouri, USA). Immediately after homogenization, 200 µl of homogenate was mixed with 600 µl of solution containing 0.375 % TBA (Sigma), 0.25 M HCl and 15 % solution of trichloroacetic acid (TCA). The mixture was incubated for 15 min at 95 °C, cooled, centrifuged shortly at 1 000 × *g* (5417R, *Eppendorf centrifuge*, Hamburg, Germany), + 4 °C, and supernatant absorbance was determined at 532 nm using spectrophotometer Ultrospec 3300 pro (Amersham Bioscience, Piscataway, New Jearsy, USA). As per the MDA standard, 1,1,3,3-tetramethoxipropane (Sigma-Aldrich) in the concentration range 0.1-1 µM was used.

Statistical analysis and data presentation

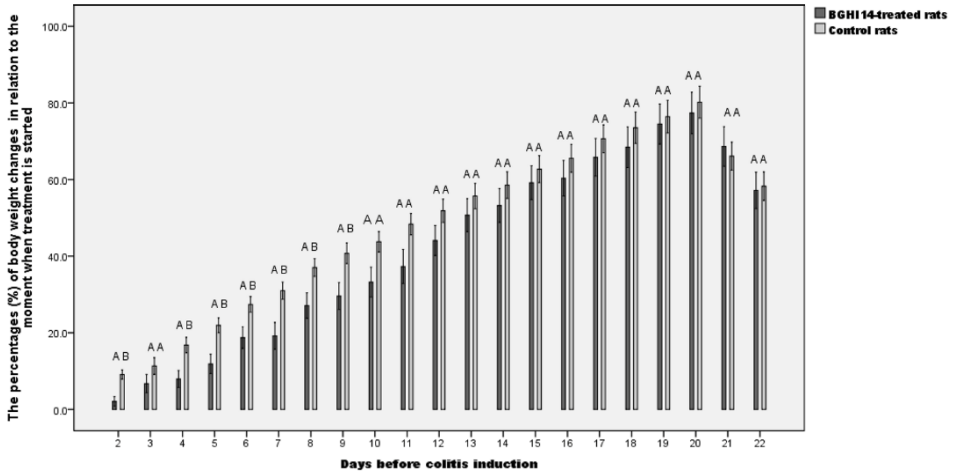
The results are presented graphically, using bar charts representing the mean value of the group with standard errors. Differences between groups were considered to be statistically different if the p value was lower than 0.05 ($p < 0.05$). Statistical differences are marked above bars, with different letters representing statistical significance between groups. Data were analyzed using the Mann-Whitney test. All statistical analyses and graph drawing were performed in SPSS 18.0 software for Windows.

Results

Body weight of rats during treatment

Percentages of body weight changes in relation to the moment when the treatment started and to the moment of colitis induction are shown in Figure 2. Prior to colitis induction, significantly lower percentages of body mass changes in BGHI14-treated rats compared to untreated rats were observed on days 2^{nd}, 4^{th}, 5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} from the feeding onset. After colitis induction, reduction of weights was detected in colitic compared to healthy rats, with statistical significance reached on days 3 and 4 for preventively treated rats, days 2, 3, 4 and 6 for therapeutically treated rats, and all days until sacrifice for continuously treated and control colitic rats. Additionally, on day 7 from TNBS instillation, continuously treated rats demonstrated a significantly lower percentage of body weight changes relative to preventively treated rats.

A



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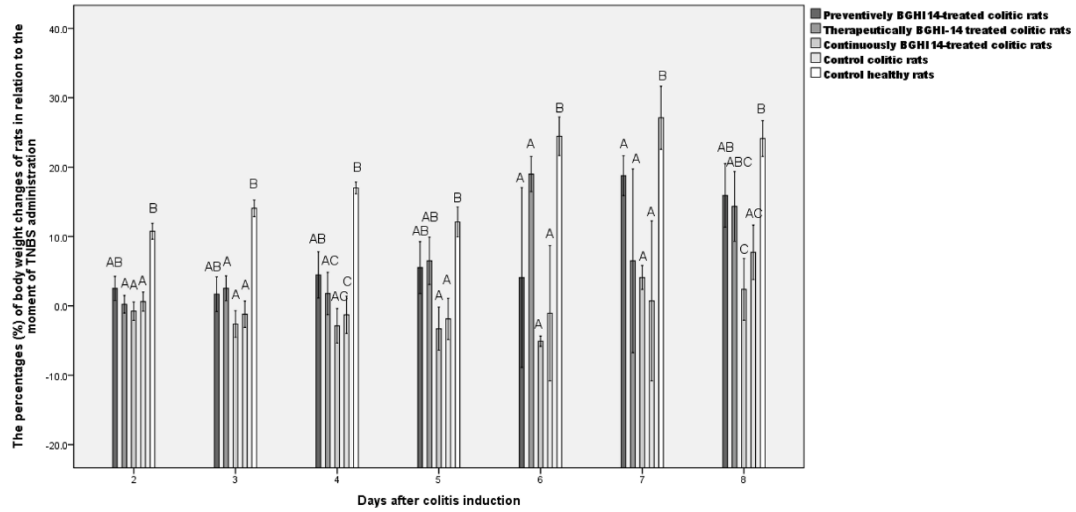


Figure 2. **The percentages of body weight changes of rats treated with *Limosilactobacillus fermentum* BGHI14 and of control rats (A) before colitis induction in relation to the moment when treatment is started; (B) after colitis induction in relation to the moment of TNBS administration. Different letters above the bars indicate statistically significant difference between the treatments (n = 9).**

Slika 2. **Procenti promene telesne težine pacova tretiranih sa *Limosilactobacillus fermentum* BGHI14 i kontrolnih pacova (A) pre indukcije kolitisa u odnosu na početak tretmana; (B) posle indukcije kolitisa u odnosu na trenutak primene TNBS. Različita slova iznad grafikona ukazuju na statistički značajne razlike između tretmana (n = 9).**

Histological scoring of damage and inflammation in colon tissue

Colitis induction resulted in a statistically significant increase in pathohistological scores in the late acute phase of disease (Figure 3). Preventively BGHI14-treated rats showed significantly lower scores compared to continuously treated rats, though the significance is marginal (p = 0.05). In colitic rats, remains of necrotic tissue were evident, as well as absorptive surface decrease, crypt and submucosal muscle hypertrophy, and transmural infiltrates of polymorphonuclear cells, monocytes and eosinophils (Figure 4).

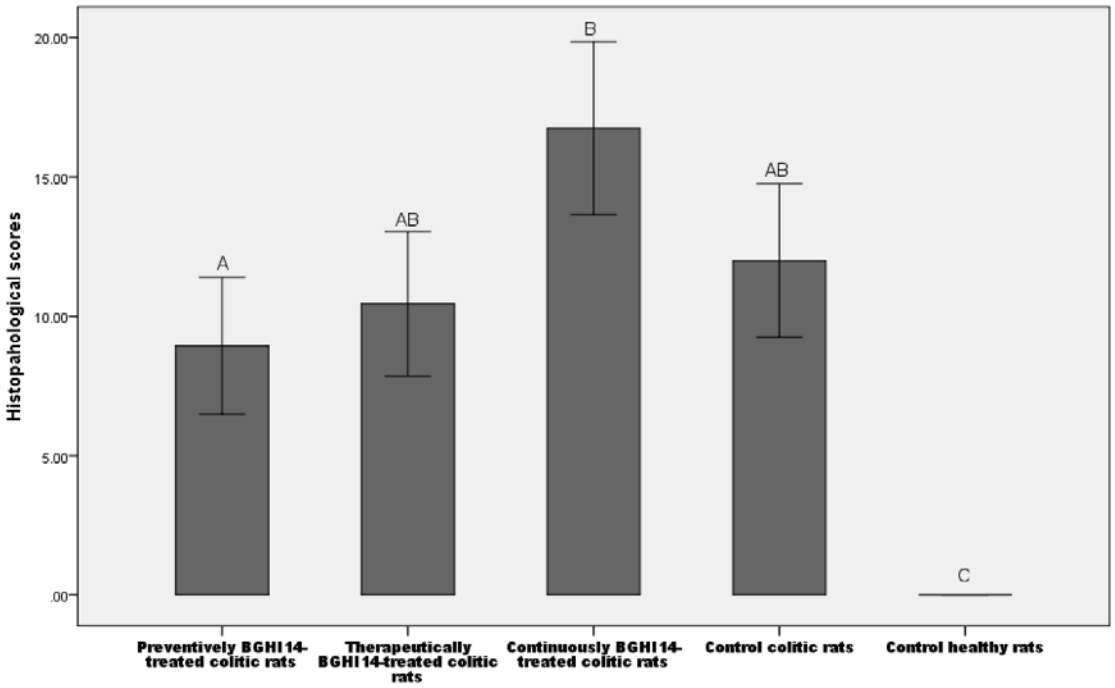


Figure 3. **Pathohistological scores measuring damage and inflammation in colon tissue of colitic and healthy rats. Different letters above the bars indicate statistically significant difference between the treatments (n = 9).**

Slika 3. **Patohistološki rezultati merenja oštećenja i zapaljenja u tkivu debelog creva pacova sa kolitisom i zdravih pacova. Različita slova iznad grafikona ukazuju na statistički značajne razlike između tretmana (n = 9).**

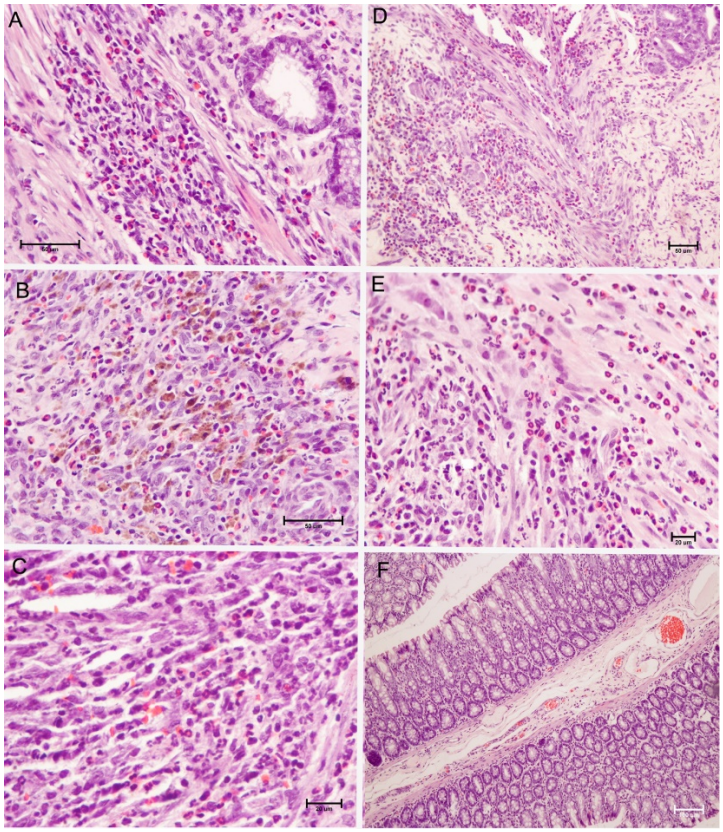


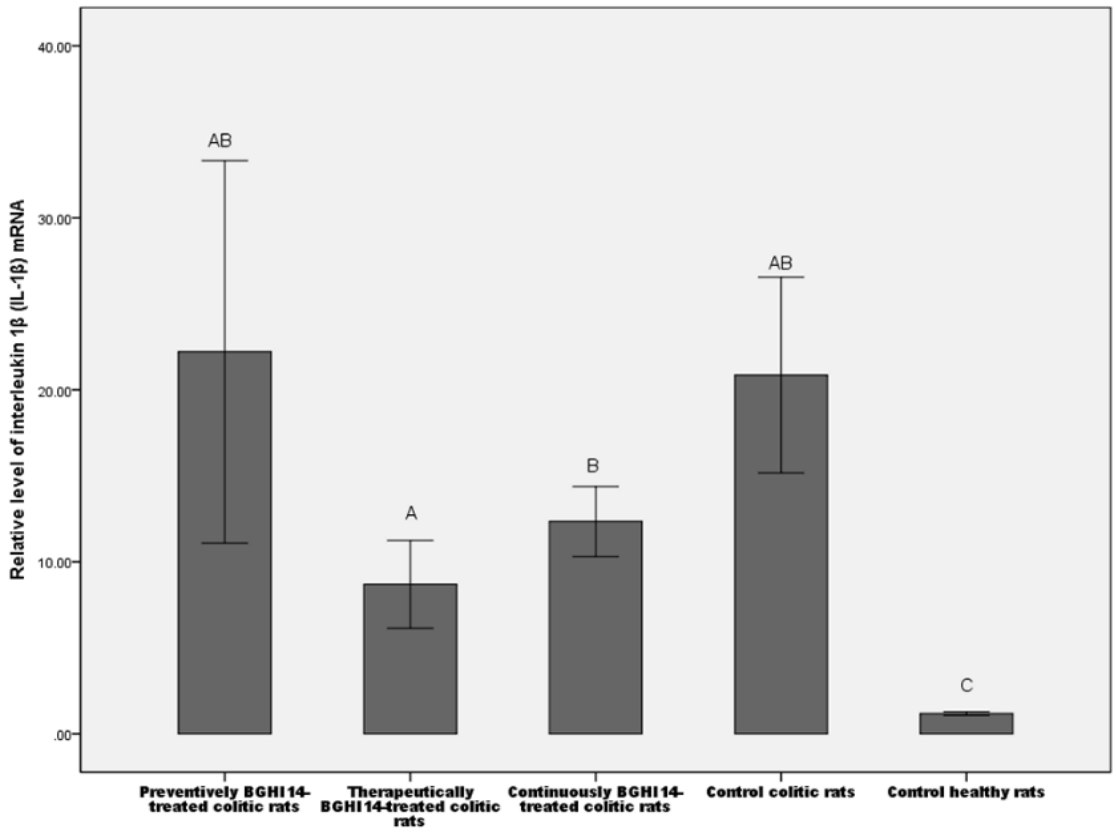
Figure 4. **Cross sections of colon tissue: (A) (400×) mononuclear and neutrophil infiltrate of mucosal muscle layer in a colon of preventively treated colitic rats; (B) (400×) neutrophils, mononuclear cells and eosinophils in submucosal infiltrate in a therapeutically treated colitic rat; additionally macrophages with haemoglobin content are visible indicating abundant bleeding in displayed area; (C) (600×) and (D) (200×) inflammation of submucosal muscles with mononuclear cells and sporadic neutrophils and eosinophils in a colon of continuously treated rats; (E) (400×) section of tissue of control colitic rats depicting monocytes, neutrophils and eosinophils in mucosal muscles and in submucosa; (F) (100×) tissue of a healthy control rat.**

Slika 4. **Poprečni preseci tkiva debelog creva: (A) (400×) mononuklearni i neutrofilni infiltrat mukoznog mišićnog sloja u debelom crevu preventivno lečenih pacova sa kolitisom; (B) (400×) zastupljenost neutrofila, mononuklearnih ćelija i eozinofila u submukoznom infiltratu kod terapeutski tretiranih pacova sa kolitisom; dodatno vidljivi makrofagi sa sadržajem hemoglobina ukazuju na obilno krvarenje u prikazanom području; (C) (600×) i (D) (200×) zapaljenje submukoznih mišića sa mononuklearnim ćelijama i sporadičnim neutrofilima i eozinofilima u debelom crevu stalno tretiranih pacova; (E) (400×) presek tkiva kontrolnih pacova sa kolitisom koji prikazuje monocite, neutrofile i eozinofile u mukoznim mišićima i u submukozi; (F) (100×) tkivo zdravog kontrolnog pacova.**

Transcription of genes in colon tissue

Transcription of genes coding for molecular markers of inflammation (tumour necrosis factor alpha, TNFα and interleukin 1 beta, IL-1β) and epithelial cell markers (tight junction protein 1, Tjp1) in colon tissue was assessed for evaluation of BGHI14 effects in colitic rats. Colitis induction caused a significant increase in *IL-1β* mRNA levels. Among colitic rats, therapeutically BGHI14-treated group showed significantly lower *IL-1β* mRNA levels relative to continuously treated rats (Figure 5A), though very low ($p = 0.046$) On the other hand, *TNFα* transcription was not changed in late acute phase of TNBS colitis relative to healthy rats, irrespective of the BGHI14 treatment regime (not shown). A statistically significant decrease in *Tjp1* mRNA level was observed after colitis induction, with the exception of therapeutically BGHI14-treated animals. Moreover, therapeutically treated rats showed significantly higher *Tjp1* mRNA transcript levels compared to continuously treated ($p = 0.019$) and nontreated ($p = 0.031$) colitic rats (Figure 5B).

A



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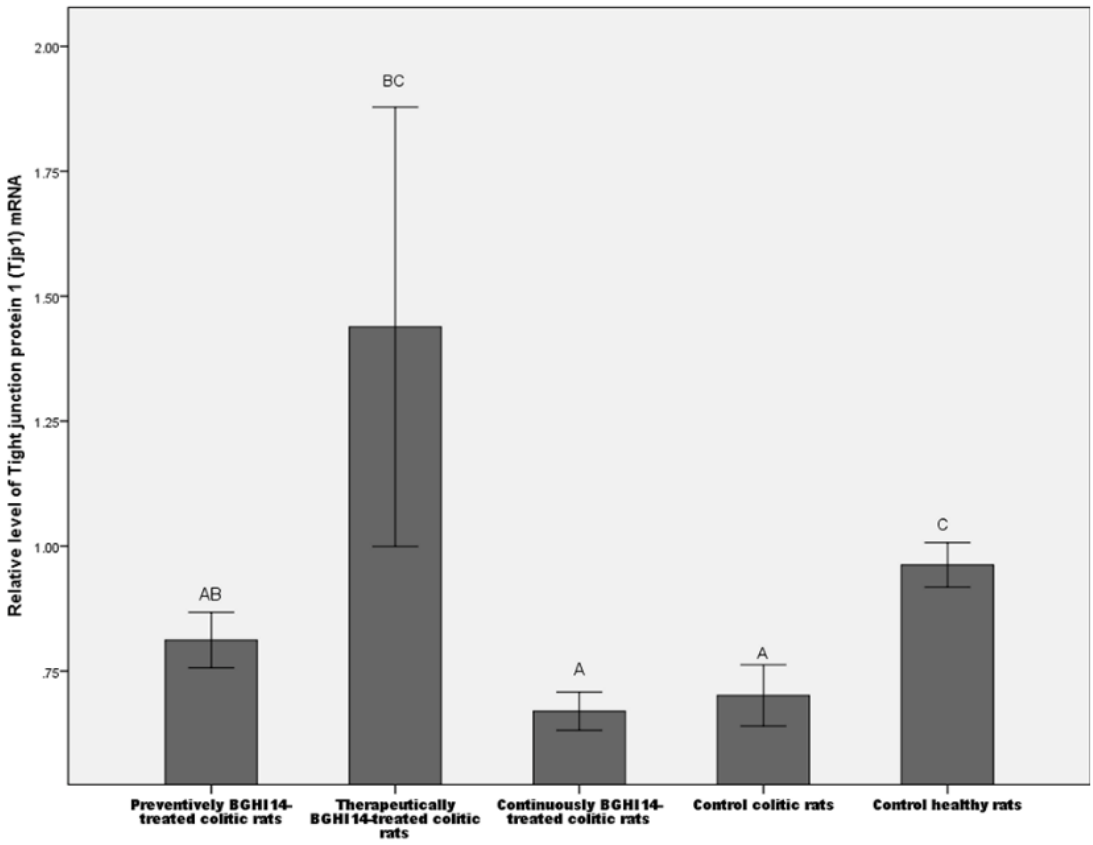

Figure 5. **Impact of different *Limosilactobacillus fermentum* BGHI14 treatment regimens on mRNA expression of: (A) *IL-1β*, and (B) *Tjp1* genes in colon tissue of colitic rats. Different letters above the bars indicate statistically significant difference between the treatments (n = 9).**

Slika 5. **Uticaj različitih režima primene *Limosilactobacillus fermentum* BGHI14 na ekspresiju iRNK: (A) IL-1β i (B) Tjp1 gena u tkivu debelog creva pacova sa kolitisom. Različita slova iznad grafikona ukazuju na statistički značajne razlike između tretmana (n = 9).**

Lipid peroxidation in colon tissue

Levels of lipid peroxidation are presented as the amount of MDA in μmoles per gram of the tissue (Figure 6). TNBS administration did not increase MDA levels seven days after colitis induction. BGHI14 affected lipid peroxidation levels only after therapeutical administration, when a significant decrease of MDA levels was observed compared to control colitic rats ($p = 0.04$).

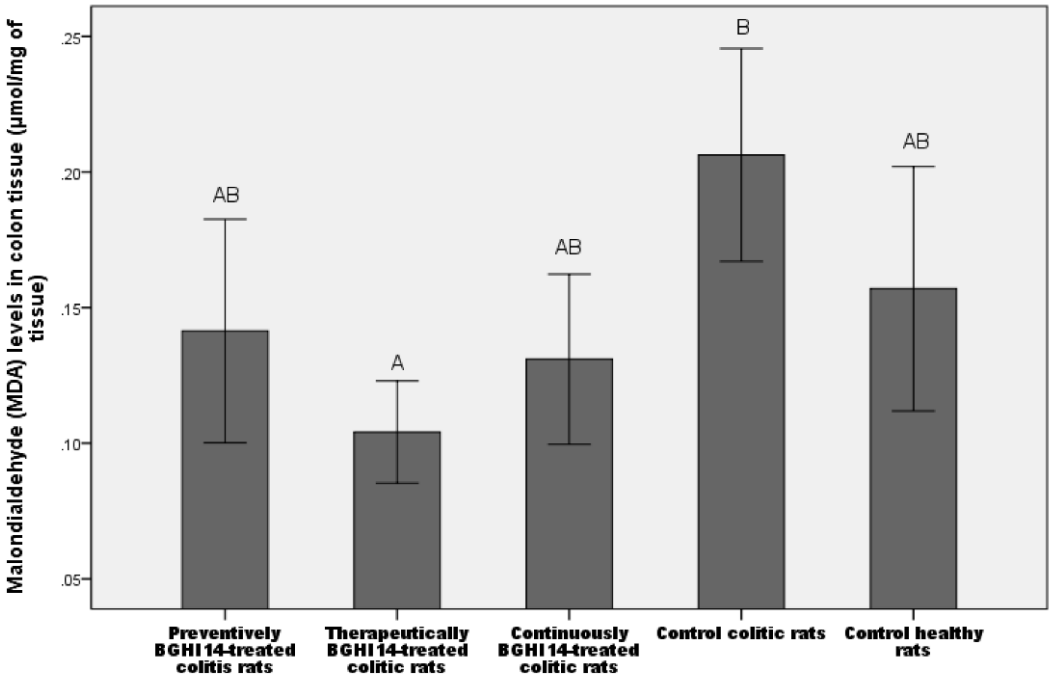

Figure 6. **Malondialdehyde (MDA) levels in colon tissue of colitic and healthy rats. Different letters above the bars indicate statistically significant difference between the treatments (n = 9).**

Slika 6. **Nivo malondialdehida (MDA) u tkivu debelog creva pacova sa kolitisom i zdravih pacova. Različita slova iznad grafikona ukazuju na statistički značajne razlike između tretmana (n = 9).**

Discussion

CD occurs as a result of a weakened immune response in the intestinal mucosa to luminal bacteria. Similar mechanisms are responsible for the progression of TNBS-colitis in rats. There is a growing body of evidence reporting positive effect of lactobacilli in the case of TNBS-colitis in animal models (22). We previously demonstrated probiotic potential and immunostimulatory activity of *Limosilactobacillus fermentum* BGHI14 strain (19, 23, 24). The same strain was tested in current research for eventual protection in TNBS-colitis in Wistar rats.

Tracking of the animals' body weights during treatment showed that the preventive and therapeutic treatments were the most effective in alleviating the consequences of the administration of TNBS. Histopathological observations revealed the reduction of damage of the colon tissue only in the case of preventively treated rats. However, therapeutically treated rats showed increased levels of *Tjp1* gene expression compared to untreated and continuously treated colitic rats. Tight junctions are important for the maintenance of intestinal epithelial cell polarization and for preventing the entry of

luminal substances in intestinal tissue (25). We assume that these results reflect the degree of tissue damage that was the least pronounced in the case of therapeutically treated rats. In addition, the level of proinflammatory *IL-1β* cytokine mRNA was decreased in therapeutically treated animals compared to continuously treated rats, indicating that the therapeutic treatment was the most effective in reducing inflammation. The results obtained in qPCR for therapeutic treatment were also supported by the level of malondialdehyde in the tissue of the colon in colitic rats, which was the lowest in this treatment group. Reactive species produced by phagocytes during inflammation can cause oxidative damage to intestinal tissue (26). However, in our study, colitis induction did not lead to an increase of lipid peroxidation, which implies that other mechanisms might be causing the damage, such as matrix metalloproteinases, as proposed by Schepens et al. (27).

Regardless of the immunostimulating effect of strain BGHI14 in healthy rats, the same strain showed a tendency to decrease the damage of colon tissue in rats with colitis when introduced before or after the induction of disease. Such action of BGHI14 can be explained by referring to the model outlined by Eckman et al. (28). In accordance with this model, the activation of NF-κB signalling pathway, which is the main pathway that is activated in eukaryotic cells after interaction with microorganisms, has different effects on the progression of damage, depending on whether it is activated in the acute or in the chronic phase of inflammation (28, 29). Epithelial restitution in acute injury could be achieved by nuclear factor kappa B (NF-κB) activation involving epithelial and myeloid cells (28). However, NF-κB signalization during the course of existing inflammation would not be beneficial, because epithelial cells have already elevated cytoprotective molecule levels so there is no benefit from additional NF-κB activation. Moreover, it could lead to the aggravation of disease scores by additionally activating myeloid cells and adaptive immune response. The presence of strain BGHI14 at the moment of induction of damage in therapeutic treatment could lead to the release of cytoprotective factors by epithelial cells and protect against damage. Similarly, in the case of preventive treatment, the level of cytoprotective factors could be increased, making epithelium prepared for the insult that followed. However, in the case of continuous treatment with BGHI14, additional activation of myeloid cells could undo the positive effects that resulted from the synthesis of cytoprotective factors. Similarly to our results, Geier et al. (30) observed no effect of continuous *Lb. fermentum* administration to colitic rats.

To summarize, the presented research has demonstrated beneficial effects of *L. fermentum* BGHI14 in the amelioration of experimental colitis in Wistar rats, when BGHI14 was applied either before or after colitis induction. This was contrasted with the continuous (before and after) administration regime of BGHI14, which did not show any beneficial effects. This study is preliminary in terms of detecting the existence of an effect of BGHI14 in the case of a single experimental model of inflammatory bowel disease (IBD). In order to make the results relevant for eventual clinical application, the inclusion of other IBD models would be needed, along with detailed mechanistic studies to confirm the above provided hypothesis behind the protective effects of BGHI14. In the first place,

future studies should test the degree of activation of myeloid cells, as well as the levels of cytoprotective factors in the intestinal mucosa BGHI14 treated animals, without and with disease induction. Furthermore, the results presented here imply the potential for use of BGHI14 in treatment of other intestinal pathologies, including infections, which would broaden the utilization potential of the tested species.

Acknowledgments

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Conflicts of interest

The strain *L. fermentum* BGHI14 is deposited in the BCCM/LMG Bacterial Culture Collection, Belgium under patent deposition, and is subject to the license agreement between the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia and Invetlab d.o.o. Belgrade, Serbia. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The authors have not declared any conflicts of interest.

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Efekat imunostimulišućeg soja roda *Limosilactobacillus* kod pacova sa kolitisom izazvanim trinitrobenzensulfonatom (TNBS)

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Kratak sadržaj

Cilj ovog istraživanja je bio da se ispita potencijal imunostimulišućeg soja *Limosilactobacillus fermentum* BGHI14 da smanji oštećenje tkiva debelog creva kod pacova sa kolitisom izazvanim 2,4,6-trinitrobenzensulfonskom kiselinom (TNBS). Pacovi Wistar soja su tretirani sojem *L. fermentum* BGHI14 u režimu preventivnog, terapijskog i kontinuiranog tretmana 22 dana pre i/ili 7 dana nakon primene TNBS. Nakon žrtvovanja, uzorci debelog creva su sakupljeni za izolaciju RNK, analizu ekspresije gena, histopatološke analize i merenje malondialdehida. Na osnovu telesnih težina pacova, histopatoloških rezultata, nivoa malondialdehida i transkripcije IL-1β citokina i proteina tesnih međućelijskih veza (Tjp-1), preventivni i terapijski tretman su se pokazali kao najefikasniji u primenjenim uslovima. S druge strane, kontinuirano lečenje nije uticalo na intenzitet oštećenja tkiva. Uzimajući u obzir ove rezultate, razmotreni su mogući mehanizmi koji stoje iza zaštitnog delovanja imunostimulišućih probiotičkih bakterija u slučaju oštećenja mukozne barijere.

Ključne reči: kolitis, *Limosilactobacillus fermentum*, probiotik, pro-inflamatorni citokini, lipidna peroksidacija