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Medical hypothesis

CRISPR-EDITED COMMENSALS OF THE NASOPHARYNX AS PLATFORM FOR DEVELOPING NEXT-GENERATION MICROBIAL PROBIOTICS

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The nasopharynx is a critical mucosal barrier where commensals sustain immune balance and block pathogen colonization. *Corynebacterium accolens* and *Dolosigranulum pigrum* are linked to reduced pneumococcal carriage, fewer viral–bacterial coinfections, and modulation of host immunity. Their metabolites—short-chain fatty acids, nitric oxide, bacteriocins—reinforce epithelial integrity and regulate NF-κB, JAK/STAT, TGF-β/STAT3, promoting Treg cells. Natural effects remain insufficient for therapy. CRISPR/Cas now enables programming these commensals into "living probiotics" that deliver therapeutic molecules in a context-specific manner.

Objective: To evaluate *C. accolens and D. pigrum* as microbial therapy candidates and propose a CRISPR/Cas9 framework for engineering probiotics to modulate mucosal immunity, exclude pathogens, and transmit anti-inflammatory signals.

Methods: A systematic review (2005–2025) was conducted in PubMed, Scopus, Web of Science, EMBASE, Google Scholar using terms: "nasopharyngeal microbiome," "*C. accolens*," "*D. pigrum*," "CRISPR editing," "probiotics," "mucosal immunity." Eligible studies covered genomic, proteomic, metabolomic features, immune interactions, CRISPR editing in probiotics, and preclinical/in silico models. Excluded: unrelated niches, insufficient methods, and non-CRISPR work. Data were synthesized into five topics: ecological roles, molecular/secretome features, CRISPR advances, biosafety design, and validation strategies.

Results: Both taxa emerged as keystone members of the nasopharyngeal microbiome. Genomics revealed stable chromosome organization, no virulence genes, and pathways for SCFA biosynthesis, lipid metabolism, and adhesion. Proteomic and metabolomic studies confirmed secretion of antimicrobial peptides, bacteriocins, and nitric oxide, reducing pathogens and modulating immunity. Functional assays showed TLR2/6 activation, NF-κB inhibition, STAT3/TGF-β promotion, and IL-10 induction, strengthening barriers, enhancing IgA, and favoring regulatory immunity. A CRISPR design was proposed for C. accolens with an IL-10 cassette under an inflammation-inducible promoter plus kill-switches, auxotrophy, and self-limiting nuclease activity. In silico modeling confirmed ecological compatibility and circuit stability. Validation was outlined across in silico prediction, in vitro epithelium–MALT co-cultures, and in vivo murine models with checkpoints for genomic stability, tolerance, and absence of systemic spread. Personalization included microbiome-guided strain selection, prebiotic co-formulation, and biofilm assays, ensuring therapeutic expression only under inflammation.

Conclusions: CRISPR-edited nasopharyngeal commensals are a promising therapeutic platform. *C. accolens* and *D. pigrum* combine stability, safety, and intrinsic immunomodulatory capacity, amplified by precise engineering. Inflammation-inducible IL-10 circuits enable localized, context-specific mucosal immunomodulation. Biosafety modules minimize ecological risk and secure evolutionary stability. Such engineered strains may deliver robust, personalized, clinically relevant interventions for infectious and inflammatory airway diseases.

***Keywords*:** CRISPR, nasopharyngeal microbiome, *Corynebacterium accolens*, *Dolosigranulum pigrum*, microbial therapy, functional probiotics.

Introduction

The nasopharyngeal microbiome is a complex and dynamic ecosystem that plays an important role in maintaining local immune balance and protecting the body from pathogenic agents, particularly viruses and bacteria that cause upper respiratory tract infections [1-4]. Under normal conditions, commensal microorganisms not only coexist with the host organism but also actively participate in the formation of colonization resistance, stimulate antiviral and antibacterial immunity, and can influence the course of inflammatory processes, acting, so to speak, as an external agent of the mucosa-associated lymphoid tissue (MALT) immune system [4-7]. For this reason, the hypothesis has emerged regarding the possibility of therapeutic modification of the microbiome as an alternative or complement to traditional treatment methods. This is particularly relevant in the context of increasing antibiotic resistance, the limited effectiveness of classical antiviral agents, and the growing frequency of chronic and recurrent respiratory diseases. [4; 8-16] In this context, commensal bacteria of the nasopharynx are considered not only as passive inhabitants of the mucous membranes but as a potential therapeutic structural platform that can be purposefully modified to achieve a desired biological effect [17; 18]. One of the key tools that has made it possible to transform microorganisms into functional next-generation probiotics is the CRISPR technology (Clustered Regularly Interspaced Short Palindromic Repeats) [18-20]. These are specific DNA regions that naturally occur in bacteria and archaea and are a component of their adaptive immune system [18-22]. Due to their precision, versatility, and relative ease of use, CRISPR systems allow editing of bacterial genomes, providing them with new functions—from the secretion of antimicrobial peptides to the production of immunomodulatory or antiviral compounds [18-22].

The hypothesis of the approach presented in this study involves using the natural properties of commensal bacteria—the ability to stably colonize the mucosa, compatibility with the human immune system, and absence of pathogenicity—in combination with the possibilities of CRISPR editing to endow them with new therapeutic functions. As a result, living, locally active probiotics may be created, acting directly at the site of pathogen entry, minimizing systemic effects and side effects. This approach potentially allows for the individualization of therapy according to the patient's microbiome profile. CRISPR-commensals constitute the foundation of next-generation microbial therapy, where bacteria act as active agents programmed to perform specific biological functions: secretion of antimicrobial or immunomodulatory compounds, inhibition of pathogens, modulation of immune responses, or counteraction of viral invasion [23; 24]. Unlike traditional drugs, they act from within the microbial ecosystem, adapting to changes in the mucosal environment [18-26]. Despite the fact that this field is currently at an early stage, the first prototypes of engineered probiotics for the prevention and treatment of upper respiratory tract infections, including acute respiratory viral infections, chronic rhinosinusitis, and post-viral inflammation, are already being developed. [18; 26] This approach opens the prospect not only for treatment but also for the maintenance of a stable and harmonious microbial environment, which is especially relevant in conditions of declining effectiveness of traditional pharmacotherapy.

Aim of the study: To conduct a systematic characterization of nasopharyngeal commensal microorganisms as a potential platform for microbial therapy, focusing on their molecular structure, immunomodulatory potential, and possibilities for functionalization through CRISPR/Cas9 technology. To develop a hypothetical model for obtaining controlled and effective next-generation probiotic strains capable of personalized colonization with subsequent experimental implementation of local immune response regulation and counteraction of pathogens in the upper respiratory tract.

Materials and Methods

To test the scientific hypothesis, a systematic literature review was conducted using modern methods of source search and selection. The search was carried out in the PubMed, Scopus, Web of Science, EMBASE, and Google Scholar databases, which made it possible to cover peer-reviewed articles, preprints, and grey literature. The main time interval was 2005 – June 2025, with emphasis on the last decade, when nasopharyngeal commensals, their molecular properties, immunomodulatory effects, and the use of CRISPR/Cas9 in the creation of functionalized probiotics were actively studied. The search strategy included relevant keywords and their combinations with Boolean operators (AND, OR), the use of quotation marks for exact search, as well as filters by language (English, Ukrainian) and document type (original research, reviews, in vitro/in vivo experiments, clinical trials). Among the queries: "nasopharyngeal commensals AND CRISPR/Cas9 engineering", "*Corynebacterium accolens* OR *Dolosigranulum pigrum* AND genomics OR immunomodulation", "mucosal immunity AND probiotic therapy AND

synthetic biology", etc. The inclusion criteria provided for the selection of publications highlighting the molecular characteristics of the main representatives of the nasopharyngeal microbiome (*Corynebacterium accolens*, *Dolosigranulum pigrum*) with genomic, proteomic, and metabolic data. Also considered were studies on CRISPR/Cas9 editing of probiotic strains (integration of therapeutic genes, safety, stability of colonization) and the effects of commensal secretomes (SCFA, NO, bactericidal peptides). Exclusion criteria included publications without full text, with insufficient methodological description, studies of only pathogenic strains without commensal context, as well as materials on other microbiomes (gut, urogenital). Editorial articles, commentaries, popular literature, and outdated data were also excluded. Selection was conducted in two stages: first, screening of titles and abstracts, then full-text analysis by two independent researchers with subsequent consensus in case of discrepancies. Artificial intelligence tools were used for linguistic verification and refinement of translated text fragments. Both language versions of the text were thoroughly examined to confirm their consistency.

Results

Identification and molecular characterization of *Corynebacterium accolens* and *Dolosigranulum pigrum* as key commensals for bioengineering. An analytical review of the current scientific literature on the topic under investigation. *Corynebacterium accolens* and *Dolosigranulum pigrum* are typical representatives of the healthy nasopharyngeal microbiome, and their stable colonization of the mucosa influences the reduction of the incidence of viral-bacterial upper respiratory tract infections, as confirmed by the studies of Brugger et al. (2020), Clark (2020), and Flynn et al. (2022) [27–29]. From a microbiological point of view, both species are Gram-positive facultative anaerobes capable of forming dense colonies that participate in the formation of colonization resistance, competitive exclusion of pathogens, and immune tolerance through a number of molecular mechanisms. [27–30] The main characteristics of *Corynebacterium accolens* and *Dolosigranulum pigrum* are summarized and systematized in Table 1.

Genomics and Proteomics. Literature data indicate that complete genome sequencing of *Corynebacterium accolens* has identified at least five key functional clusters. [31-33] The most important of them are responsible for lipid metabolism, synthesis of adhesive structures, and modulation of the immune response. [28; 31; 34] The main genes – lipA, fadD, pld – encode lipases, acyl-CoA synthetases, and phospholipases that hydrolyze triglycerides of skin sebum with the formation of free fatty acids (lauric, oleic, palmitic). [34-38] These metabolites have bacteriostatic and bactericidal effects on Gram-positive pathogens by inhibiting type III transpeptidases and disrupting the electrostatic balance of their membranes. [34-42]

Proteomic analysis revealed the presence of fimbriae-like proteins (mainly of SpaD-type) and collagen-binding proteins (CbpA, SlpX), which provide specific adhesion to glycosylated receptors of mucosal surfaces (mucins MUC5AC, MUC2). [43-49] Lipoproteins capable of interacting with TLR2/6 and reducing activation of the NF-κB signaling cascade, thereby limiting host inflammatory responses, were also identified. [50; 51] Comparative genomics with pathogenic *Corynebacterium* showed the absence in *C. accolens* of mobile elements, invasion factors, hemolysins, and plasmids with antibiotic resistance genes, as well as the presence of a stable GC-rich genome that reduces the risk of horizontal DNA transfer. [52-53] **The genome of *Dolosigranulum pigrum*** is compact but contains clusters for the synthesis of short-chain fatty acids (SCFA). [54-56] The main genes – ackA, pta, prpE, ldh – form the propionate-acetate cycle, the products of which (acetate and propionate) serve as a carbon source for other commensals and immunomodulate MALT cells [7; 57-59]. **Proteomics of *Dolosigranulum pigrum*** revealed a number of secreted proteins with potential antimicrobial activity, including serine proteases and peptidoglycan hydrolases. [56; 60; 61] Genomic studies of some strains also showed the presence of sortase A enzyme and biosynthetic clusters of lantibiotic-like peptides, which likely provide inhibition of Gram-positive pathogen growth through disruption of cell wall biosynthesis or lipid transport. [56; 60-62]. **Transcriptomics. Transcriptomic analysis of *Corynebacterium accolens*** in co-culture with nasopharyngeal epithelial cells revealed induced expression of lipolysis genes (lipF, lipA) and increased transcription of adhesion proteins (cbpA, slpX, spaD). [34,50,51,63-67,68-70] Modulation of the TLR2/6 cascade was also observed, with suppression of pro-inflammatory cytokines IL-6, IL-8 and an increase in IL-10 and TGF-β levels in dendritic cells. [34; 50; 51; 63-70] For Dolosigranulum pigrum, a stable increase in the expression of SCFA-biosynthesis enzymes (ackA, pta, ldh) was characteristic in response to epithelial contact signals (mucins, sIgA). [34; 50; 51; 63-67; 68-70].

Table 1

Key Commensals for Bioengineering: Identification and Molecular Characterization of *Corynebacterium accolens* and *Dolosigranulum pigrum*

Aspect	**Corynebacterium accolens**	
Role in microbiome	Typical commensal of healthy nasopharyngeal microbiome; stable colonization associated with low respiratory viral-bacterial infections.	Typical commensal of healthy nasopharyngeal microbiome; contributes to immune modulation and metabolic cross-feeding.
Microbiology	Gram-positive facultative anaerobe; forms dense colonies contributing to colonization resistance and pathogen exclusion.	Gram-positive facultative anaerobe; forms colonies with adhesive properties, producing immunomodulatory metabolites.
Genomics	Complete genome sequencing revealed ≥5 key functional clusters, including genes for lipid metabolism (lipA, lipG, fadD, fadE, pld), surface adhesins, and immune modulation.	Compact genome (1.7–1.9 Mbp) with metabolic clusters for short-chain fatty acid (SCFA) production: ackA, pta, prpE, ldh.
Key genes/functions	Lipases and phospholipases hydrolyze skin sebum triglycerides to free fatty acids (lauric, oleic, palmitic acids) with bacteriostatic/cidal effects on Gram-positive pathogens.	SCFA biosynthesis genes form propionate-acetate metabolic cycle; acetate and propionate serve as carbon sources and immune modulators.
Proteomics	Presence of fimbriae-like proteins (SpaD-type), collagen-binding surface proteins (CbpA, SlpX), mediating specific binding to epithelial mucins (MUC5AC, MUC2).	Adhesins MapA (mucin-associated protein A), sortase A anchor proteins to cell wall; high affinity to sialylated glyco-conjugates on epithelium.
Expression and Production	Expression of lipoproteins mimicking TLR components; interact with TLR2/6 receptor complexes to downregulate NF-κB signaling.	Production of lantibiotic-like peptides-modified low-molecular-weight antimicrobials disrupting lipid transporter function in pathogens.
Comparative genomics	Lacks mobile genetic elements, hemolysins, invasion factors, and antibiotic resistance plasmids; stable karyotype with high GC content (>60%) indicating low horizontal gene transfer risk.	Compact genome but enriched in metabolic and antimicrobial gene clusters supporting niche colonization and pathogen suppression.
Transcriptomics	In vitro co-culture with nasopharyngeal epithelial cells induces upregulation of lipolysis genes (lipF, lipA) and surface adhesion genes (cbpA, slpX, spaD) within 3–6 hours.	Stable upregulation of SCFA biosynthesis enzymes (ackA, pta, ldh) triggered by epithelial contact signals (mucins, sIgA).
Modulation and Induction	Modulates mucosal TLR signaling: activates TLR2/6, suppresses pro-inflammatory cytokines (IL-6, IL-8), and increases anti-inflammatory cytokines (IL-10, TGF-β) in dendritic cells.	Induces foxp3 expression in regulatory T cells in MALT co-culture, indicating immune tolerance promotion; suppresses nfkb1 expression in macrophages and epithelial cells, reducing inflammation.

Secretomes and Signaling Molecules Affecting MALT. The secretome of nasopharyngeal microorganisms is a complex of active molecules that bacteria release into the colonization environment. [5; 70; 71] In the nasopharynx, it interacts with the mucosal epithelium, intercellular matrix, and immune structures of the mucosa-associated

lymphoid tissue (MALT). [73] To understand the mechanism of action of the secretome, three layers of interaction are distinguished: the surface mucus layer, the epithelial barrier, and the subepithelial immune zone with follicular structures, macrophages, T- and B-lymphocytes, and dendritic cells. [71; 72] The secretome penetrates through these layers and acts through specific molecular targets. [74; 75] Based on the analysis, the main mechanisms of key molecules of the nasopharyngeal microorganism secretome were determined (Table 2).

Table 2

Key signaling molecules and their immunomodulatory mechanisms in the context of MALT

Molecule Type	Examples	Primary Actions
Short-Chain Fatty Acids (SCFA)	Acetate, Propionate	- Activation of Treg cells via FOXP3 expression- Suppression of IL-6 and TNF-α
Nitric Oxide (NO)	-	- Vasodilation and anti-inflammatory effect- Modulation of macrophages and dendritic cells
Lipid Metabolites	Bacterial lipids, FFA derivatives	- Bind to TLR2/6 receptors- Regulation of innate immune responses
Antimicrobial Peptides	Bacteriocins, Lantibiotics	- Selective inhibition of *S. aureus*, *S. pneumoniae* colonization
MALT Modulation Mechanisms	-	- Treg activation- Decreased IL-6/IL-8 secretion- Indirect pathogen inhibition

At the bacterial level, secretome products are formed as a result of enzymatic substrate degradation (lipases, glycosidases, proteases), denitrification of nitrogen compounds, and synthesis of signaling peptides (lantibiotics), which are exported via Sec/Tat systems. [71-73] After release, they enter the mucus or come into contact with the epithelium. [71-73] Nasopharyngeal mucus consists of mucins (MUC5AC, MUC5B), within which secretome diffusion occurs: SCFA (acetate, propionate) penetrate rapidly, while peptides bind to glycoconjugates, prolonging their action. [71-73] Subsequently, the secretome reaches the ciliated columnar epithelium with goblet cells, M cells, and intraepithelial lymphocytes. [75-81] Epithelial cell receptors include TLR2/6 (lipids), GPCR GPR41/43 (SCFA), guanylate cyclase C (NO), and Siglec (glycopeptides). [76-81] In response, ICAM-1 expression is activated, defensin and cathelicidin secretion occurs, and tight junctions (claudin, occludin, ZO-1) are strengthened. [68-84] Some molecules (SCFA, NO, peptides) penetrate through the epithelium or are transported by M cells into MALT, where they affect dendritic cells, macrophages (shift M1→M2), and T cells (SCFA induce FOXP3 and Treg). [68-84] B cells, under the influence of TGF-β and IL-10, switch antibodies to IgA. [67; 71-87] Molecular regulation includes suppression of NF-κB by NO, activation of STAT3 (TGF-β, IL-10), MAPK/ERK control over chemokines and MHC II, as well as JAK/STAT induction of Treg and suppression of Th17. [85-94] This limits pathogen colonization. Commensals additionally produce bacteriocins and lantibiotics that inhibit *Staphylococcus aureus*, *Streptococcus pneumoniae*, and other microorganisms. [88-94] Strengthening of the epithelium and reduction of receptors for adhesins (PsaA, CbpA) decrease the risk of invasion, while Treg enhancement maintains immune tolerance and microbiome homeostasis. [88-94]

Development of an original model of a multi-stage assessment of safety and efficacy. Based on analysis of the literature data [1–94], a summary of modern methods for evaluating genetically modified microorganisms is presented. Drawing on this review material, the work proposes a conceptual multi-stage approach for assessing the safety and efficacy of a functionalized probiotic with modulated CRISPR/Cas9 expression. The approach includes sequential application of in silico, in vitro, and in vivo methods. Its structural scheme is presented in Figure 1.

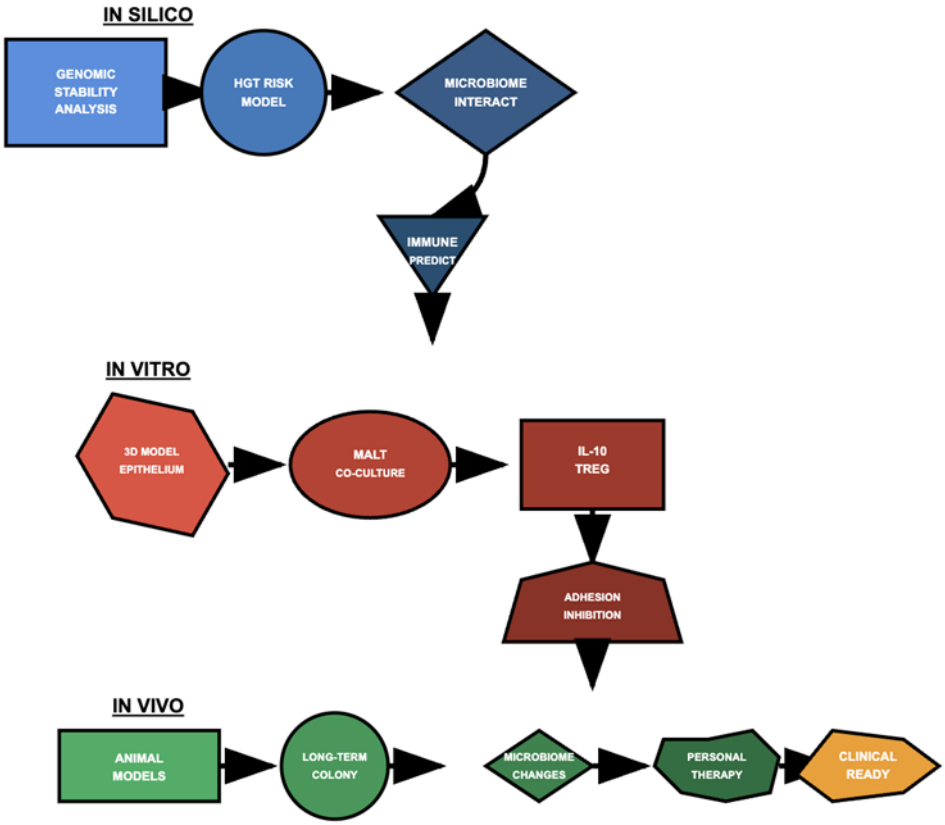

Figure 1. Multistage safety and efficacy assessment

At the *in silico* stage, the authors of the study propose conducting a detailed computational analysis of the strain's genomic stability, taking into account the risks of horizontal gene transfer, which theoretically enables the prediction of potential genetic alterations and helps avoid undesirable mutations. Additionally, it is recommended to perform modeling of probiotic interactions with the existing microbiome, which should help to assess its biocompatibility and possible impact on the nasopharyngeal ecosystem. Special attention is advised to be given to predicting the immunogenic activity of the strain in order to minimize the risk of excessive immune responses.

At the *in vitro* stage, we propose to use three-dimensional models of the nasopharyngeal epithelium that most accurately reproduce the physiological conditions of the mucosal surface. Co-cultivation with immune cells of the lymphoid tissue located in the nasopharynx (MALT) should allow investigation of the interaction of the probiotic with local immunity. Assessment of anti-inflammatory molecules such as IL-10, as well as activation of regulatory T cells (Treg), is expected to demonstrate the immunomodulatory properties of the strain. At the same time, it is recommended to perform tests on bacterial adhesion to epithelial cells and their ability to suppress the growth of potential pathogens, which would indicate the competitive potential of the created strain.

The transition to *in vivo* studies in animal models simulating the human nasopharyngeal environment represents the final stage, enabling the study of long-term probiotic colonization and its effects on the host's immunological state under real biological conditions. In addition to assessing local immune responses, the impact on microbiome composition and the potential for personalized therapy depending on the type of baseline flora should be investigated. Conducting the proposed studies using the developed model will allow for the simultaneous identification of potential side effects and the confirmation of the long-term safety of the probiotic's application. Thus, the presented multistep approach ensures comprehensive validation and increases the likelihood of successful implementation of the functional probiotic strain in clinical practice.

Technical justification of the authors' model for the implementation of the experiment. Based on the preliminary review of relevant literature sources [1–94], which outlines the current state of the studied problem, a hypothesis

was formulated according to which the authors' model of the technical implementation of the experiment is proposed (Fig. 2).

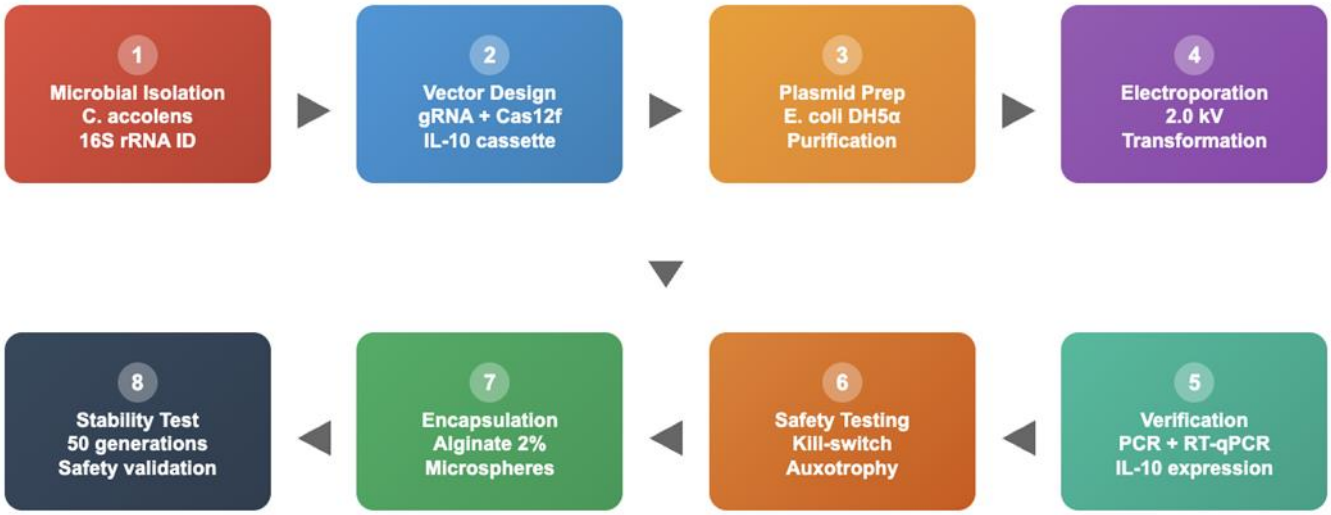

Figure 2. CRISPR/Cas9-Modulated Probiotic Development

Technical implementation of expression control modulated by the in vitro CRISPR/Cas9 system in the functionalized commensal probiotic. At the initial stage of the study, microbiological isolation of the target strain is planned. For this, mucus must be collected from the nasopharynx of a healthy donor using a sterile flocked swab. The swab must be immediately placed in a sterile tube containing 1 mL of phosphate-buffered saline (PBS, pH 7.4). Within 10 minutes at room temperature, resuspension is carried out by rotating the swab in the buffer. From the obtained suspension, we propose to take 100 μL and spread it evenly across the surface of a Columbia Blood Agar (CBA) Petri dish using a sterile L-shaped spreader. The plate should be incubated in a thermostat at 37 °C and 5 % CO_2 for 24 hours. The next day, inspection of the plate under illumination is required. Typical *Corynebacterium accolens* colonies are creamy, slightly shiny, round, dense, 1–2 mm in diameter. [34; 102] Such colonies should be replated individually onto a new CBA dish in order to obtain a pure strain. After an additional 24 hours of incubation, identification is performed by preparing a lysate from 1 colony in 50 μL of water, boiling for 10 minutes, centrifuging for 2 minutes at 12 000 g, and using the supernatant as a template for PCR of the 16S rRNA gene (primers 27F/1492R). The amplicon must be sequenced and compared with the NCBI databases. The next step is to proceed to vector system design for CRISPR/Cas9 editing. To do this, a neutral site in the bacterial genome should be determined, usually between two metabolically inactive regions or near pseudogenes, which should be verified through comparative analysis of the full genomic sequence (e.g., *C. accolens* strain ATCC 49725) [84-94]. Then a synthetic single guide RNA (gRNA) is created that targets a ~20 bp region and contains a PAM sequence (e.g., TTTV for Cas12f). The next stage involves synthesis by in vitro transcription or integration into a vector. Subsequently, a plasmid is constructed (e.g., based on pJIR750 or pCas12f-syn), which includes: (1) Cas12f (codon-optimized for Gram-positive bacteria); (2) a gRNA cassette; (3) an HDR template with homologous flanks of 500 nucleotides; (4) a sequence of an inducible promoter (e.g., SynNFκB with two NF-κB response elements and a basal σ^{70} promoter); (5) an open reading frame of the IL-10 gene (a human gene with a Gram-positive RBS may be used); (6) an rrnB terminator; (7) a self-regulated gRNA directed to a partial region of Cas12f after integration is achieved; (8) a kill-switch cassette – a promoter activated by abnormal pH (e.g., pH < 6.0), which activates expression of the ccdB gene; (9) an auxotrophy system, which includes deletion of the dapA gene via double crossover, verified using a flippase cassette. After assembly, the construct-containing vector must be amplified in *E. coli* DH5α. The transformation process is carried out by chemical induction of competence ($CaCl_2$) or electroporation (1.8 kV, 5 ms). The plasmid is purified from an overnight culture grown on LB + ampicillin (100 μg/mL) using a kit (Qiagen MiniPrep), the concentration is measured (NanoDrop), and plasmid integrity is checked on a 0.8 % agarose gel. Then electrocompetent *C. accolens* cells must be prepared. For this, 50 mL of culture are grown in BHI medium to OD_{600} = 0.5. The culture is cooled on ice, centrifuged at 5000 g for 10 minutes at 4 °C, and washed three times in 10 % sterile glycerol. Cells are then concentrated to 100 μL. To 100 μL, 1–2 μg of plasmid are added, the mixture is transferred

to a cold sterile electroporation cuvette (2 mm), and electroporation is performed on a Gene Pulser (2.0 kV, 25 μF, 200 Ω). Immediately after the pulse, 900 μL of BHI + 0.5 M sucrose are added, and the culture is incubated for 3 h at 37 °C without antibiotic. After recovery, 100 μL are plated onto BHI agar supplemented with chloramphenicol (10 μg/mL) and DAP (0.3 mM), and plates are incubated for 48 hours at 37 °C. Colonies are checked for insertion by colony PCR. For this, one colony is resuspended in 20 μL of water, and 1 μL is taken for PCR. Primers flank the left and right boundaries of the insertion; positive amplicons are sequenced. It is also proposed to verify IL-10 transcription via RT-qPCR (after coculture with cells), determine Cas expression to validate feedback, and assess the presence of in-frame integration. For functional testing, modified bacteria must be cocultured with the RPMI-2650 cell line, which should first be stimulated with IL-1β (10 ng/mL). Cells should be seeded in a 12-well plate at 2×10^5 cells/well 24 h before coculture. Bacteria are then added at an MOI of 10:1 and incubated for 6–8 hours. Supernatant is collected for IL-10 ELISA, and cells for RT-qPCR on Cas12f, IL-10, and control genes. To validate the kill-switch, incubate bacteria in MRS without inducers at pH 5.5 or 42 °C – growth should stop. For auxotrophy, plate bacteria on medium without DAP: growth should not be observed, which should be checked by serial plating and monitoring colony formation. The next step is to encapsulate bacteria in 2 % sodium alginate. To do this, mix the culture with alginate at a 1:1 ratio, add the suspension dropwise into 100 mM $CaCl_2$, forming microspheres of ~300–500 μm in diameter. Then wash the capsules with PBS and incubate with a 3D model of ciliated epithelium. Adhesive properties are determined using fluorescent dyes (e.g., SYTO9), and expression of cbpA/slpX is assessed by RT-qPCR. The final stage is to verify genetic stability. Passage the culture for 50 generations in medium without antibiotic, and every 10 passages perform PCR analysis. To check for horizontal transfer, coculture with *S. pneumoniae* or *S. aureus*, then screen recipients for the presence of the vector cassette. Also perform whole-genome sequencing to assess genome integrity and model in silico the risk of conjugation through OriT/ICE. In our opinion, the application of this method will make it possible to obtain a self-regulated probiotic strain capable of responding only to local inflammation, expressing the therapeutic gene solely in the area of infection, independently limiting itself outside the nasopharynx, not transferring its genes to other bacteria, and remaining functionally stable during long-term use.

Safety, colonization potential, symbiotic interaction. The use of CRISPR-modified commensal bacteria as live biotherapeutic agents significantly expands the framework of traditional understanding of microbial engineering system safety and requires an in-depth analysis of their genetic, ecological, and ethical characteristics. Unlike classical probiotics, these commensals function within the complex ecosystem of the human microbiome, where they can influence the structure and dynamics of microbial communities. A key prerequisite for biosafety is ensuring the genetic stability of the therapeutic cassette and the absence of its horizontal mobility. Particular attention must be given to the risk of unpredictable interactions between the CRISPR system and mobile genetic elements — transposons, plasmids, or phage vectors — which may potentially contribute to the transfer of therapeutic modules to other representatives of the microbiome. After successful introduction of the therapeutic cassette and confirmation of its functional activity, the next critically important step is assessing the safety, colonization stability, and symbiotic behavior of the modified strain under conditions that mimic the complex ecology of the nasopharyngeal microbiome. Four aspects are central in this phase: (1) preservation of the genetic construct without horizontal transfer; (2) stability of the expression system; (3) colonization competitiveness; (4) interaction with the natural microbiota and potential symbiotic support through prebiotics. These parameters determine the ability of the engineered strain to function in a physiologically relevant environment without destabilizing the local microbial ecosystem. An important aspect is the ecological compatibility of the modified strain — its ability to integrate into the microbiome structure without disrupting ecosystem balance. CRISPR modifications may alter metabolic profiles, adhesive properties, and immunomodulatory mechanisms, potentially leading to the displacement of ecologically important commensals. Engineered strains enter a network of interspecies interactions involving competition for resources, quorum sensing, and metabolite exchange. This creates the need for multilevel biocontainment mechanisms — auxotrophy systems, inducible regulatory circuits, and programmable self-elimination mechanisms, which must demonstrate long-term stability even under ecological stress. In-depth biosafety analysis is inseparable from ethical reflection on the introduction of CRISPR-modified commensals into medical practice. Ethical considerations encompass fundamental principles of predictability and proportionality of engineered intervention. Stability of the genetic construct, minimization of evolutionary risks, and prevention of the emergence of new adaptive traits form the basis of ethical acceptability of such systems. The principle of ecological responsibility requires considering not only individual but also population-level consequences: changes in microbiome structure, the risk of creating evolutionary "stress nodes," and the possibility of long-term persistence. The principle of non-interference with heredity implies that therapeutic modules must not contain components capable of recombining with human genetic

structures or spreading uncontrollably within microbial populations. Adherence to these principles forms the foundation for public trust, necessary for clinical and regulatory acceptance of living genetically modified systems.

At the next stage of the study check the stability of the genetic cassette should be assessed by serial passaging of the transformed culture on both selective and non-selective media. The C. accolens::IL10 culture must be grown on BHI agar without antibiotics for 50 consecutive generations (10 passages every 24 hours) to simulate long-term persistence in the biotope without therapeutic pressure. Every 10 passages, 5–10 colonies should be screened by PCR for the presence of Cas12f, the therapeutic gene (IL-10), the kill-switch cassette, and the auxotrophy marker. In parallel, evaluate the integrity of the integration flanks. To detect possible cassette elimination or deletion events, additionally perform Southern blot using DIG-labeled probes targeting the HDR-insertion flanks.

For the assessment of horizontal transfer of genetic elements, it is proposed to conduct an in vitro study by co-cultivating the transformed strain with potential recipients—specifically Streptococcus pneumoniae, Staphylococcus aureus, and Haemophilus influenzae. To do this, co-cultures must be prepared at a 1:1 ratio in antibiotic-free medium and incubated at 37 °C under microaerophilic conditions for 24–48 hours. After that, the bacterial populations should be separated by serial dilution on selective media: plasmids or inserted cassettes should be present only in the original strain. Recipient colonies are tested by PCR for the Cas cassette, IL-10, the cat marker, or any other vector element. The absence of horizontal transfer is confirmed by the absence of a positive signal.

In a parallel *in silico* assessment, use the ICEfinder platform to analyze for the presence of integrative-conjugative elements (ICE), oriT sites, and mobile integrases within the HDR flanks or vector residues. Only upon confirmed genetic isolation of the construct, i.e. absence of mobile genetic elements, should the project be recognized as biosafely integrated. Determine colonization potential by evaluating adhesive ability to ciliated epithelium, competitiveness against local flora, and efficiency of attachment to the mucosal surface.

Creation of an in vitro adhesion model. A monolayer culture of RPMI-2650 cells is seeded onto sterile cover slips placed in 24-well plates. After 24 h of cultivation, add the modified bacterium at a concentration of 10^7 CFU/mL. After 2 hours of incubation, wash the plates three times with PBS, fix with 4 % paraformaldehyde, and stain with SYTO9 or an Alexa Fluor 488 conjugate. Count the number of adhered bacteria per 100 cells using fluorescence microscopy. In parallel, compare with non-modified *C. accolens* and competitors (*S. aureus*, *S. pneumoniae*) to analyze attachment advantage. Determine competitiveness in mixed conditions by coculturing the engineered strain with commensals (*Dolosigranulum pigrum*, *Lactobacillus* spp.) and pathogens. Mix cultures at a 1:1:1 ratio and incubate in BHI or MRS for 24 h, then perform selective platings for each strain, count CFUs, and determine the colonization share. Simultaneously analyze the expression of adhesion genes (*cbpA*, *spaD*, *slpX*) by RT-qPCR. Upon increased expression of these genes in the modified strain, evaluate better adaptation to the epithelial microenvironment. Within the strategy of enhancing colonization stability, apply a synbiotic approach involving inclusion in the therapeutic formulation of a specific pre-probiotic – i.e. a substance that selectively stimulates the growth or attachment of exactly the modified strain. As candidates, use sialylated oligosaccharides (e.g. 3′-sialyllactose), mucin-mimicking glycans, or short-chain fatty acids (butyrate), which support local nutrition of the bacterium in the nasopharyngeal area. By adding such pre-probiotics into the formulation (e.g. a nasal gel), achieve enhanced probiotic fixation on the mucosa. **In parallel**, carry out an analysis of transcriptional activity under natural microbiome conditions – for this, use ex vivo biofilm models where the modified strain is subjected to mixed coculture with a fragmented human nasopharyngeal microbiome. After 48 h of coculture, perform total RNA extraction followed by RT-qPCR for the *Cas*, *IL-10*, regulatory gRNA, and kill-switch genes. If the system is not activated outside the induction zone (i.e. in the absence of proinflammatory signals), this indicates high specificity and a safe background. **The potential for personalized colonization** is substantiated by comprehensive determination of individual nasopharyngeal microbiome profiles using 16S rRNA sequencing, allowing precise identification of dominant bacterial taxa and assessment of their relative ratios in a particular recipient. [55] Based on the obtained data, select optimal CRISPR/Cas9-edited probiotic strains or their combinations that best match the microbiota specificity of each individual, which is key to increasing therapeutic efficacy.

To predict the likelihood of successful colonization and the therapeutic effect of probiotic strains, it is proposed to apply machine learning methods, taking into account the composition of the microbiome ecosystem and the specific features of the nasopharyngeal immune response. Profile the nasopharyngeal microbiota, including quantitative analysis of key representatives such as *Corynebacterium accolens* and *Dolosigranulum pigrum*, and identify potential pathogenic competitors, which will allow rational selection of probiotic strains capable of ensuring stable

colonization and modulating the local immune response. Apply such an individualized approach to enhance the safety and efficacy of probiotic therapy, opening prospects for the development of personalized microbiome-regulating strategies.

In vivo experimental model for testing the CRISPR/Cas9-modulated commensal probiotic *Corynebacterium accolens*::IL-10. The development of this experimental model was based on an analysis of literary sources [1–94], which made it possible to determine key parameters for further validation. Within the framework of our proposed original experimental approach, it is planned to assess the efficacy and safety of the created probiotic strain *Corynebacterium accolens*::IL-10 in SPF C57BL/6 mice aged 8–10 weeks. Before the start of the study, it is recommended that the animals be housed in sterile individually ventilated cages (IVC) for 7 days for acclimatization, ensuring standardized conditions: 22 ± 1 °C, relative humidity 50–60%, sterile food and water ad libitum.

During this period, screen the baseline nasopharyngeal microflora of each mouse: perform a nasal lavage with 50 μL of sterile PBS in each nostril and plate the material on Columbia Blood Agar (CBA). After 24–48 h of incubation, in the absence of colonies of pathogenic species (*Staphylococcus aureus*, *Streptococcus pneumoniae*), admit the animals to the study. This is necessary to avoid cross-interference of the probiotic with undesirable competing strains. The obtained data are schematically shown in Figure 3.

In parallel, prepare a suspension of the edited strain. Wash the colonies of Corynebacterium accolens::IL-10 grown according to the previous protocol in PBS, centrifuge for 5 min at 4000 g, and wash twice to remove medium residues. Standardize the concentration to 10^8 CFU/mL (OD_{600} ~0.5), corresponding to a physiologically relevant dose for intranasal mucosal colonization. At this dose the bacterium can form dense microcolonies on the nasopharyngeal epithelium without inducing a local toxic reaction.

Divide mice into 4 groups: (1) PBS control, (2) wild-type C. accolens, (3) modified C. accolens::IL-10, (4) modified strain with subsequent infectious challenge with S. pneumoniae. This allows simultaneous assessment of the safety, colonization potential, and protective effect of the probiotic upon pathogen infection.

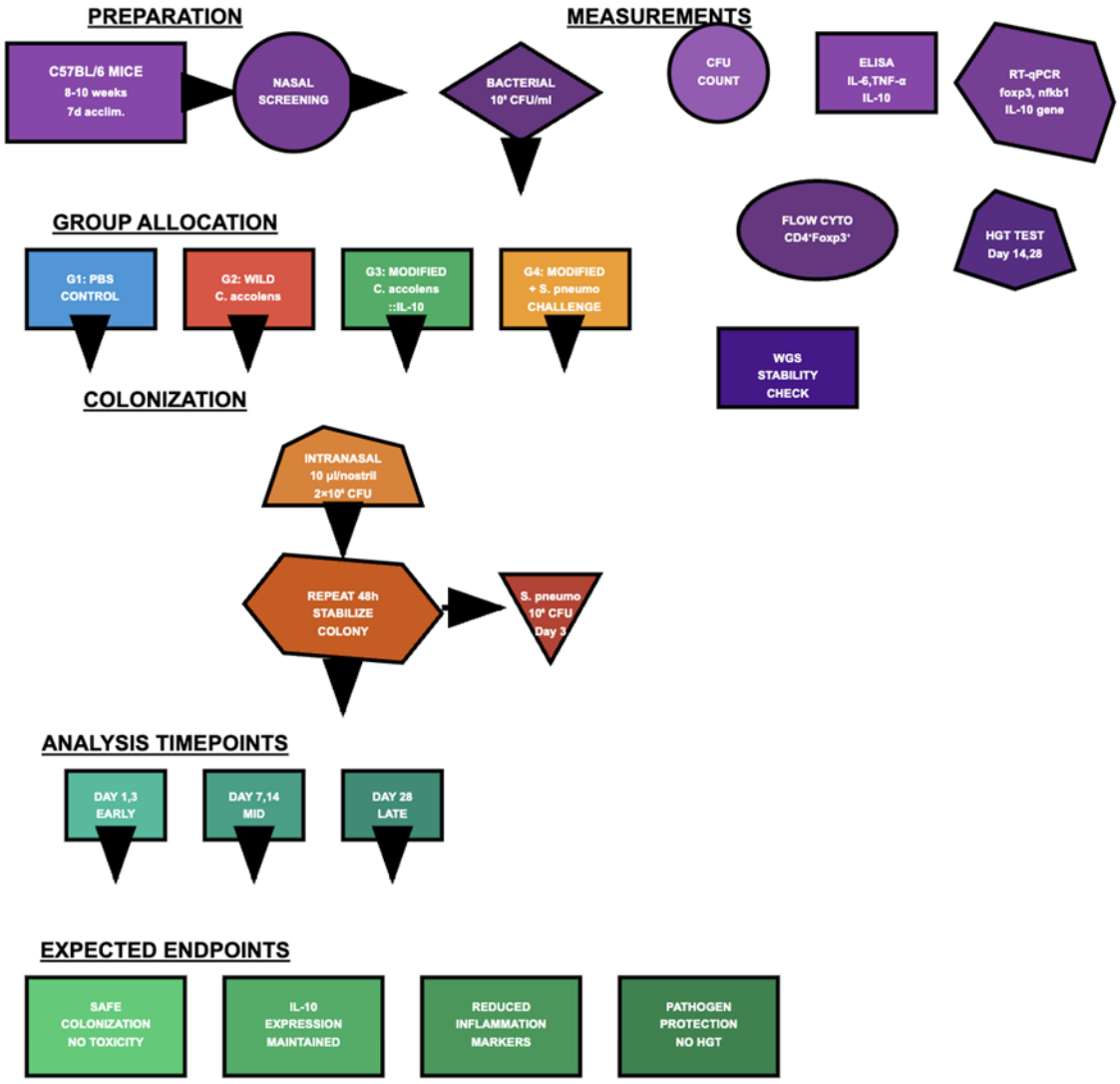

Figure 3. In vivo experimental model - *C. accolens::IL*-10 testing

Perform colonization under light inhalation anesthesia (isoflurane 1.5–2 %) to prevent fluid aspiration and reduce animal stress. Place the mouse on its back, fix the head in a slightly elevated position. Administer 10 µL of bacterial suspension into each nostril using a micropipette (single dose 20 µL = 2×10^6 CFU). Upon reaching the mucosa, according to our model, the probiotic must diffuse along the ciliated epithelium, adhere to mucin receptors (MUC5AC, MUC2), securing itself by adhesion proteins (*cbpA*, *spaD*). The bacterium must begin to metabolize lipids and produce short-chain fatty acids, creating a favorable microenvironment for its own colonization. Repeat administration after 48 h to enhance settlement and achieve stable colonization by day 7. At various time points (days 1, 3, 7, 14, 28), sample material. For this, repeat light anesthesia, instill 50 µL PBS into the nostril and collect the lavage for analysis. Plate part of the sample on selective medium to count CFU of the edited strain and confirm its presence (PCR for *IL-10*, *Cas12f*). Use another part for ELISA (IL-6, TNF-α, IL-10) to assess the balance of pro- and anti-inflammatory signals [94] at the colonization site. At the same time, euthanize a subset of mice (isoflurane + cervical dislocation) to obtain nasopharyngeal biopsies. Mechanically mince tissues, homogenize and extract RNA for RT-qPCR analysis of immune markers such as *foxp3* (Treg activation), *nfkb1* (pro-inflammatory activation), as well as local expression of the therapeutic gene *IL-10*. **To assess protective effect in group G4**, on day 3 after colonization administer the pathogen *Streptococcus pneumoniae* (10^6 CFU/20 µL). The pathogen attaches to the mucosal epithelium and triggers an inflammatory cascade through Toll-like receptor activation, stimulating the release of IL-1β, IL-6 and TNF-α. It is expected that the modified probiotic, through local secretion of IL-10, will induce differentiation of Treg cells, reduce NF-κB activity and decrease the severity of inflammation while simultaneously competing with the pathogen for adhesion sites. **In parallel with these analyses, perform flow**

cytometry of MALT cells isolated from nasopharyngeal lymphoid tissue. Determine numbers of $CD4^+Foxp3^+$ regulatory T cells and M1- and M2-macrophages, which enables pathophysiological assessment of the balance between pro- and anti-inflammatory immune responses. Measure cytokine levels in lavages and serum by ELISA, determining the probiotic's impact on systemic and local immune responses. **On days 14 and 28**, collect all recovered bacteria from lavages and test for horizontal transfer of therapeutic genes by coculturing them with recipient strains (*S. aureus*, *S. pneumoniae*) and perform PCR for the *IL-10* cassette. Use whole-genome sequencing of the edited strain after colonization to confirm integration stability and absence of unwanted mutations. The final outcome of the experiment should demonstrate that *C. accolens::IL-10* safely colonizes the murine nasopharyngeal mucosa, maintains functional expression of the therapeutic gene, reduces pro-inflammatory markers, and effectively limits pathogen colonization, without causing systemic infection or transferring its genes to other bacteria.

Discussion

The obtained results of systemic analysis indicate a high potential of CRISPR-edited nasopharyngeal commensal bacteria as a basis for creating next-generation functional probiotics. *Corynebacterium accolens* and *Dolosigranulum pigrum* possess a number of key characteristics that highlight their potential as bioengineering platforms: the ability for stable mucosal colonization, absence of pathogenic determinants, and secretion of immunomodulatory and antimicrobial metabolites, which is consistent with the findings of Stubbendieck, R. M., Hurst, J. H., & Kelly, M. S. (2024) and Menberu, M. A. et al. (2021), who also note these strains as promising probiotic candidates for maintaining upper respiratory tract microbiome homeostasis. [38; 55]

Stubbendieck, R. M., Hurst, J. H., & Kelly, M. S. (2024) in their study state that *D. pigrum* is a little-studied lactic acid bacterium increasingly considered a mutualist of the human airway. Although early reports suggested its possible pathobiont nature, more recent data indicate that this species is a common component of the human respiratory microbiota, rarely isolated from infected samples and most often found in mixed cultures with established human pathobionts. *D. pigrum* exerts its beneficial effects on the airways both via direct interaction with bacterial respiratory pathobionts and via modulation of host immune and inflammatory responses. The authors further note that, due to its numerous predicted auxotrophies and potential dependence on cooperation with other commensal bacteria in producing certain antimicrobial factors, *D. pigrum* may prove insufficiently effective as a monostrain probiotic, instead requiring application within a probiotic consortium to achieve a pronounced positive effect. Despite the need for further research, *D. pigrum* possesses a number of key characteristics that make it a highly promising candidate for a nasal probiotic to prevent or treat acute respiratory infections (ARI) and enhance respiratory health in humans. [55]

In turn, Menberu, M. A., Liu, S., Cooksley, C., Hayes, A. J., Psaltis, A. J., Wormald, P. J., & Vreugde, S. (2021) note that the combined antimicrobial activity of *Corynebacterium accolens* strains and their secreted proteins against *Staphylococcus aureus* and clinical MRSA isolates in planktonic and biofilm forms may be useful for preventing overgrowth of *S. aureus* in the nasal microbiota. The authors further state that this opens a prospect for protective use of *Corynebacterium* against colonization of the nasal cavity by antibiotic-resistant *S. aureus* in a complex microbial environment. The obtained results have potential clinical relevance for the development of personalized probiotic therapies and may help restore a disrupted nasal microbiome in chronic rhinosinusitis (CRS) [38].

The CRISPR/Cas9 technology opens new opportunities for endowing these bacteria with specific therapeutic functions, including expression of anti-inflammatory cytokines (e.g., IL-10) and induction of a local regulatory immune response. [20] Liu, L., Helal, S. E., & Peng, N. (2023) note that CRISPR/Cas9 technology is a powerful tool for highly precise genome editing of probiotic microorganisms. Depending on the DNA repair pathways in cells – NHEJ, MMEJ, or HDR – it allows deletions, insertions, or point modifications of genes, with HDR providing the greatest accuracy due to the use of repair templates [20]. The authors further add that combining CRISPR/Cas9 with recombination systems and new Cas nuclease variants expands the possibilities of probiotic genetic engineering, opening prospects for creating functional strains for therapeutic application. [20] Compared to traditional pharmacological agents, this approach offers advantages in the form of local action, minimization of systemic side effects, and the possibility of personalized therapy tailored to the patient's individual microbiome. [26] At the same time, the implementation of CRISPR-engineered probiotics is accompanied by a number of challenges. First of all, a comprehensive safety assessment of the edited strains is required: absence of horizontal transfer of therapeutic genes, long-term genetic stability, and controlled activity of modified cassettes [18].

A significant limitation of the presented study is the lack of original experimental data – the results are based on literature review and theoretical models. Further preclinical in vitro and in vivo studies are required to confirm the effectiveness of the proposed concept, optimize regulation systems of therapeutic gene expression, and verify safety and long-term colonization of the modified probiotics. In view of current trends of increasing antibiotic resistance and limited effectiveness of classical treatment methods for upper respiratory tract infections, CRISPR-edited commensals may become a promising direction for the development of individualized microbial therapy. Their implementation into clinical practice will require a comprehensive strategy – from the development of biosafe constructs to the creation of regulatory protocols and evaluation of long-term consequences for patient health and the microbiome ecosystem.

In conclusions: CRISPR/Cas9-edited nasopharyngeal commensals are a promising platform for local microbial therapy due to their ability to combine probiotic properties with targeted expression of therapeutic genes. *Corynebacterium accolens* and other physiological strains may form the basis for the creation of new biopreparations capable of restoring the microbiome, regulating inflammation, and potentially influencing neuro-glio-capillary mechanisms of immune response. However, the absence of original experimental data limits the practical conclusions of this work, highlighting the need for further preclinical studies to verify the efficacy and safety of the proposed approach.

References

1. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. Nat Rev Microbiol. 2017;15(5):259-70. doi:10.1038/nrmicro.2017.14

2. Li X, Chen M, Chen T, et al. The intricate interplay among microbiota, mucosal immunity, and viral infection in the respiratory tract. J Transl Med. 2025;23:488. doi:10.1186/s12967-025-06433-2

3. Kang HM, Kang JH. Effects of nasopharyngeal microbiota in respiratory infections and allergies. Clin Exp Pediatr. 2021;64(11):543-51. doi:10.3345/cep.2020.01452

4. Bondarenko Ya, Shushliapina N, Starkova I. Features of clinical course and diagnosis in pediatric otitis media with ARVI. Ukr J Med Biol Sport. 2025;10(2):17–28. doi:10.63341/ujmbs/2.2025.17

5. Di Stadio A, Costantini C, Renga G, Pariano M, Ricci G, Romani L. The Microbiota/Host Immune System Interaction in the Nose to Protect from COVID-19. Life (Basel). 2020;10(12):345. doi:10.3390/life10120345

6. Nesbitt H, Burke C, Haghi M. Manipulation of the Upper Respiratory Microbiota to Reduce Incidence and Severity of Upper Respiratory Viral Infections: A Literature Review. Front Microbiol. 2021;12:713703. doi:10.3389/fmicb.2021.713703

7. Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. Toxicol Pathol. 2006;34(5):599–608. doi:10.1080/01926230600865531

8. Mayorga-Ramos A, Zúñiga-Miranda J, Carrera-Pacheco SE, Barba-Ostria C, Guamán LP. CRISPR-Cas-Based Antimicrobials: Design, Challenges, and Bacterial Mechanisms of Resistance. ACS Infect Dis. 2023;9(7):1283–1302. doi:10.1021/acsinfecdis.2c00649

9. Sen D, Mukhopadhyay P. Antimicrobial resistance (AMR) management using CRISPR-Cas based genome editing. Gene Genome Editing. 2024;36:100031. doi:10.1016/j.ggedit.2024.100031

10. Bondarenko YD, Kulyk DE, Sivak PM, Pustova NO, Makieieva NI, Bilovol AM. Management of patients with upper respiratory tract infections accompanied by Exanthem Subitum. Intermed J. 2025;(2):119–27. doi:10.32782/2786-7684/2025-2-22

11. Bondarenko Y, Tsyko O. Coxsackievirus: practical recognition of hand, foot, and mouth disease syndrome in children. Collect Sci Pap «ΛΌГΟΣ». 2025 Jun 6;509–20. doi:10.36074/logos-06.06.2025.102

12. Bondarenko Y, Biriukova M. Clinical and diagnostic significance of the color and consistency of upper and lower respiratory tract secretions in assessing the bacterial or viral etiology of the disease. Young Sci. 2025;2(133):25–31. doi:10.32839/2304-5809/2025-2-133-33

13. Bondarenko Y, Kulyk D, Pustova N, Bilovol A, Makieieva N. Viral exanthems in children: etiopathogenesis, clinical features, patient management in outpatient practice. Grail Sci. 2025;(51):888–906. doi:10.36074/grail-of-science.18.04.2025.119

14. Bondarenko Y, Kulyk D, Biriukova M. Clinical and therapeutic aspects of influenza in children: viral manifestation, features of viral exanthem, and possible management strategies for fever. Grail Sci. 2025;(51):848–61. doi:10.36074/grail-of-science.18.04.2025.115

15. Bondarenko Y, Birjukova M. Etiopathogenetic mechanisms of recurrent tonsillitis development in children in organized groups. Grail Sci. 2025;(49):1109–19. doi:10.36074/grail-of-science.21.02.2025.158

16. Bondarenko Y, Zhuravel Y, Ognev V. Determinants of health in preschool and school-age children: challenges of recurrent tonsillitis. Grail Sci. 2025;(49):1089–100. doi:10.36074/grail-of-science.21.02.2025.157

17. Hidalgo-Cantabrana C, O'Flaherty S, Barrangou R. CRISPR-based engineering of next-generation lactic acid bacteria. Curr Opin Microbiol. 2017;37:79–87. doi:10.1016/j.mib.2017.05.015

18. Ramachandran G, Bikard D. Editing the microbiome the CRISPR way. Philos Trans R Soc Lond B Biol Sci. 2019;374(1772):20180103. doi:10.1098/rstb.2018.0103

19. Neil K, Allard N, Roy P, Grenier F, Menendez A, Burrus V, Rodrigue S. High-efficiency delivery of CRISPR-Cas9 by engineered probiotics enables precise microbiome editing. Mol Syst Biol. 2021;17:e10335. doi:10.15252/msb.202110335

20. Liu L, Helal SE, Peng N. CRISPR-Cas-Based Engineering of Probiotics. Biodes Res. 2023;5:0017. doi:10.34133/bdr.0017

21. Sorek R, Lawrence CM, Wiedenheft B. CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem. 2013;82:237–66. doi:10.1146/annurev-biochem-072911-172315

22. Terns MP, Terns RM. CRISPR-based adaptive immune systems. Curr Opin Microbiol. 2011;14(3):321–7. doi:10.1016/j.mib.2011.03.005

23. Tiwari A, Krisnawati DI, Susilowati E, Mutalik C, Kuo TR. Next-Generation Probiotics and Chronic Diseases: A Review of Current Research and Future Directions. J Agric Food Chem. 2024;72(50):27679–700. doi:10.1021/acs.jafc.4c08702

24. Goh YJ, Barrangou R. Harnessing CRISPR-Cas systems for precision engineering of designer probiotic lactobacilli. Curr Opin Biotechnol. 2019;56:163–71. doi:10.1016/j.copbio.2018.11.009

25. Mousavinasab F, Karimi R, Taheri S, Ahmadvand F, Sanaaee S, Najafi S, et al. Microbiome modulation in inflammatory diseases: Progress to microbiome genetic engineering. Cancer Cell Int. 2023;23(1):271. doi:10.1186/s12935-023-03095-2

26. Ali N, Vora C, Mathuria A, Kataria N, Mani I. Advances in CRISPR-Cas systems for gut microbiome. Prog Mol Biol Transl Sci. 2024;208:59–81. doi:10.1016/bs.pmbts.2024.07.008

27. Flynn M, Lyall Z, Shepherd G, Lee ONY, Da Fonseca IM, Dong Y, et al. Interactions of the bacteriome, virome, and immune system in the nose. FEMS Microbes. 2022;3:xtac020. doi:10.1093/femsmc/xtac020

28. Clark SE. Commensal bacteria in the upper respiratory tract regulate susceptibility to infection. Curr Opin Immunol. 2020;66:42–9. doi:10.1016/j.coi.2020.03.010

29. Brugger SD, Eslami SM, Pettigrew MM, Escapa IF, Henke MT, Kong Y, Lemon KP. *Dolosigranulum pigrum* Cooperation and Competition in Human Nasal Microbiota. mSphere. 2020;5(5):e00852-20. doi:10.1128/mSphere.00852-20

30. de Steenhuijsen Piters WA, Bogaert D. Unraveling the Molecular Mechanisms Underlying the Nasopharyngeal Bacterial Community Structure. mBio. 2016;7(1):e00009–16. doi:10.1128/mBio.00009-16

31. Tran TH, Escapa IF, Roberts AQ, Gao W, Obawemimo AC, Segre JA, et al. Metabolic capabilities are highly conserved among human nasal-associated Corynebacterium species in pangenomic analyses. bioRxiv. 2023. doi:10.1101/2023.06.05.543719

32. Naqvi M, Utheim TP, Charnock C. Whole genome sequencing and characterization of Corynebacterium isolated from the healthy and dry eye ocular surface. BMC Microbiol. 2024;24:368. doi:10.1186/s12866-024-03517-9

33. Popowitch EB, Tran TH, Escapa IF, Bhatt E, Sozat AK, Ahmed N, et al. Description of two novel Corynebacterium species isolated from human nasal passages and skin. bioRxiv. 2024 Nov 21. doi:10.1101/2024.11.21.624533

34. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon KP. *Corynebacterium accolens* releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. mBio. 2016;7(1):e01725-15. doi:10.1128/mBio.01725-15

35. Burkovski A. Cell envelope of corynebacteria: structure and influence on pathogenicity. ISRN Microbiol. 2013;2013:935736. doi:10.1155/2013/935736

36. Horn KJ, Jaberi Vivar AC, Arenas V, Andani S, Janoff EN, Clark SE. Corynebacterium species inhibit Streptococcus pneumoniae colonization and infection of the mouse airway. Front Microbiol. 2022;12:804935. doi:10.3389/fmicb.2021.804935

37. Aquino de Sá MDC, Gouveia GV, Krewer CDC, Veschi JL, de Mattos-Guaraldi AL, da Costa MM. Distribution of PLD and FagA, B, C and D genes in Corynebacterium pseudotuberculosis isolates from sheep and goats with caseus lymphadenitis. Genet Mol Biol. 2013;36(2):265–8. doi:10.1590/S1415-47572013005000013

38. Menberu MA, Liu S, Cooksley C, Hayes AJ, Psaltis AJ, Wormald PJ, Vreugde S. *Corynebacterium accolens* has antimicrobial activity against Staphylococcus aureus and methicillin-resistant S. aureus pathogens isolated from the sinonasal niche of chronic rhinosinusitis patients. Pathogens. 2021;10(2):207. doi:10.3390/pathogens10020207

39. Tan SW, Yoon BK, Jackman JA. Membrane-disruptive effects of fatty acid and monoglyceride mitigants on E. coli bacteria-derived tethered lipid bilayers. Molecules. 2024;29(1):237. doi:10.3390/molecules29010237

40. Casillas-Vargas G, Ocasio-Malavé C, Medina S, Morales-Guzmán C, Del Valle RG, Carballeira NM, Sanabria-Ríos DJ. Antibacterial fatty acids: an update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. Prog Lipid Res. 2021;82:101093. doi:10.1016/j.plipres.2021.101093

41. Yoon BK, Jackman JA, Valle-González ER, Cho N-J. Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. Int J Mol Sci. 2018;19(4):1114. doi:10.3390/ijms19041114

42. Fischer CL. Antimicrobial activity of host-derived lipids. Antibiotics. 2020;9(2):75. doi:10.3390/antibiotics9020075

43. Rogers EA, Das A, Ton-That H. Adhesion by pathogenic corynebacteria. Adv Exp Med Biol. 2011;715:91–103. doi:10.1007/978-94-007-0940-9_6

44. Marques da Silva W, Seyffert N, Silva A, Azevedo V. A journey through the Corynebacterium pseudotuberculosis proteome promotes insights into its functional genome. PeerJ. 2021;9:e12456. doi:10.7717/peerj.12456

45. Burkovski A. Proteomics of toxigenic corynebacteria. Proteomes. 2023;12(1):2. doi:10.3390/proteomes12010002

46. Finke S, Fagerlund A, Smith V, Krogstad V, Zhang MJ, Saragliadis A, et al. Bacillus thuringiensis CbpA is a collagen binding cell surface protein under c-di-GMP control. Cell Surf. 2019;5:100032. doi:10.1016/j.tcsw.2019.100032

47. Mays ZJS, Chappell TC, Nair NU. Quantifying and engineering mucus adhesion of probiotics. ACS Synth Biol. 2020;9(2):356–67. doi:10.1021/acssynbio.9b00356

48. Ott L, Höller M, Gerlach RG, Hensel M, Rheinlaender J, Schäffer TE, Burkovski A. Corynebacterium diphtheriae invasion-associated protein (DIP1281) is involved in cell surface organization, adhesion and internalization in epithelial cells. BMC Microbiol. 2010;10:2. doi:10.1186/1471-2180-10-2

49. Abril AG, Quintela-Baluja M, Villa TG, Calo-Mata P, Barros-Velázquez J, Carrera M. Proteomic characterization of virulence factors and related proteins in Enterococcus strains from dairy and fermented food products. Int J Mol Sci. 2022;23(18):10971. doi:10.3390/ijms231810971

50. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A. 2000;97(25):13766–71. doi:10.1073/pnas.250476497

51. Shin HS, Xu F, Bagchi A, Herrup E, Prakash A, Valentine C, et al. Bacterial lipoprotein TLR2 agonists broadly modulate endothelial function and coagulation pathways in vitro and in vivo. J Immunol. 2011;186(2):1119–30. doi:10.4049/jimmunol.1001647

52. Schröder J, Maus I, Meyer K, Wördemann S, Blom J, Jaenicke S, et al. Complete genome sequence, lifestyle, and multi-drug resistance of the human pathogen Corynebacterium resistens DSM 45100 isolated from blood samples of a leukemia patient. BMC Genomics. 2012;13:141. doi:10.1186/1471-2164-13-141

53. Nasim F, Dey A, Qureshi IA. Comparative genome analysis of Corynebacterium species: the underestimated pathogens with high virulence potential. Infect Genet Evol. 2021;93:104928. doi:10.1016/j.meegid.2021.104928

54. Renz A, Widerspick L, Dräger A. First genome-scale metabolic model of *Dolosigranulum pigrum* confirms multiple auxotrophies. Metabolites. 2021;11(4):232. doi:10.3390/metabo11040232

55. Stubbendieck RM, Hurst JH, Kelly MS. Dolosigranulum pigrum: A promising nasal probiotic candidate. PLoS Pathog. 2024;20(2):e1011955. doi:10.1371/journal.ppat.1011955

56. Flores Ramos S, Brugger SD, Escapa IF, Skeete CA, Cotton SL, Eslami SM, et al. Genomic stability and genetic defense systems in Dolosigranulum pigrum, a candidate beneficial bacterium from the human microbiome. mSystems. 2021;6(5):e0042521. doi:10.1128/mSystems.00425-21

57. Hosmer J, McEwan AG, Kappler U. Bacterial acetate metabolism and its influence on human epithelia. Emerg Top Life Sci. 2024;8(1):1–13. doi:10.1042/ETLS20220092

58. Tang CF, Paz-Alvarez M, Pudney PDA, Lane ME. Characterization of piroctone olamine for topical delivery to the skin. Int J Cosmet Sci. 2023;45(3):345–53. doi:10.1111/ics.12839

59. Pettinato E, Steiner TM, Cassens EA, Geisberger T, Seitz C, König S, et al. Propionate metabolism in Desulfurella acetivorans. Front Microbiol. 2025;16:1545849. doi:10.3389/fmicb.2025.1545849

60. Sozat AK, Popowitch EB, Hurst JH, Kelly MS. Screening *Dolosigranulum pigrum* strains for secreted factors that inhibit Staphylococcus aureus. J Pediatric Infect Dis Soc. 2023;12(Suppl 1):S8–9. doi:10.1093/jpids/piad070.017

61. De Boeck I, Wittouck S, Martens K, Spacova I, Cauwenberghs E, Allonsius CN, et al. The nasal mutualist *Dolosigranulum pigrum* AMBR11 supports homeostasis via multiple mechanisms. iScience. 2021;24(9):102978. doi:10.1016/j.isci.2021.102978

62. Cascioferro S, Totsika M, Schillaci D. Sortase A: an ideal target for anti-virulence drug development. Microb Pathog. 2014;77:105–12. doi:10.1016/j.micpath.2014.10.007

63. Islam MA, Albarracin L, Melnikov V, Andrade BGN, Cuadrat RRC, Kitazawa H, Villena J. *Dolosigranulum pigrum* modulates immunity against SARS-CoV-2 in respiratory epithelial cells. Pathogens. 2021;10(6):634. doi:10.3390/pathogens10060634

64. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25− T cells. J Clin Invest. 2003;112(9):1437–43. doi:10.1172/JCI19441

65. Chen CC, Manning AM. TGF-β1, IL-10 and IL-4 differentially modulate the cytokine-induced expression of IL-6 and IL-8 in human endothelial cells. Cytokine. 1996;8(1):58–65. doi:10.1006/cyto.1995.0008

66. National Center for Biotechnology Information. Dolosigranulum pigrum. National Library of Medicine. [cited 2025 Aug 5]. Available from: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=29394

67. Kauk O, Bondarenko Y, Kulyk D. Prognostic value of inflammatory biomarkers (CRP, IL-6, procalcitonin) in patients with ischemic stroke in intensive care settings. Psychiatry Neurol Med Psychol. 2025;12(3(29)):344–56. doi:10.26565/2312-5675-2025-29-05

68. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Sig Transduct Target Ther. 2017;2:17023. doi:10.1038/sigtrans.2017.23

69. Pereira SG, Oakley F. Nuclear factor-kappaB1: regulation and function. Int J Biochem Cell Biol. 2008;40(8):1425–30. doi:10.1016/j.biocel.2007.05.004

70. Popowitch EB, Boiditswe SC, Patel MZ, Aquino JN, Sozat AK, Caiazzo AJ, et al. Dolosigranulum savutiense sp. nov., isolated from human upper respiratory samples collected in Botswana. Int J Syst Evol Microbiol. 2024;74(8):006498. doi:10.1099/ijsem.0.006498

71. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. Cell Res. 2020;30:492–506. doi:10.1038/s41422-020-0332-7

72. Kim DY, Fukuyama S, Nagatake T, Takamura K, Kong IG, Yokota Y, et al. Implications of nasopharynx-associated lymphoid tissue (NALT) in the development of allergic responses in an allergic rhinitis mouse model. Allergy. 2012;67(4):502–9. doi:10.1111/j.1398-9995.2011.02782.x

73. Mancabelli L, Taurino G, Ticinesi A, Ciociola T, Vacondio F, Milani C, et al. Disentangling the interactions between nasopharyngeal and gut microbiome and their involvement in the modulation of COVID-19 infection. Microbiol Spectr. 2023;11:e02194-23. doi:10.1128/spectrum.02194-23

74. Suzuki M, Cooksley C, Suzuki T, Ramezanpour M, Nakazono A, Nakamaru Y, et al. TLR Signals in Epithelial Cells in the Nasal Cavity and Paranasal Sinuses. Front Allergy. 2021;2:780425. doi:10.3389/falgy.2021.780425

75. Ma J, Piao X, Mahfuz S, Long S, Wang J. The interaction among gut microbes, the intestinal barrier and short chain fatty acids. Anim Nutr. 2021;9:159-74. doi:10.1016/j.aninu.2021.09.012

76. Steinbach EC, Plevy SE. The role of macrophages and dendritic cells in the initiation of inflammation in IBD. Inflamm Bowel Dis. 2014;20(1):166-75. doi:10.1097/MIB.0b013e3182a69dca

77. Takeuchi T, Nakanishi Y, Ohno H. Microbial metabolites and gut immunology. Annu Rev Immunol. 2024;42:153-78. doi:10.1146/annurev-immunol-090222-102035

78. Akhtar M, Chen Y, Ma Z, Zhang X, Shi D, Khan JA, et al. Gut microbiota-derived short chain fatty acids are potential mediators in gut inflammation. Anim Nutr. 2021;8:350-60. doi:10.1016/j.aninu.2021.11.005

79. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nat Rev Microbiol. 2018;16(6):355-67. doi:10.1038/s41579-018-0001-8

80. Siegel SJ, Weiser JN. Mechanisms of Bacterial Colonization of the Respiratory Tract. Annu Rev Microbiol. 2015;69:425-44. doi:10.1146/annurev-micro-091014-104209

81. Hillmer EJ, Zhang H, Li HS, Watowich SS. STAT3 signaling in immunity. Cytokine Growth Factor Rev. 2016;31:1-15. doi:10.1016/j.cytogfr.2016.05.001

82. Hutchins AP, Diez D, Miranda-Saavedra D. The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. Brief Funct Genomics. 2013;12(6):489-98. doi:10.1093/bfgp/elt028

83. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:17023. doi:10.1038/sigtrans.2017.23

84. Siednienko J, Nowak J, Moynagh PN, Gorczyca WA. Nitric oxide affects IL-6 expression in human peripheral blood mononuclear cells involving cGMP-dependent modulation of NF-κB activity. Cytokine. 2011;54(3):282-8. doi:10.1016/j.cyto.2011.02.015

85. Liu BM, Beck EM, Fisher MA. The Brief Case: Ventilator-Associated *Corynebacterium accolens* Pneumonia in a Patient with Respiratory Failure Due to COVID-19. J Clin Microbiol. 2021;59:e00137-21. doi:10.1128/jcm.00137-21

86. Neubauer M, Sourek J, Ryc M, Boháček J, Mara M, Münková J. *Corynebacterium accolens* sp. nov., a gram-positive rod exhibiting satellitism, from clinical material. Syst Appl Microbiol. 1991;14:46-51.

87. Lerat E, Ochman H. Recognizing the pseudogenes in bacterial genomes. Nucleic Acids Res. 2005;33(10):3125-32. doi:10.1093/nar/gki631

88. Douglas GM, Shapiro BJ. Pseudogenes act as a neutral reference for detecting selection in prokaryotic pangenomes. Nat Ecol Evol. 2024;8(2):304-14. doi:10.1038/s41559-023-02268-6

89. ATCC. *Corynebacterium accolens* (ATCC® 49725™) genome assembly and annotation. ATCC Genome Portal. 2020 Dec 21. Available from: https://genomes.atcc.org/genomes/bcb5707d03a7431d

90. Park DE, Aziz M, Salazar JE, Pham T, Nelson SG, Villani J, et al. The nasal microbiome modulates risk for SARS-CoV-2 infection. EBioMedicine. 2025;115:105660. doi:10.1016/j.ebiom.2025.105660

91. Brugger SD, Eslami SM, Pettigrew MM, Escapa IF, Henke MT, Kong Y, et al. *Dolosigranulum pigrum* Cooperation and Competition in Human Nasal Microbiota. mSphere. 2020;5(5):e00852-20. doi:10.1128/mSphere.00852-20

92. Nazir A, Hussain FHN, Raza A. Advancing microbiota therapeutics: the role of synthetic biology in engineering microbial communities for precision medicine. Front Bioeng Biotechnol. 2024;12:1511149. doi:10.3389/fbioe.2024.1511149

93. Flores Ramos S, Brugger SD, Escapa IF, Skeete CA, Cotton SL, Eslami SM, et al. Genomic Stability and Genetic Defense Systems in Dolosigranulum pigrum, a Candidate Beneficial Bacterium from the Human Microbiome. mSystems. 2021;6(5):e00425-21. doi:10.1128/mSystems.00425-21

94. Bondarenko YaD, Kauk OI, Stetsenko SO, Pliten OM. Neuro-glio-capillary dysfunction in children with respiratory infections: early clinical markers and the role of outpatient screening. Psychiatry, Neurology and Medical Psychology. 2025;12(4(30)):449–471. https://doi.org/10.26565/2312-5675-2025-30-03