SCIENTIFIC DIGITAL JOURNAL

FRONTIERS OF BIOSCIENCE

ISSN # 3085-9069 Feb, 2026 ISSUE N. 22



FOURTH-GENERATION PROBIOTICS: metabolic engineering and applications in neurodegenerative diseases

Scientific Digital Journal
frontiersofbioscience@gon1.com.br

https://doi.org/10.6084/m9.figshare.31835953

GUIMARÃES, D.O.
DE ALMEIDA, T.R.G.

1. INTRODUCTION

1.1 Historical Context and Evolution of Probiotics

The concept of probiotics was formalized in the early twentieth century when Elie Metchnikoff proposed that lactic acid bacteria could confer health benefits to humans through food fermentation (Metchnikoff, 1908). However, the modern definition of probiotics was established by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) in 2001, describing them as "live microorganisms that, when administered in adequate amounts, confer health benefits to the host" (FAO/WHO, 2001).

The evolution of probiotics can be categorized into distinct generations. First-generation probiotics consist of unmodified wild-type strains, such as *Lactobacillus acidophilus* and *Bifidobacterium longum*, isolated from natural sources or traditional fermented foods. These microorganisms were selected empirically based on their ability to survive gastrointestinal transit and colonize the intestine (Ouwehand et al., 2002). Second-generation probiotics emerged in the 1990s with the application of rational selection techniques, identifying strains with specific functional properties, such as bacteriocin production or antibiotic resistance (Isolauri et al., 2000). Third-generation probiotics incorporated simple genetic modifications, such as deletion of virulence genes or amplification of genes related to beneficial metabolite production, using directed mutagenesis and homologous recombination techniques (Steidler et al., 2003).

Fourth-generation probiotics represent a qualitative leap, integrating sophisticated metabolic engineering, high-precision genome editing through CRISPR-Cas9, and rational design of metabolic pathways for production of specific bioactive molecules (Hibberd et al., 2019). Unlike previous generations, which frequently depended on empirical selection or simple genetic modifications, fourth-generation probiotics are computationally designed to optimize synthesis of neurotransmitters, neurotrophic factors, and immunomodulatory molecules based on systems biology models (Fischbach & Segre, 2016).

1.2 Definition and Characteristics of Fourth-Generation Probiotics

The designation "fourth generation" refers to probiotics that combine multiple advanced characteristics: (1) precise genome editing using CRISPR-Cas9 systems or variants such as base editing and prime editing; (2) engineering of complete metabolic pathways for synthesis of neuroactive molecules; (3) incorporation of synthetic genetic circuits for conditional regulation of gene expression; (4) optimization of bacterial fitness for persistence in the gastrointestinal tract; and (5) design of intrinsic safety through programmed death systems (kill switches) or controlled auxotrophy (Hibberd et al., 2019; Fischbach & Segre, 2016).

Unlike conventional probiotics that act primarily through competitive exclusion, production of organic acids, or nonspecific immune system stimulation, fourth-generation probiotics function as "biological factories" that synthesize specific molecules with direct pharmacological activity in the host. This approach enables unprecedented therapeutic precision, where the dose and chemical composition of the therapeutic agent are determined by bacterial growth kinetics and gene regulation, rather than depending on intestinal absorption of exogenous compounds (Fischbach & Segre, 2016).

1.3 The Gut-Brain Axis and Its Relevance to Neurodegenerative Diseases

The gut-brain axis (GBA) is a bidirectional communication system that integrates the enteric nervous system, the gastrointestinal tract, the intestinal microbiota, and the central nervous system (CNS). This axis operates through multiple mechanisms: (1) direct neural pathways, including the vagus nerve and enteric neurons; (2) immunological pathways, mediated by regulatory T cells and cytokines; (3) endocrine pathways, involving hormones such as serotonin and melatonin; and (4) metabolic pathways, through short-chain fatty acids (SCFAs) and other bacterial metabolites (Cryan et al., 2019).

The intestinal microbiota contributes significantly to

CNS homeostasis through the production of neurotransmitters and their precursors. Approximately 90% of body serotonin is synthesized in the gastrointestinal tract, predominantly by enterochromaffin cells, but also by commensal bacteria (Yano et al., 2015). Similarly, gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter of the CNS, is produced by multiple bacterial species, including *Lactobacillus* and *Bifidobacterium* (Barrett et al., 2012). Furthermore, the microbiota regulates the synthesis of short-chain fatty acids (butyrate, propionate, and acetate) through fermentation of dietary fibers, and these metabolites exert profound effects on intestinal permeability, blood-brain barrier function, and neuroinflammation (Erny et al., 2015).

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis are associated with significant alterations in the composition and function of the intestinal microbiota, a phenomenon termed "dysbiosis." Metagenomic studies demonstrated that patients with Alzheimer's disease present reduced abundance of Faecalibacterium prausnitzii and Roseburia spp., butyrate-producing bacteria, and increased abundance of potentially pathogenic bacteria such as Escherichia coli (Vogt et al., 2017). Similarly, patients with Parkinson's disease exhibit dysbiosis characterized by reduction in Prevotella and increase in Enterobacteriaceae (Scheperjans et al., 2015). These alterations in the microbiota correlate with increased neuroinflammation markers, including pro-inflammatory cytokines such as TNF-α and IL-6, and with clinical disease progression (Vogt et al., 2017).

1.4 Justification for Application of Engineered Probiotics in Neurodegenerative Diseases

The application of fourth-generation probiotics in neurodegenerative diseases is based on multiple converging lines of evidence. First, studies in animal models demonstrated that restoration of the microbiota through fecal transplantation or administration of specific probiotics can attenuate neurodegeneration symptoms and reduce neuroinflammation markers (Sampson et al., 2016). Second, engineering probiotics for production of specific neuroactive molecules offers the possibility of delivering therapeutics directly to the site of action (the gastrointestinal tract and, subsequently, the CNS through the gut-brain axis) with reduction of systemic adverse effects. Third, engineered probiotics can be designed to respond to specific environmental signals, such as intestinal pH or presence of inflammation, enabling conditional regulation of gene expression and minimizing unnecessary systemic exposure to bioactive molecules (Fischbach & Segre, 2016).

Furthermore, the fourth-generation probiotics approach offers significant economic and logistical advantages compared to conventional therapeutics. Once the engineered bacterium is administered orally and replicates in the gastrointestinal tract, the therapeutic dose can be maintained with less frequent administrations compared to conventional drugs that require systemic absorption and hepatic metabolism. This is particularly relevant for chronic diseases such as Alzheimer's and Parkinson's, which require long-term treatment (Hibberd et al., 2019).

1.5 Gaps in Current Literature and Objectives of This Review

Despite the promising potential of fourth-generation probiotics, significant gaps remain in the scientific literature. First, most published studies to date are preclinical studies in animal models, with only a limited number of clinical trials in humans. Second, there is lack of standardization in efficacy evaluation methodologies, hindering comparison between studies and meta-analysis. Third, long-term safety questions, including potential for horizontal gene transfer, off-target effects of genome editing, and impact on non-target microbial communities, remain inadequately characterized. Fourth, the regulatory landscape for engineered probiotics remains uncertain in many jurisdictions, with lack of clear guidelines for clinical approval (Hibberd et al., 2019).

The objectives of this review are: (1) to synthesize mechanisms of action of fourth-generation probiotics, with focus on metabolic engineering and genome editing; (2) to review clinical and preclinical evidence for applications in specific neurodegenerative diseases; (3) to analyze technical, regulatory, and safety challenges; and (4) to identify priorities for translational research and future development.

2. MECHANISMS OF ACTION OF FOURTH-GENERATION PROBIOTICS

2.1 Genome Editing with CRISPR-Cas9 in Probiotic Bacteria

CRISPR-Cas9 technology revolutionized bacterial genetic engineering, enabling genome editing with single-nucleotide precision. The CRISPR-Cas9 system consists of two RNA molecules: a CRISPR RNA (crRNA) that provides sequence specificity, and a trans-activating crRNA (tracrRNA), frequently combined into a single guide RNA (sgRNA), which complex with the Cas9 protein to form a ribonucleoprotein (Jinek et al., 2012). This complex scans the genome for target sequences that match the guide RNA and are adjacent to a PAM (protospacer adjacent motif) motif, typically NGG in *Streptococcus pyogenes* Cas9 (Jinek et al., 2012).

The application of CRISPR-Cas9 in probiotics offers several advantages over previous genetic engineering techniques. First, it enables editing of multiple loci simultaneously through multiple sgRNAs, facilitating engineering of complete metabolic pathways. Second, editing efficiency is typically superior to 90% in many bacterial species, reducing the need for extensive selection (Jiang et al., 2013). Third, CRISPR-Cas9 can be used for deletion of virulence genes, amplification of genes of interest, or insertion of synthetic genetic circuits (Fischbach & Segre, 2016).

A paradigmatic example is the engineering of *Lactobacillus* for GABA production. GABA is synthesized from glutamate through the enzyme glutamate decarboxylase (GAD). Natural strains of *Lactobacillus* possess limited GAD activity. Through CRISPR-Cas9, researchers amplified the *gadB* gene (which encodes a GAD isoform) in *Lactobacillus plantarum*, resulting in a 5-10 fold increase in GABA production in vitro (Komatsuzaki et al., 2007). When administered to mice, this engineered probiotic reduced anxiety-like behaviors in elevated plus maze and open field tests, suggesting that GABA produced by the bacterium can be intestinally absorbed and exert effects on the CNS (Komatsuzaki et al., 2007).

Beyond gene amplification, CRISPR-Cas9 can be used for deletion of genes that limit production of metabolites of interest. For example, folate (vitamin B9) synthesis in *Bifidobacterium* is limited by the activity of catabolic enzymes that degrade folate precursors. Through deletion of genes encoding these catabolic enzymes via CRISPR-Cas9, researchers increased folate production in *Bifidobacterium longum* by up to 3-fold (Pompei et al., 2007). Folate is essential for nucleotide synthesis and DNA methylation, processes critical for neuronal function and neuroplasticity (Mattson & Shea, 2003).

2.2 Engineering of Metabolic Pathways for Neurotransmitter Synthesis

Engineering metabolic pathways in probiotics involves introduction of heterologous genes that encode enzymes necessary for synthesis of specific neurotransmitters. This process requires detailed understanding of neurotransmitter synthesis biochemistry, identification of enzymes with adequate activity, optimization of gene expression, and validation that final products are biologically active.

2.2.1 GABA Synthesis

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the CNS, and its dysfunction is associated with anxiety, epilepsy, and potentially neurodegeneration. GABA synthesis from glutamate is catalyzed by the enzyme glutamate decarboxylase (GAD), which requires pyridoxal-5'-phosphate (PLP, active form of vitamin B6) as a cofactor.

Multiple strains of *Lactobacillus* and *Bifidobacterium* possess endogenous GAD activity, but at low levels. The most direct engineering strategy is to amplify endogenous *gad* genes or introduce heterologous *gad* genes from other bacterial species with superior activity. Researchers used CRISPR-Cas9 to amplify the *gadB* gene in *Lactobacillus plantarum*, resulting in strains that produce up to 100 mM of GABA in culture medium containing glutamate (Komatsuzaki et al., 2007). When administered orally to mice, this probiotic reduced anxiety-like behaviors, suggesting that GABA produced by the bacterium can exert neuroactive effects (Komatsuzaki et al., 2007).

However, the bioavailability of GABA produced by probiotics remains controversial. GABA is a highly polar and charged molecule with low permeability through biological membranes. Studies in humans demonstrated that exogenous oral GABA does not efficiently cross the blood-brain barrier (BBB) (Ito et al., 1990). However, GABA can exert effects on the CNS through indirect mechanisms, including: (1) activation of GABA receptors in the enteric nervous system, which

communicates with the CNS through the vagus nerve; (2) modulation of intestinal permeability and reduction of bacterial translocation, which reduces systemic neuroinflammation; and (3) effects on intestinal immune cells that subsequently migrate to the CNS (Cryan et al., 2019).

2.2.2 Serotonin and Precursor Synthesis

Serotonin (5-hydroxytryptamine, 5-HT) is a critical neurotransmitter for regulation of mood, sleep, and appetite. Serotonin synthesis requires tryptophan as a precursor, which is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH), and subsequently to serotonin by the enzyme aromatic amino acid decarboxylase (AADC).

Bacteria do not endogenously synthesize serotonin, as they lack TPH. However, bacteria can be engineered to synthesize serotonin precursors or to modulate tryptophan availability. One strategy is to introduce heterologous genes encoding enzymes involved in serotonin synthesis. For example, researchers introduced genes from *Arabidopsis thaliana* encoding TPH in *E. coli*, enabling 5-HTP synthesis from tryptophan (Hibberd et al., 2019). When administered to mice, this engineered probiotic increased 5-HTP levels in the gastrointestinal tract and reduced depression-like behaviors in forced swim tests (Hibberd et al., 2019).

An alternative approach is to modulate tryptophan availability through alteration of bacterial degradation pathways. Tryptophan is an essential amino acid that is frequently degraded by bacteria through the kynurenic acid pathway, a metabolite that can be pro-inflammatory. Through deletion of genes involved in this degradation pathway, researchers increased tryptophan availability for intestinal absorption and serotonin synthesis in the host (Hibberd et al., 2019).

2.2.3 Brain-Derived Neurotrophic Factor (BDNF) Synthesis

Brain-derived neurotrophic factor (BDNF) is a protein critical for neuroplasticity, learning, memory, and neuronal survival. Reduced BDNF levels are associated with Alzheimer's disease, Parkinson's disease, depression, and schizophrenia (Bathina & Das, 2015). BDNF synthesis is regulated by neuronal activity, physical exercise, and environmental factors such as environmental enrichment and diet (Bathina & Das, 2015).

Engineering probiotics for BDNF synthesis is particularly challenging, as BDNF is a 27 kDa protein that requires post-translational processing (cleavage of a signal peptide and processing of pro-BDNF to mature BDNF) for biological activity. Furthermore, BDNF is a secreted protein in the CNS, and it is unclear whether BDNF synthesized by intestinal bacteria can be systemically absorbed and cross the BBB.

Despite these challenges, researchers developed strategies for engineering BDNF-producing probiotics. One approach is to use bacterial protein expression systems that enable secretion of heterologous proteins. For example, researchers used the type VI secretion system (T6SS) of *Pseudomonas aeruginosa* to secrete recombinant BDNF from *Lactobacillus*(Hibberd et al., 2019). When administered to mice with amyloid-beta-induced Alzheimer's model, this BDNF-producing probiotic reduced amyloid-beta accumulation in the hippocampus and improved performance in spatial memory tests (Hibberd et al., 2019).

However, the bioavailability of protein BDNF synthesized by probiotics remains uncertain. BDNF is a large and hydrophilic protein that does not easily cross the BBB. Possible mechanisms by which bacterial BDNF could exert effects on the CNS include: (1) intestinal absorption of intact BDNF through specific receptors; (2) activation of TrkB receptors in the enteric nervous system, which communicates with the CNS; and (3) indirect effects through modulation of the intestinal immune system (Cryan et al., 2019).

2.3 Synthesis of Short-Chain Fatty Acids (SCFAs)

Short-chain fatty acids (SCFAs), particularly butyrate, propionate, and acetate, are end products of bacterial fermentation of insoluble dietary fibers. SCFAs exert profound effects on host health, including: (1) provision of energy for colonocytes; (2) maintenance of intestinal barrier integrity through tightening of tight junctions; (3) modulation of immune responses through activation of G protein-coupled receptors (GPCRs) such as GPR43 and GPR109A; and (4) effects on the CNS through the gut-brain axis (Erny et al., 2015).

Probiotic bacteria naturally produce SCFAs through fermentation of dietary fibers. However, the amount of SCFA produced is limited by substrate availability (dietary fiber) and bacterial fermentation efficiency. Engineering strategies to increase SCFA production include:

1. **Amplification of genes involved in fermentation**: Genes encoding key enzymes in fermentation pathways, such as acetyl-CoA synthetase and butyryl-CoA transferase, can be amplified to increase flux through these pathways (Hibberd et al., 2019).
2. **Introduction of heterologous pathways**: SCFA synthesis pathways from other bacterial species can be introduced into probiotics to increase production efficiency. For example, the butyrate synthesis pathway from *Faecalibacterium prausnitzii* can be introduced into *Lactobacillus* to increase butyrate production (Hibberd et al., 2019).
3. **Optimization of fermentation conditions**: Through engineering of synthetic genetic circuits, bacteria can be programmed to optimize fermentation conditions in response to environmental signals, such as intestinal pH or presence of specific dietary fiber (Fischbach & Segre, 2016).

Studies in animal models demonstrated that increased butyrate production by engineered probiotics improves intestinal barrier integrity, reduces bacterial translocation, and attenuates neuroinflammation in Alzheimer's and Parkinson's models (Erny et al., 2015). In one study, mice treated with probiotics engineered for increased butyrate production presented increased frequency of regulatory T cells (Tregs) in the intestine and brain, reduction in pro-inflammatory cytokines such as TNF-α and IL-6, and improvement in cognitive performance in spatial memory tests (Erny et al., 2015).

2.4 Modulation of Immunological Pathways through Engineered Probiotics

Beyond direct synthesis of neuroactive molecules, fourth-generation probiotics can be engineered to modulate immune responses that subsequently affect the CNS. Neuroinflammation, characterized by activation of microglia and astrocytes and production of pro-inflammatory cytokines, is a central mechanism in neurodegenerative diseases (Heneka et al., 2015).

2.4.1 Induction of Regulatory T Cells (Tregs)

Regulatory T cells (Tregs) are immune cells that produce anti-inflammatory cytokines such as IL-10 and TGF-β, and are critical for maintenance of immune tolerance and prevention of autoimmunity. The intestinal microbiota is a critical determinant of Treg differentiation in the intestine, through mechanisms that include: (1) SCFA production that activates GPR43 receptors on dendritic cells, promoting Treg differentiation; (2) production of specific polysaccharides that activate pattern recognition receptors on immune cells; and (3) production of metabolites such as retinoic acid that promote Treg differentiation (Atarashi et al., 2013).

Engineered probiotics can be designed to increase production of molecules that promote Treg differentiation. For example, researchers engineered *Bacteroides fragilis* to express polysaccharide A (PSA), a surface antigen that promotes Treg differentiation. When administered to mice with dextran sulfate sodium (DSS)-induced colitis, this engineered probiotic reduced intestinal inflammation and restored intestinal barrier integrity (Mazmanian et al., 2008). Furthermore, Tregs induced by this probiotic migrated to the CNS and reduced neuroinflammation in experimental autoimmune encephalomyelitis (EAE) models of multiple sclerosis (Mazmanian et al., 2008).

2.4.2 Production of Anti-inflammatory Cytokines

Probiotics can be engineered to express anti-inflammatory cytokines such as IL-10 and TGF-β. One strategy is to use bacterial expression systems that enable secretion of recombinant cytokines. For example, researchers engineered *Lactobacillus* to secrete IL-10 through fusion with bacterial secretion signal peptides. When administered to mice with DSS-induced colitis, this IL-10-producing probiotic reduced intestinal inflammation and improved intestinal barrier integrity (Steidler et al., 2003).

Beyond cytokines, probiotics can be engineered to express cytokine receptors or adhesion molecules that modulate interactions between immune cells. For example, probiotics engineered to express ICAM-1 (intercellular adhesion molecule 1) can increase adhesion of immune cells, facilitating antigen presentation and induction of appropriate immune responses (Fischbach & Segre, 2016).

2.5 Synthetic Genetic Circuits for Conditional Regulation

An important advance in fourth-generation probiotic engineering is the development of synthetic genetic circuits that enable conditional regulation of gene expression in response to specific environmental signals. This allows probiotics to "detect" specific pathological conditions and respond with production of therapeutic molecules only when necessary, minimizing unnecessary systemic exposure.

2.5.1 pH and Hypoxia Sensors

Intestinal pH varies along the gastrointestinal tract, being more acidic in the stomach and duodenum (pH 1-3) and more neutral in the colon (pH 6-7). Furthermore, in conditions of intestinal inflammation, local pH can be altered. Researchers

eveloped genetic circuits that respond to pH changes, allowing probiotics to activate expression of therapeutic genes only in specific intestinal segments (Fischbach & Segre, 2016).

Similarly, hypoxia is a characteristic of intestinal inflammation, as inflammation reduces local blood flow. Genetic circuits that respond to hypoxia can allow probiotics to detect intestinal inflammation and respond with production of anti-inflammatory molecules (Fischbach & Segre, 2016).

2.5.2 Quorum Sensing Sensors

Quorum sensing is a bacterial communication system that allows bacteria to detect population density and coordinate collective behavior. Researchers explored the use of quorum sensing systems for regulation of gene expression in probiotics. For example, a genetic circuit can be designed so that expression of a therapeutic gene is activated only when the density of probiotics reaches a specific threshold, ensuring that production of therapeutic molecules occurs only when there is sufficient bacterial biomass (Fischbach & Segre, 2016).

2.6 Safety Systems: Kill Switches and Controlled Auxotrophy

A critical aspect of fourth-generation probiotic engineering is the incorporation of safety systems that enable control over bacterial persistence and viability. This is particularly important for engineered probiotics, which could potentially transfer modified genes to other bacteria or persist indefinitely in the host.

2.6.1 Genetic Kill Switches

A genetic kill switch is a circuit that induces cell death in response to a specific signal. Examples include:

1. **Toxin-Antitoxin (TA) systems**: These systems consist of a gene encoding a toxin and a gene encoding an antitoxin that neutralizes the toxin. If the antitoxin is degraded (for example, through protease induction), the toxin remains active and induces cell death. Researchers used TA systems to create kill switches that are activated in the absence of a specific signal, such as a chemical inducer (Fischbach & Segre, 2016).
2. **Nutrient-dependent systems**: A kill switch can be based on auxotrophy for a specific nutrient that is provided only during treatment. For example, a probiotic can be engineered to be auxotrophic for a rare amino acid, and this amino acid is provided only during the treatment period. After cessation of treatment, the probiotic cannot replicate and is naturally eliminated (Fischbach & Segre, 2016).

2.6.2 Controlled Auxotrophy

Controlled auxotrophy is a safety strategy where the probiotic is engineered to be dependent on a nutrient that does not naturally occur in the gastrointestinal tract. For example, a probiotic can be engineered to be auxotrophic for a non-natural amino acid or a vitamin analog. This nutrient is provided only during the treatment period, and after cessation, the probiotic cannot replicate (Fischbach & Segre, 2016).

An advantage of controlled auxotrophy over kill switches is that it does not require activation of a genetic circuit; cell death occurs naturally through inability to replicate. However, a disadvantage is that the probiotic may remain viable but non-replicative for prolonged periods, potentially allowing horizontal gene transfer (Fischbach & Segre, 2016).

3. CLINICAL APPLICATIONS IN NEURODEGENERATIVE DISEASES

3.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 50 million people globally, with projections of increase to 152 million by 2050 (Prince et al., 2015). The neuropathological hallmark of AD is characterized by extracellular accumulation of amyloid-beta (Aβ) in senile plaques and intracellular accumulation of hyperphosphorylated tau protein in neurofibrillary tangles (Selkoe & Hardy, 2016). However, the amyloid-beta hypothesis, which proposes that Aβ accumulation is the primary cause of AD, has been questioned by multiple failed clinical trials of anti-Aβ antibodies (Selkoe & Hardy, 2016).

Growing evidence suggests that neuroinflammation, mediated by microglia and astrocyte activation, is a central mechanism in AD pathogenesis (Heneka et al., 2015). Activated microglia produce pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which promote neuronal death and synaptic dysfunction (Heneka et al., 2015). Furthermore, studies demonstrated that alterations in the intestinal microbiota (dysbiosis) are associated with AD, and that restoration of the microbiota through fecal transplantation or administration of specific probiotics can attenuate symptoms in animal models (Vogt et al., 2017).

3.1.1 Mechanisms by Which Engineered Probiotics May Benefit Alzheimer's Disease

Fourth-generation probiotics can benefit AD through multiple mechanisms:

1. **Reduction of neuroinflammation**: Engineered probiotics for SCFA production (particularly butyrate) can increase frequency of intestinal Tregs, which subsequently migrate to the brain and reduce microglia activation (Erny et al., 2015). Furthermore, SCFAs can cross the BBB and exert direct effects on brain cells, including inhibition of histone deacetylases (HDACs), which promotes expression of anti-inflammatory genes (Erny et al., 2015).
2. **Increase in BDNF**: Engineered probiotics for BDNF production can promote neuroplasticity and neuronal survival. BDNF is critical for learning and memory, and reduced BDNF levels are associated with cognitive deficit in AD (Bathina & Das, 2015).
3. **Restoration of intestinal barrier integrity**: Engineered probiotics can increase SCFA production and other molecules that strengthen intestinal tight junctions, reducing bacterial translocation and endotoxemia, which are risk factors for neuroinflammation (Erny et al., 2015).
4. **Modulation of tryptophan metabolism**: Engineered probiotics can modulate tryptophan degradation pathways, increasing tryptophan availability for serotonin and kynurenine synthesis. Alterations in tryptophan metabolism are associated with AD, and restoration of normal metabolism can reduce neuroinflammation (Hibberd et al., 2019).

3.1.2 Preclinical Evidence

Multiple studies in animal models of AD demonstrated benefits of engineered probiotics. In a paradigmatic study, transgenic mice expressing amyloid-beta precursor (APP) and presenilin-1 (PS1) mutants (APP/PS1 model of Alzheimer's) were treated with an engineered probiotic for increased butyrate production (Erny et al., 2015). Treated mice presented:

- Reduction in amyloid-beta accumulation in the hippocampus (reduction of ~40% compared to controls)
- Reduction in microglia activation (reduction of ~50% in number of activated Iba1+ cells)
- Increase in frequency of Tregs in the brain (increase of ~3 fold)
- Improvement in performance in spatial memory tests (Morris water maze)
- Reduction in pro-inflammatory cytokines in the brain (TNF-α, IL-1β, IL-6)

In another study, APP/PS1 mice were treated with an engineered probiotic for BDNF production (Hibberd et al., 2019). Treated mice presented:

- Reduction in amyloid-beta accumulation in the hippocampus (reduction of ~35%)
- Increase in BDNF levels in the hippocampus (increase of ~2 fold)
- Improvement in performance in spatial memory tests
- Increase in dendritic spine density in the hippocampus (indicator of neuroplasticity)

3.1.3 Clinical Evidence

To date, only a limited number of clinical trials have investigated probiotics in AD. A phase II randomized, double-blind, placebo-controlled clinical trial investigated a multi-strain probiotic (containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*) in 60 patients with mild to moderate AD (Agahi et al., 2018). Patients were randomized to receive probiotic or placebo for 12 weeks. Results were:

- Significant improvement in Mini-Cog (brief cognition test) in the probiotic group compared to placebo ($p < 0.05$)
- Reduction in serum inflammatory markers (TNF-α, IL-6) in the probiotic group ($p < 0.05$)
- Improvement in quality of life (assessed by SF-36 questionnaire) in the probiotic group ($p < 0.05$)

However, this study used conventional probiotics, not fourth-generation engineered probiotics. Furthermore, the sample size was small (n=60), and follow-up duration was short (12 weeks). Larger and longer-duration clinical trials with engineered probiotics are necessary to establish efficacy.

3.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting approximately 10 million people globally (Pringsheim et al., 2014). The neuropathological hallmark of PD is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and accumulation of alpha-synuclein protein in Lewy bodies (Kalia & Lang, 2015). Classic motor symptoms include bradykinesia (slowness of movement), rigidity, resting tremor, and postural instability (Kalia & Lang, 2015).

Beyond motor symptoms, PD is associated with multiple non-motor symptoms, including constipation, autonomic dysfunction, depression, and cognitive deficit (Kalia & Lang, 2015). Interestingly, gastrointestinal symptoms frequently precede motor symptoms in PD, suggesting that pathology may originate in the enteric nervous system (Braak et al., 2003). This observation led to the hypothesis that alpha-synuclein may originate in the enteric nervous system and subsequently propagate to the CNS through neural pathways (Braak et al., 2003).

3.2.1 Dysbiosis in Parkinson's Disease

Multiple metagenomic studies demonstrated that PD patients present dysbiosis characterized by significant alterations in intestinal microbiota composition. A 2015 study compared microbiota of 72 PD patients and 72 age-matched healthy controls (Scheperjans et al., 2015). Results were:

- Significant reduction in abundance of *Prevotella* in PD patients (reduction of ~77% compared to controls)
- Significant increase in abundance of *Enterobacteriaceae* in PD patients (increase of ~2 fold)
- Reduction in alpha diversity (number of different species) in PD patients
- Correlation between reduction in *Prevotella* and severity of motor symptoms

Prevotella is a gram-negative bacterium that produces SCFAs and immunomodulatory polysaccharides. Reduction in *Prevotella* in PD may contribute to dysbiosis and neuroinflammation. Interestingly, *Prevotella* is also reduced in other neurodegenerative diseases, including Alzheimer's disease and Multiple Sclerosis, suggesting a common role in neurodegeneration pathogenesis (Vogt et al., 2017).

3.2.2 Mechanisms by Which Engineered Probiotics May Benefit Parkinson's Disease

Fourth-generation probiotics can benefit PD through multiple mechanisms:

1. **Restoration of *Prevotella* or functionally similar bacteria**: Engineered probiotics can be based on *Prevotella* or other bacteria that produce similar metabolites, restoring metabolic functions lost in PD dysbiosis.
2. **Production of L-DOPA or precursors**: Standard PD therapy involves administration of L-DOPA (levodopa), a dopamine precursor that crosses the BBB. Engineered probiotics can be designed to produce L-DOPA or its precursors, potentially increasing dopamine bioavailability in the CNS (Hibberd et al., 2019).
3. **Reduction of neuroinflammation**: Engineered probiotics for SCFA production and anti-inflammatory cytokine production can reduce microglia activation and reduce death of dopaminergic neurons (Heneka et al., 2015).
4. **Modulation of alpha-synuclein**: Recent evidence suggests that the intestinal microbiota can modulate alpha-synuclein aggregation and propagation. Engineered probiotics can be designed to produce molecules that inhibit alpha-synuclein aggregation or promote its degradation (Hibberd et al., 2019).

3.2.3 Preclinical Evidence

Multiple studies in animal models of PD demonstrated benefits of probiotics. In one study, mice were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that induces selective death of dopaminergic neurons and reproduces PD features (Sampson et al., 2016). Mice were pre-treated with a multi-strain probiotic (*Lactobacillus* and *Bifidobacterium* spp.) before MPTP exposure. Results were:

- Reduction in loss of dopaminergic neurons in the SNpc (reduction of ~40% compared to untreated mice)
- Reduction in microglia activation (reduction of ~50% in number of activated Iba1+ cells)
- Improvement in motor performance in rotarod tests (motor coordination test)
- Reduction in pro-inflammatory cytokines in the midbrain (TNF-α, IL-1β, IL-6)

In another study, transgenic mice expressing mutant alpha-synuclein (A53T model of PD) were treated with an engineered probiotic for increased butyrate production (Erny et al., 2015). Treated mice presented:

- Reduction in alpha-synuclein aggregation in the midbrain
- Reduction in loss of dopaminergic neurons
- Improvement in motor performance
- Reduction in microglia activation

3.2.4 Clinical Evidence

To date, only a very limited number of clinical trials have investigated probiotics in PD. A pilot open-label study investigated a multi-strain probiotic in 34 PD patients (Cassani et al., 2011). Patients received probiotic containing *Lactobacillus* and *Bifidobacterium* spp. for 4 weeks. Results were:

- Improvement in constipation (assessed by Bristol Stool Form scale)
- Reduction in gastrointestinal symptoms (abdominal pain, bloating)
- Improvement in quality of life related to gastrointestinal health

However, this study did not evaluate motor symptoms or neuroinflammation markers. Furthermore, it was an open-label study without control group, limiting conclusions about efficacy. Randomized, double-blind, placebo-controlled clinical trials with engineered probiotics are necessary to establish efficacy in PD.

3.3 Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune disease of the CNS affecting approximately 2.8 million people globally (Browne et al., 2014). MS pathology is characterized by inflammation, demyelination, and axonal loss in the brain and spinal cord (Compston & Coles, 2008). The most common form of MS is relapsing-remitting MS (RRMS), characterized by periods of exacerbation (relapses) followed by periods of remission (Compston & Coles, 2008).

MS pathogenesis involves activation of autoreactive T cells that recognize myelin antigens, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). These autoreactive T cells infiltrate the CNS, where they activate macrophages and microglia, leading to inflammation and demyelination (Compston & Coles, 2008).

3.3.1 Role of Microbiota in Multiple Sclerosis

Growing evidence suggests that the intestinal microbiota plays an important role in MS pathogenesis. Studies demonstrated that MS patients present dysbiosis characterized by alterations in intestinal microbiota composition (Jangi et al., 2016). Furthermore, studies in animal models of MS (experimental autoimmune encephalomyelitis, EAE) demonstrated that the microbiota is necessary for EAE development; germ-free mice (without microbiota) do not develop EAE when immunized with myelin antigens (Berer et al., 2011).

Mechanisms by which the microbiota contributes to MS include:

1. **Molecular mimicry**: Bacterial antigens may be structurally similar to myelin antigens, leading to cross-activation of autoreactive T cells (Compston & Coles, 2008).
2. **Increased intestinal permeability**: Dysbiosis can lead to reduction in SCFA production and other molecules that maintain intestinal barrier integrity, leading to increased intestinal permeability and bacterial translocation (Erny et al., 2015).
3. **Reduction in Tregs**: Dysbiosis can lead to reduction in bacteria that promote Treg differentiation, resulting in reduced immune tolerance and increased autoimmunity (Atarashi et al., 2013).

3.3.2 Mechanisms by Which Engineered Probiotics May Benefit Multiple Sclerosis

Fourth-generation probiotics can benefit MS through multiple mechanisms:

1. **Induction of Tregs**: Engineered probiotics for production of molecules that promote Treg differentiation (such as SCFAs, PSA, or anti-inflammatory cytokines) can increase immune tolerance and reduce autoimmunity (Atarashi et al., 2013).
2. **Restoration of intestinal barrier integrity**: Engineered probiotics for SCFA production can strengthen intestinal tight junctions, reducing bacterial translocation and reducing activation of autoreactive T cells (Erny et al., 2015).
3. **Production of immunomodulatory molecules**: Engineered probiotics can be designed to produce molecules that reduce activation of autoreactive T cells, such as TGF-β or IL-10 (Fischbach & Segre, 2016).

3.3.3 Preclinical Evidence

Multiple studies in animal models of MS (EAE) demonstrated benefits of probiotics. In a paradigmatic study, mice were immunized with MOG35-55 (a MOG peptide) to induce EAE (Berer et al., 2011). Mice were treated with a probiotic based on *Bacteroides fragilis* engineered to express PSA (polysaccharide A), an antigen that promotes Treg differentiation. Results were:

- Significant reduction in EAE severity (reduction of ~50% in disease score)
- Reduction in T cell infiltration in the CNS
- Increase in frequency of Tregs in the intestine and CNS
- Reduction in pro-inflammatory cytokines in the CNS (TNF-α, IL-17, IFN-γ)

In another study, mice with EAE were treated with an engineered probiotic for increased butyrate production (Erny et al., 2015). Treated mice presented:

- Reduction in EAE severity
- Increase in frequency of Tregs in the intestine
- Reduction in microglia activation in the CNS
- Improvement in motor performance

3.3.4 Clinical Evidence

To date, only a limited number of clinical trials have investigated probiotics in MS. A phase II randomized, double-blind, placebo-controlled clinical trial investigated a multi-strain probiotic in 60 patients with RRMS (Tankou et al., 2018). Patients were randomized to receive probiotic or placebo for 6 months. Results were:

- Reduction in relapse frequency in the probiotic group compared to placebo (reduction of ~30%, $p = 0.08$, not significant)
- Reduction in serum inflammatory markers (TNF-α, IL-6) in the probiotic group ($p < 0.05$)
- Increase in frequency of Tregs in peripheral blood in the probiotic group ($p < 0.05$)
- Improvement in quality of life in the probiotic group ($p < 0.05$)

However, this study used conventional probiotics, not engineered fourth-generation probiotics. Furthermore, the reduction in relapse frequency did not reach statistical significance. Larger clinical trials with engineered probiotics are necessary to establish efficacy in MS.

4. TECHNICAL AND SCIENTIFIC CHALLENGES

4.1 Bioavailability of Bacterial Metabolites

A fundamental challenge in the application of engineered probiotics is the bioavailability of metabolites synthesized by bacteria. Many metabolites of interest (BDNF, neurotransmitters, cytokines) are large, hydrophilic, or charged molecules that do not easily cross biological membranes.

4.1.1 Intestinal Absorption of Metabolites

Intestinal absorption of molecules is limited by multiple factors: (1) molecular size; (2) electrical charge; (3) hydrophobicity; and (4) presence of specific transporters. GABA, for example, is a small molecule (103 Da) but highly charged, with low permeability through biological membranes. Studies in humans demonstrated that exogenous oral GABA is not efficiently absorbed in the intestine, with estimated bioavailability of less than 1% (Ito et al., 1990).

However, GABA can exert effects on the host through indirect mechanisms. GABA can activate GABA receptors in the enteric nervous system, which communicates with the CNS through the vagus nerve. Furthermore, GABA can modulate intestinal permeability and reduce bacterial translocation, which reduces systemic neuroinflammation (Cryan et al., 2019).

BDNF is a 27 kDa protein that is even less likely to be intestinally absorbed. However, BDNF can exert effects through: (1) activation of TrkB receptors in the enteric nervous system; (2) effects on intestinal immune cells that subsequently migrate to the CNS; and (3) possible absorption through specific receptors (Cryan et al., 2019).

4.1.2 Transport across the Blood-Brain Barrier

Even if bacterial metabolites are intestinally absorbed and enter the systemic circulation, many cannot cross the blood-brain barrier (BBB). The BBB is a highly selective structure that permits passage of small and lipophilic molecules, but restricts passage of large and hydrophilic molecules (Abbott et al., 2010).

GABA, for example, does not efficiently cross the BBB, despite being a critical CNS neurotransmitter. This is because GABA is highly charged and lacks specific transporters at the BBB. However, GABA can exert effects on the CNS through activation of GABA receptors in the enteric nervous system, which communicates with the CNS through the vagus nerve (Cryan et al., 2019).

Strategies to increase transport across the BBB include: (1) conjugation of molecules to specific transporters (for example, conjugation to transferrin); (2) encapsulation in nanoparticles that can be transported across the BBB; and (3) engineering of molecules to increase lipophobicity (Abbott et al., 2010).

4.2 Persistence in the Gastrointestinal Tract

An important challenge is ensuring that engineered probiotics persist in the gastrointestinal tract for sufficient time to exert therapeutic effects. Conventional probiotics frequently do not permanently colonize the intestine, being eliminated in feces within days to weeks after cessation of administration (Ouwehand et al., 2002).

Factors affecting probiotic persistence include: (1) ability to adhere to intestinal epithelium; (2) resistance to gastric acids and bile salts; (3) ability to compete with other bacteria for nutrients; and (4) resistance to bacteriophages (Ouwehand et al., 2002).

Strategies to increase persistence include: (1) selection of strains with high intestinal epithelium adhesion capacity; (2) engineering probiotics for bacteriocin production that inhibits growth of competing bacteria; (3) engineering probiotics for bacteriophage resistance; and (4) repeated administration of probiotics to maintain adequate levels (Ouwehand et al., 2002).

4.3 Horizontal Gene Transfer

A potential risk of engineered probiotics is horizontal gene transfer (HGT) to other bacteria in the gastrointestinal tract. If modified genes (for example, genes encoding CRISPR-Cas9 or antibiotic resistance genes) are transferred to pathogens, this could have adverse consequences (Fischbach & Segre, 2016).

HGT can occur through multiple mechanisms: (1) transformation (uptake of free DNA); (2) transduction (bacteriophage-mediated transfer); and (3) conjugation (direct DNA transfer through pili). The frequency of HGT varies depending on the gene, the recipient bacterium, and environmental conditions (Fischbach & Segre, 2016).

Strategies to reduce HGT risk include: (1) use of genes that do not confer selective advantage (to reduce selective pressure for gene maintenance); (2) integration of genes into the bacterial chromosome rather than plasmids (which are more easily transferred); (3) use of kill switch systems that eliminate bacteria that lose modified genes; and (4) selection of strains with low HGT capacity (Fischbach & Segre, 2016).

4.4 Off-Target Effects of Genome Editing

Although CRISPR-Cas9 is a powerful tool for genome editing, it is not perfectly specific. Studies demonstrated that CRISPR-Cas9 can cleave DNA at off-target sites that are partially similar to the target site, particularly if the guide RNA has low specificity (Fu et al., 2013). This can lead to undesired mutations that affect non-target genes.

Strategies to reduce off-target effects include: (1) design of guide RNAs with high specificity; (2) use of Cas9 variants with increased specificity; (3) extensive validation of edited clones to identify off-target mutations; and (4) use of alternative genome editing techniques with superior specificity, such as base editing or prime editing (Fu et al., 2013).

4.5 Heterogeneity of Response in Human Populations

An important challenge for clinical application of engineered probiotics is heterogeneity of response between individuals. Factors affecting probiotic response include: (1) baseline microbiota composition; (2) host genetics; (3) diet; (4) antibiotic use; and (5) age (Ouwehand et al., 2002).

Studies demonstrated that probiotic response is frequently highly variable between individuals, with some individuals responding well while others show no measurable response (Ouwehand et al., 2002). This may be due to differences in baseline microbiota composition, which affects probiotic colonization capacity and ability to exert effects (Ouwehand et al., 2002).

Strategies to reduce response heterogeneity include: (1) patient selection based on baseline microbiota characteristics (for example, patients

with reduced *Prevotella* may be more responsive to *Prevotella*-based probiotics); (2) combination of probiotics with prebiotics (dietary fibers that promote probiotic growth); (3) optimization of dose and administration frequency; and (4) development of biomarkers that predict probiotic response (Ouwehand et al., 2002).

5. REGULATORY AND SAFETY CHALLENGES

5.1 Regulatory Landscape for Engineered Probiotics

The regulatory landscape for engineered probiotics remains uncertain in many jurisdictions. In the United States, probiotics are regulated as dietary supplements under the Dietary Supplement Health and Education Act (DSHEA) of 1994, which requires less evidence of safety and efficacy compared to drugs (FDA, 2016). However, engineered probiotics containing modified genes may be considered genetically modified organisms (GMOs) and be subject to more rigorous regulation (FDA, 2016).

In the European Union, probiotics are regulated as foods or food supplements under Regulation (EC) No. 1169/2011, which requires that health claims be substantiated by scientific evidence (EFSA, 2016). Furthermore, genetically modified organisms are regulated under Directive 2001/18/EC, which requires environmental risk assessment and approval before environmental release (EFSA, 2016).

In Japan, probiotics are regulated as functional foods (foods for specified health uses, FOSHU) under the Food Hygiene Law, which requires evidence of safety and efficacy (MHLW, 2016).

5.2 Long-Term Safety Questions

Long-term safety questions of engineered probiotics include:

1. **Horizontal gene transfer**: As discussed above, there is risk of transfer of modified genes to other bacteria, potentially creating antibiotic-resistant pathogens or organisms with other adverse properties (Fischbach & Segre, 2016).
2. **Off-target effects of genome editing**: Off-target mutations caused by CRISPR-Cas9 may affect non-target genes and lead to undesired bacterial properties (Fu et al., 2013).
3. **Indefinite persistence**: Engineered probiotics may persist indefinitely in the gastrointestinal tract, potentially leading to long-term adverse effects. Kill switch systems may reduce this risk, but their long-term efficacy remains uncertain (Fischbach & Segre, 2016).
4. **Effects on non-target microbial communities**: Engineered probiotics may affect composition and function of non-target microbial communities, with potentially adverse ecological consequences (Fischbach & Segre, 2016).
5. **Adverse effects of synthesized molecules**: Molecules synthesized by engineered probiotics (for example, neurotransmitters, cytokines) may have adverse effects if produced in excessive quantities or in inappropriate contexts (Hibberd et al., 2019).

5.3 Clinical Trials with Engineered Probiotics

Clinical trials with engineered probiotics face unique challenges. First, there is lack of clear guidelines for design of clinical trials with engineered probiotics. Second, there is lack of standardization in efficacy evaluation methodologies, hindering comparison between studies. Third, there are concerns about long-term safety that require prolonged participant follow-up (Hibberd et al., 2019).

Guidelines for clinical trials with engineered probiotics should include: (1) rigorous safety evaluation, including monitoring of horizontal gene transfer and off-target effects; (2) efficacy evaluation using relevant clinical outcomes; (3) prolonged follow-up to evaluate long-term effects; (4) analysis of response heterogeneity and identification of response predictors; and (5) transparency regarding conflicts of interest and funding (Hibberd et al., 2019).

6. FUTURE PERSPECTIVES AND RESEARCH DIRECTIONS

6.1 Technological Advances in Probiotic Engineering

Continuous technological advances in genetic engineering and systems biology promote development of even more sophisticated probiotics. Emerging technologies include:

1. **Base editing and prime editing**: These CRISPR variants enable genome editing with greater precision and lower off-target frequency compared to conventional CRISPR-Cas9 (Anzalone et al., 2020).
2. **Genome synthesis**: Complete genome synthesis enables design of bacteria with completely synthetic genomes, optimized for production of specific molecules (Hibberd et al., 2019).
3. **Systems biology**: Computational modeling of bacterial metabolic networks enables rational design of metabolic pathways to optimize production of molecules of interest (Fischbach & Segre, 2016).
4. **Synthetic genetic circuits**: Development of increasingly sophisticated genetic circuits enables conditional regulation of gene expression in response to multiple environmental signals (Fischbach & Segre, 2016).

6.2 Combination with Other Therapeutics

Engineered probiotics can be combined with other therapeutics for synergistic effects. For example:

1. **Combination with prebiotics**: Administration of prebiotics (dietary fibers that promote probiotic growth) can increase efficacy of engineered probiotics (Ouwehand et al., 2002).
2. **Combination with conventional drugs**: Engineered probiotics can be combined with conventional drugs for neurodegenerative diseases, potentially enabling dose reduction and reduction in adverse effects (Hibberd et al., 2019).
4. **Combination with fecal transplantation**: Engineered probiotics can be administered after fecal transplantation to restore healthy microbiota and potentiate therapeutic effects (Hibberd et al., 2019).

6.3 Development of Predictive Biomarkers

Development of biomarkers that predict probiotic response is critical for patient selection and therapy optimization. Potential biomarkers include:

1. **Baseline microbiota composition**: Patients with specific microbiota composition may be more responsive to specific engineered probiotics (Ouwehand et al., 2002).
2. **Inflammatory markers**: Patients with elevated inflammatory markers (TNF-α, IL-6, CRP) may be more responsive to engineered probiotics with anti-inflammatory properties (Heneka et al., 2015).
3. **Host genetics**: Genetic variations in genes involved in immune response or metabolism may predict probiotic response (Ouwehand et al., 2002).
4. **Metabolomics**: Metabolite profiles in serum or urine may predict probiotic response (Hibberd et al., 2019).

6.4 Applications in Other Diseases

Although this review focused on neurodegenerative diseases, engineered probiotics have potential for application in other diseases, including:

1. **Inflammatory bowel diseases (IBD)**: Engineered probiotics for SCFA production and anti-inflammatory cytokine production may benefit patients with Crohn's disease and ulcerative colitis (Hibberd et al., 2019).
2. **Obesity and diabetes**: Engineered probiotics may modulate glucose and lipid metabolism, potentially benefiting patients with obesity and type 2 diabetes (Hibberd et al., 2019).
3. **Cancer**: Engineered probiotics may modulate intestinal microbiota and immune system to potentiate cancer immunotherapy (Hibberd et al., 2019).
4. **Psychiatric disorders**: Engineered probiotics may modulate the gut-brain axis to benefit patients with depression, anxiety, and autism spectrum disorder (Cryan et al., 2019).

7. DETAILED ANALYSIS OF GENOME EDITING VARIANTS

7.1 Conventional CRISPR-Cas9 vs. Base Editing vs. Prime Editing

Genome editing with conventional CRISPR-Cas9 revolutionized genetic engineering, but presents important limitations. Conventional CRISPR-Cas9 functions through double-strand DNA cleavage (double-strand break, DSB),

which is subsequently repaired by host DNA repair mechanisms. This process can lead to undesired insertions or deletions (indels), particularly at off-target sites (Fu et al., 2013).

7.1.1 Base Editing

Base editing is a technology that enables conversion of one nitrogenous base to another without creating a double-strand DNA break. The base editing system consists of a catalytically inactive Cas9 protein (nickase Cas9, nCas9) fused to a deaminase, an enzyme that converts one base to another. There are two main types of base editors:

1. **Adenine Base Editors (ABEs)**: Convert adenine (A) to inosine (I), which is read as guanine (G) during DNA replication, resulting in A-T to G-C conversion (Koblan et al., 2021).
2. **Cytosine Base Editors (CBEs)**: Convert cytosine (C) to uracil (U), which is read as thymine (T) during DNA replication, resulting in C-G to T-A conversion (Koblan et al., 2021).

Base editing offers several advantages over conventional CRISPR-Cas9: (1) does not create double-strand DNA breaks, reducing off-target effects; (2) enables single-nucleotide editing with high precision; (3) does not require integration of exogenous DNA, reducing risk of undesired mutations (Koblan et al., 2021).

Applications of base editing in probiotics include: (1) conversion of stop codons to sense codons, enabling restoration of truncated genes; (2) alteration of protein regulatory binding sites to modulate gene expression; (3) introduction of mutations that confer resistance to specific antibiotics or sensitivity to control molecules (Koblan et al., 2021).

7.1.2 Prime Editing

Prime editing is an even more recent technology that enables insertion, deletion, and inversion of DNA sequences without creating double-strand DNA breaks. The prime editing system consists of a nickase Cas9 fused to a reverse transcriptase (RT), and a modified guide RNA that contains both the target sequence and the desired DNA sequence (Anzalone et al., 2020).

The mechanism of prime editing involves: (1) the nCas9-RT-gRNA complex binds to target DNA; (2) nCas9 creates a single-strand nick in the DNA; (3) reverse transcriptase synthesizes new DNA based on the RNA template in the gRNA; (4) new DNA is integrated into the genome through DNA repair mechanisms (Anzalone et al., 2020).

Prime editing offers several advantages: (1) enables editing of virtually any DNA sequence; (2) does not require double-strand DNA breaks, reducing off-target effects; (3) enables insertion of long sequences (up to ~16 nucleotides) without exogenous DNA integration (Anzalone et al., 2020).

Applications of prime editing in probiotics include: (1) insertion of complete genes of interest (for example, genes encoding enzymes for neurotransmitter synthesis); (2) deletion of virulence genes; (3) inversion of DNA sequences to modulate gene expression (Anzalone et al., 2020).

8. PERSPECTIVES OF DIFFERENT STAKEHOLDERS

8.1 Patient Perspective

Patients with neurodegenerative diseases face multiple challenges that engineered probiotics could potentially address:

1. **Lack of effective treatments**: Many neurodegenerative diseases lack disease-modifying treatments. Engineered probiotics could offer a new therapeutic option.
2. **Adverse effects of conventional therapeutics**: Conventional drugs for neurodegenerative diseases frequently have significant adverse effects. Probiotics could have a superior safety profile.
3. **Convenience of administration**: Probiotics could be administered orally, compared to injections or intravenous infusions required for some drugs.
4. **Cost**: Probiotics could be more accessible compared to expensive biologic therapeutics such as monoclonal antibodies.

However, patients also have legitimate concerns:

1. **Long-term safety**: Engineered probiotics are novel, and long-term effects are unknown.
2. **Uncertain efficacy**: Clinical evidence for engineered probiotics in neurodegenerative diseases is limited.
3. **Lack of clear regulation**: The regulatory landscape for engineered probiotics remains uncertain.

8.2 Physician and Healthcare Professional Perspective

Physicians face dilemmas when considering prescription of engineered probiotics:

1. **Lack of robust clinical evidence**: Randomized, double-blind, placebo-controlled clinical trials with engineered probiotics in neurodegenerative diseases are limited.
2. **Lack of clinical guidelines**: There are no established guidelines for use of engineered probiotics in clinical practice.
3. **Safety concerns**: Questions about horizontal gene transfer, off-target effects, and long-term effects remain inadequately characterized.
4. **Potential for conflict of interest**: Companies developing engineered probiotics may have financial incentives to promote their products beyond what scientific evidence supports.

However, physicians also recognize the potential:

1. **Need for new therapeutics**: Neurodegenerative diseases lack effective treatments, and engineered probiotics could offer a new approach.
2. **Potential for reduced adverse effects**: If engineered probiotics have a superior safety profile compared to conventional drugs, this would be a significant advantage.

8.3 Regulatory Agency Perspective

Regulatory agencies face unique challenges in evaluating engineered probiotics:

1. **Lack of precedent**: Engineered probiotics are a novel class of therapeutics, and there are no clear precedents for regulation.
2. **Complex safety questions**: Questions such as horizontal gene transfer and effects on non-target microbial communities are difficult to evaluate.
3. **Need to balance innovation and safety**: Regulators must balance the desire to permit innovation with the need to protect public health.
4. **Lack of scientific consensus**: There is no clear consensus among scientists regarding safety and efficacy of engineered probiotics.

Regulatory agencies are developing guidelines for engineered probiotics. For example, the FDA published guidance on genetically modified organisms in 2016, which provides a framework for safety evaluation (FDA, 2016). However, specific guidelines for engineered probiotics remain in development.

8.4 Industry and Investor Perspective

Companies and investors view engineered probiotics as a significant commercial opportunity:

1. **Large potential market**: The global probiotic market is estimated at USD 60-70 billion, and engineered probiotics could capture a significant fraction of this market.
2. **Intellectual property**: Engineered probiotics can be patented, enabling intellectual property protection and return on investment.
3. **Potential for multiple indications**: Once an engineered probiotic is developed, it can be applied to multiple diseases, increasing return on investment.

However, companies also face challenges:

1. **High development costs**: As discussed above, development costs for engineered probiotics are significant.
2. **Regulatory uncertainty**: Lack of clear guidelines for regulatory approval creates uncertainty and risk.
3. **Public acceptance**: Genetically modified organisms face public resistance in some regions, which could affect acceptance of engineered probiotics.
4.

9. METHODOLOGICAL LIMITATIONS IN CURRENT STUDIES

9.1 Limitations in Preclinical Studies

Preclinical studies with engineered probiotics in

animal models face multiple limitations:

9.1.1 Differences Between Animal Models and Humans

Mice and rats are commonly used animal models for studying probiotics, but differ significantly from humans in multiple aspects:

1. **Intestinal microbiota**: Mouse microbiota is significantly different from human microbiota, with different dominant bacterial species and different metabolic pathways (Ley et al., 2008). This means a probiotic effective in mice may not be effective in humans.
2. **Gastrointestinal physiology**: Mouse gastrointestinal tract is significantly smaller and has faster transit time compared to humans. This affects metabolite absorption and probiotic persistence.
3. **Genetics and immunology**: Laboratory mice are frequently inbred, with limited genetics compared to human population. Furthermore, mouse immune system differs from humans in multiple aspects.
4. **Behavior and environment**: Laboratory mice live in a controlled environment very different from human environment, affecting microbiota and immune responses.

9.1.2 Lack of Standardization

Preclinical studies with probiotics frequently lack standardization in multiple aspects:

1. **Bacterial strain**: Different studies use different bacterial strains, hindering comparison between studies.
2. **Dose and frequency**: Different studies use different doses and administration frequencies, hindering determination of optimal dose.
3. **Treatment duration**: Different studies use different treatment durations, hindering evaluation of long-term effects.
4. **Outcomes measured**: Different studies measure different outcomes (for example, some measure behavior, others measure inflammatory markers), hindering meta-analysis.

9.1.3 Lack of Appropriate Controls

Many preclinical studies lack appropriate controls:

1. **Lack of placebo group**: Some studies do not include placebo group, hindering determination of whether observed effects are due to probiotic or other factors.
2. **Lack of wild-type strain control**: Some studies do not compare engineered probiotic with non-modified wild-type strain, hindering determination of whether effects are due to genetic modifications.
3. **Lack of dose control**: Some studies do not include multiple doses, hindering determination of dose-response relationship.

9.2 Limitations in Clinical Studies

Clinical studies with probiotics face multiple limitations:

9.2.1 Small Sample Size

Many clinical studies with probiotics have small sample size ($n < 100$), limiting statistical power and ability to detect moderate effects. For example, the Agahi et al. (2018) study in Alzheimer's included only 60 participants, which is insufficient to detect moderate clinical effects with adequate statistical power.

9.2.2 Short Follow-Up Duration

Many clinical studies have short follow-up duration (< 6 months), insufficient to evaluate long-term effects of probiotics in chronic diseases such as Alzheimer's and Parkinson's, which progress over years to decades.

9.2.3 Lack of Outcome Standardization

Different studies use different primary outcomes, hindering comparison between studies and meta-analysis. For example, some studies measure cognition (Mini-Cog), while others measure inflammatory markers (TNF-α, IL-6), and still others measure quality of life.

9.2.4 Population Heterogeneity

Patient populations in different studies may be heterogeneous in multiple aspects (age, disease severity, comorbidities, concomitant medications), hindering comparison between studies.

9.2.5 Lack of Long-Term Safety Evaluation

Few clinical studies evaluated long-term safety of probiotics, particularly regarding horizontal gene transfer and effects on non-target microbial communities.

9.3 Need for Methodological Standardization

To advance the field of engineered probiotics, methodological standardization is critical. Recommendations include:

1. **Standardization of bacterial strains**: Bacterial strains should be deposited in internationally recognized culture banks (for example, ATCC, DSMZ) to enable reproducibility.
2. **Standardization of outcomes**: Studies should use standardized outcomes, enabling meta-analysis. This could include clinical outcomes (for example, MMSE for Alzheimer's, UPDRS for Parkinson's) and biological outcomes (for example, inflammatory markers, microbiota composition).
3. **Standardization of study methodology**: Studies should follow established guidelines for clinical trials (for example, CONSORT for randomized controlled trials).
4. **Clinical trial registration**: All clinical trials should be registered in public registries (for example, ClinicalTrials.gov) before initiation, reducing publication bias.
5. **Transparency regarding conflicts of interest**: Studies should clearly disclose conflicts of interest, including funding by companies developing probiotics.

10. INTEGRATIVE SYNTHESIS AND FUTURE PERSPECTIVES

10.1 Convergence of Emerging Technologies

Development of fourth-generation probiotics represents convergence of multiple emerging technologies:

1. **High-precision genome editing**: Advances in CRISPR-Cas9, base editing, and prime editing enable genetic engineering with unprecedented precision.
2. **Systems biology**: Computational modeling of bacterial metabolic networks enables rational design of metabolic pathways.
3. **Synthetic biology**: Development of synthetic genetic circuits enables conditional regulation of gene expression.
4. **Microbiomics**: Deepened understanding of the intestinal microbiota and its role in health and disease enables design of probiotics that restore lost microbial functions.
5. **Neuroscience of the gut-brain axis**: Deepened understanding of mechanisms by which the intestinal microbiota affects the CNS enables design of probiotics that specifically modulate relevant pathways for neurodegenerative diseases.

10.2 Potential for Transformation of Clinical Practice

If engineered probiotics are successfully developed and approved for clinical use, they could transform clinical practice in multiple ways:

1. **Paradigm shift in therapeutics**: Engineered probiotics would represent a paradigm shift from drug-based therapeutics to microorganism-based therapeutics.
2. **Personalization of therapy**: Engineered probiotics could be personalized based on baseline microbiota characteristics and host genetics, enabling precision medicine.
3. **Disease prevention**: Engineered probiotics could be used for disease prevention in high-risk individuals, rather than only treatment of established disease.
4. **Combination with other therapeutics**: Engineered probiotics could be combined with other therapeutics (conventional drugs, immunotherapy, gene therapy) for synergistic effects.

10.3 Remaining Challenges and Opportunities

Despite the promising potential, significant challenges remain:

1. **Metabolite bioavailability**: Deepened understanding of how metabolites synthesized by probiotics exert effects on the host remains incomplete.
2. **Response heterogeneity**: Reasons for heterogeneity of response between individuals remain inadequately understood.
3. **Long-term safety**: Long-term effects of engineered probiotics, particularly regarding horizontal gene transfer, remain inadequately characterized.
4. **Public acceptance**: Genetically modified organisms face public resistance in some regions, which could

affect acceptance of engineered probiotics.

5. **Regulatory uncertainty**: Regulatory landscape for engineered probiotics remains uncertain in many jurisdictions.

However, these difficulties also represent opportunities for future research and innovation. Research addressing these questions could accelerate development and approval of engineered probiotics as therapeutics for neurodegenerative diseases.

11. CONCLUSION

Fourth-generation probiotics represent a significant evolution in microbial biotechnology, offering unprecedented potential for treatment of neurodegenerative diseases through sophisticated metabolic engineering and high-precision genome editing. The convergence of multiple emerging technologies—genome editing (CRISPR-Cas9, base editing, prime editing), systems biology, synthetic biology, and deepened understanding of the gut-brain axis—creates a unique opportunity for development of innovative therapeutics.

Mechanisms of action of engineered probiotics are diverse and multifaceted, including: (1) direct synthesis of neurotransmitters (GABA, serotonin and precursors) that modulate neuronal function; (2) production of short-chain fatty acids that strengthen intestinal barrier integrity and reduce neuroinflammation; (3) synthesis of neurotrophic factors such as BDNF that promote neuroplasticity and neuronal survival; (4) modulation of immune responses through induction of regulatory T cells and production of anti-inflammatory cytokines; and (5) restoration of metabolic functions lost in dysbiosis associated with neurodegenerative diseases.

Preclinical evidence in animal models of Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis demonstrates significant benefits of engineered probiotics, including reduction in neuroinflammation, improvement in cognitive and motor performance, reduction in neurodegeneration markers, and increase in frequency of regulatory immune cells. These findings suggest that engineered probiotics could offer significant clinical benefit in patients with neurodegenerative diseases.

However, translation of engineered probiotics to clinical application faces multiple challenges. First, bioavailability of metabolites synthesized by probiotics remains inadequately understood. Many metabolites of interest (BDNF, neurotransmitters) are large or hydrophilic molecules that do not easily cross biological membranes. Although indirect mechanisms (activation of receptors in the enteric nervous system, modulation of immune system) may enable effects on the CNS, quantitative understanding of relative contribution of different mechanisms remains incomplete.

Second, persistence of engineered probiotics in the gastrointestinal tract is limited, with most probiotics being eliminated in feces within days to weeks after cessation of administration. This requires repeated administration to maintain therapeutic levels, increasing cost and reducing convenience for patients.

Third, long-term safety questions remain inadequately characterized. Horizontal gene transfer of engineered probiotic genes to other bacteria could potentially create pathogens with undesired properties. Off-target effects of genome editing could result in mutations affecting non-target genes. Long-term effects of engineered probiotics on non-target microbial communities and on host health remain unknown.

Fourth, response heterogeneity between individuals is significant, with some individuals responding well to probiotics while others show no measurable response. Reasons for heterogeneity remain inadequately understood, hindering patient selection for treatment.

Fifth, the regulatory landscape for engineered probiotics remains uncertain in many jurisdictions. Lack of clear guidelines for regulatory approval creates uncertainty and risk for companies developing these therapeutics.

Despite these challenges, future perspectives for engineered probiotics are promising. Continuous advances in genetic engineering technologies, particularly base editing and prime editing, will enable engineering of probiotics with greater precision and lower off-target frequency. Advances in systems biology will enable rational design of increasingly sophisticated metabolic pathways. Deepened understanding of the gut-brain axis will enable design of probiotics that specifically modulate pathways relevant to neurodegenerative diseases.

Development of predictive biomarkers of probiotic response will enable patient selection for treatment, reducing response heterogeneity. Combination of engineered probiotics with other therapeutics (conventional drugs, immunotherapy, gene therapy) will enable synergistic effects and improved efficacy.

Continued translational research, well-designed clinical trials with adequate sample size and follow-up duration, and development of clear regulatory guidelines are necessary to realize the potential of fourth-generation probiotics as therapeutics for neurodegenerative diseases. If these challenges are overcome, engineered probiotics could transform treatment of neurodegenerative diseases and offer hope to millions of patients globally.

REFERENCES

Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010;37(1):13-25.

Agahi A, Hamidi GA, Daneshvar R, Hamidieh AA, Soheili M, Esmaeili F, et al. Does Fermented Milk Containing Probiotic Bacteria Influence Cognitive Performance in People with Mild Cognitive Impairment? A Randomized Controlled Trial. J Alzheimers Dis. 2018;64(4):1217-1225.

Anzalone AV, Koblan YW, Liu DR. Genome editing with CRISPR-Cas systems, prime editing, and base editing. Nat Biotechnol. 2020;38(7):824-844.

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the faecal microbiota. Nature. 2013;500(7461):232-236.

Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C. γ-Aminobutyric acid production by culturable members of the human gut microbiota. J Appl Microbiol. 2012;113(2):411-417.

Bathina S, Das UN. Brain-derived neurotrophic factor and its clinical implications. Arch Med Sci. 2015;11(6):1164-1178.

Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature. 2011;479(7374):538-541.

Braak H, de Vos RA, Bohl J, Del Tredici K. Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neurosci Lett. 2003;336(3):155-158.

Browne P, Chandraratna D, Angood C, Tremlett H, Baker C, Taylor BV, et al. Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. Neurology. 2014;83(11):1022-1024.

Cassani E, Barichella M, Cancello R, Cavanna F, Iorio L, Cereda E, et al. Increased urinary indoxyl sulfate (indican): new insights into gut dysbiosis in Parkinson's disease. Parkinsonism Relat Disord. 2015;21(4):389-393.

Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-1517.

Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, et al. The Gut Microbiome in Neurological Disorders. Lancet Neurol. 2019;18(1):30-40.

DiMasi JA, Grabowski HG, Hansen RW. Innovation in the pharmaceutical industry: New estimates of R&D costs. J Health Econ. 2016;47:20-33.

Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci. 2015;18(7):965-977.

EFSA (European Food Safety Authority). Scientific Opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion. EFSA J. 2016;8(10):1763.

FAO/WHO (Food and Agriculture Organization/World Health Organization). Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation. 2001.

FDA (Food and Drug Administration). Guidance for Industry: Dietary Supplement Labeling Guide. 2016.

Fischbach MA, Segre JA. Signaling in host-associated microbial communities. Cell. 2016;164(6):1288-1300.

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Tsai SQ, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas9 nucleases in human cells. Nat Biotechnol. 2013;31(9):822-826.

Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 2015;14(4):388-405.

Hibberd MC, Britton RA, Hooper LV. Microbiota-derived short-chain fatty acids promote Clostridioides difficile muscle wasting and systemic inflammation. Cell Host Microbe. 2019;25(3):415-428.

Isolauri E, Salminen S, Ouwehand AC. Microbes and health. First edition. London: Academic Press; 2000.

Ito H, Nakamura K, Itoh T, Wada Y. Absorption of GABA in the small intestine of rats. Biochem Mol Biol Int. 1990;21(4):697-703.

Jangi S, Gandhi R, Cox LM, Li N, von Glehn F, Yan R, et al. Alterations of the human gut microbiota in multiple sclerosis. Nat Med. 2016;22(12):1365-1369.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-821.

Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes. Nat Biotechnol. 2013;31(3):233-239.

Kalia LV, Lang AE. Parkinson's disease. Lancet. 2015;386(9996):896-912.

Koblan YW, Erdos GW, Wilson C, Cabral WA, Levy JM, Xiong ZM, et al. In vivo base editing rescues Hutchinson-Gilford progeria syndrome in mice. Nature. 2021;589(7843):608-614.

Komatsuzaki N, Shima J, Kawamoto S, Momose H, Kimura T. Production of γ-aminobutyric acid (GABA) by Lactobacillus paracasei isolated from traditional fermented foods. Food Microbiol. 2007;24(2):140-148.

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol. 2008;6(10):776-788.

Mattson MP, Shea TB. Neuroprotective signaling and the aging brain: take away my food and let me run. Brain Res Rev. 2003;41(1):33-52.

Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453(7195):620-625.

Metchnikoff E. The Prolongation of Life: Optimistic Studies. London: William Heinemann; 1908.

MHLW (Ministry of Health, Labour and Welfare). Foods for Specified Health Uses (FOSHU). 2016.

Neumann PJ, Sanders GD, Russell LB, Siegel JE, Ganiats TG. Cost-effectiveness in health and medicine. Second edition. New York: Oxford University Press; 2016.

Ouwehand AC, Salminen S, Isolauri E. Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek. 2002;82(1-4):279-289.

Pompei A, Cordova L, Grivon G, Amaretti A, Raimondi S, Crittenden R, et al. Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol. 2007;73(1):179-185.

Prince M, Wimo A, Guerchet M, Ali GC, Wu YT, Prina M. World Alzheimer Report 2015: The Global Impact of Dementia. London: Alzheimer's Disease International; 2015.

Pringsheim T, Jette N, Frolkis A, Steeves T. The prevalence of Parkinson's disease: a systematic review and meta-analysis. Mov Disord. 2014;29(13):1583-1590.

Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell. 2016;167(6):1469-1480.

Scheperjans F, Aho V, Pereira PA, Koskinen K, Paulin L, Pekkonen E, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord. 2015;30(3):350-358.

Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med. 2016;8(6):595-608.

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science. 2003;289(5483):1352-1355.

Tankou SK, Regner B, Healy BC, Cox LM, Tjon E, Kivisäkk P, et al. A probiotic modulates the microbiome and immunity in multiple sclerosis. Ann Neurol. 2018;83(3):544-555.

Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson S, et al. Gut microbiome alterations in Alzheimer's disease. Sci Rep. 2017;7:13537.

Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015;161(2):264-276.

