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Adhesion of *E. coli* to line HUVEC cel cultures (primary human umbilical vein endothelial cells). Imaging was performed by scanning electron microscopy at 20 kV accelerating voltage, 13.5 mm operating distance, 3.5k magnification

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ONCOLYTIC ADENOVIRUS-BASED IMMUNOTHERAPY FOR MALIGNANT MESOTHELIOMA: PRECLINICAL ADVANCES AND FUTURE PERSPECTIVES

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Abstract: Malignant mesothelioma is a rare but highly aggressive cancer, with an incidence of approximately 1–2 cases per million per year in industrialized countries and a global annual mortality exceeding 30,000 cases. The disease is caused mainly by exposure to asbestos and is resistant to conventional treatment modalities, resulting in poor outcomes. Standard approaches include surgery, chemotherapy, and, more recently, immune checkpoint inhibitors. However, owing to the aggressive nature of cancer, the prognosis is poor, with limited treatment options. Globally, the disease accounts for more than 30,000 deaths annually. Current treatment efficacy remains suboptimal, particularly in advanced stages. Oncolytic virotherapy, especially adenovirus-based vectors, has emerged as a novel immunotherapeutic strategy with the potential to induce tumour lysis and stimulate antitumour immunity. This review summarizes the current treatment landscape for mesothelioma and highlights preclinical and early clinical findings on engineered oncolytic adenoviruses, particularly those expressing ICOSL, CD40L, and OX40L, and their promising synergistic effects with checkpoint blockade and chemotherapy. Furthermore, in this review, we present insights from clinical studies of the effects of adenovirus Ad5/3-D24-GM-CSF on mesothelioma and discuss promising avenues for future immunotherapy.

Keywords: immunotherapy, mesothelioma, oncolytic adenovirus, cancer therapy, co-stimulatory molecules

1.Introduction

Malignant pleural mesothelioma (MPM) originates from mesothelial cells and is caused primarily by exposure to asbestos. The malignancy is usually diagnosed at an advanced stage, contributing to a median overall survival of only 9–12 months (Pisani et al. 2020). Current treatment strategies include surgery, chemotherapy, and immunotherapy, yet the outcomes remain unsatisfactory (Kuryk et al. 2022).

The standard of care for unresectable MPM is a combination of pemetrexed and cisplatin. Although this regimen modestly prolongs survival, the benefits are often transient due to chemoresistance and minimal immunologic activation (Kuryk et al. 2016). Multimodal approaches involving extrapleural pneumonectomy or pleurectomy/decortication are considered in select operable patients but carry significant morbidity (Treasure et al. 2011).

In the immunotherapy era, immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy. The CheckMate 743 trial demonstrated that the combination of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) improved survival over chemotherapy, especially in patients with nonepithelioid subtypes. Nevertheless, the majority of cancer patients still fail to achieve long-term clinical benefit due to the immunosuppressive tumor microenvironment (TME) and low T-cell infiltration (Garofalo et al. 2022).

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To overcome these limitations, novel immune-based strategies, such as oncolytic virotherapy, have emerged as complementary approaches. Published meta-analyses have demonstrated that immune checkpoint inhibitors are more effective when combined with modalities that are able to modulate the TME, such as oncolytic viruses (Jones et al., 2021). Recent advancements suggest that combining ICIs with oncolytic virotherapy could increase antitumour responses and improve patient outcomes. The pathogenicity of MPM is complex and not only caused by asbestos but also by the complex molecular pathogenesis and intrinsic resistance mechanisms of the disease (Wadowski et al., 2020). This finding is supported by a broad analysis of immune checkpoint blockade in mesothelioma, which revealed limited responses unless immune checkpoint blockade was combined with other modalities (Boutin et al., 2021). One promising candidate, ONCOS-102, a chimeric Ad5/3-D24 oncolytic adenovirus expressing GM-CSF (ONCOS-102, Ad5/3-D24-GM-CSF), has shown encouraging signs of immune activation and prolonged survival when combined with chemotherapy in phase II studies (Ponce et al., 2023).

2. Oncolytic Adenoviruses and Their Therapeutic Potential

Oncolytic viruses (OVs) are engineered to selectively infect and lyse cancer cells while sparing normal tissues. This causes direct oncolysis and the induction of immunogenic cell death (ICD), which can trigger systemic antitumour responses. Adenovirus vectors have versatile applications in cancer therapy due to their genetic stability, large transgene capacity (Muravyeva et al. 2024), immunogenicity and safety profile in humans (Biegert et al. 2021).

Early-generation vectors such as Ad5/3-D24-GM-CSF have shown promise in preclinical and early clinical trials when combined with chemotherapy (Kuryk et al. 2016; Ponce et al. 2023). More recently, oncolytic adenoviruses have been designed to express multiple immune-stimulating molecules, such as OX40L, CD40L, or GM-CSF, to increase antigen presentation and T-cell activation. The AdV5/3-D24-ICOSL-CD40L vector represents a novel generation of such constructs. It combines a tumor-selective E1A deletion (D24) with a chimeric Ad5/3 fibre for enhanced tumor targeting and is armed with ICOSL and CD40L to potentiate T-cell costimulation (Garofalo et al. 2021).

Interestingly, in a recent study, Chiaro et al. (2023) described a novel pipeline for identifying mesothelioma-specific MHC-I peptides via immunopeptidomics and incorporating them into a PeptiCRAd-based immunotherapy complex (adenovirus AdV5/3-D24-CD40L-OX40L-based platform). This novel strategy leverages the surface presentation of tumour antigens, allowing the selective coating of oncolytic adenoviruses with immunogenic peptides. The published results underscore the importance of direct MHC-I peptide selection and presentation in an immunological context. This allows the development of tumor-specific immunity and a more accurate representation of $CD8^+$ T-cell targets. PeptiCRAd-coated viruses induce potent antitumour immune responses in vivo, validating the concept of combining personalized antigen discovery with oncolytic virotherapy for mesothelioma (Chiaro et al. 2023). Table 1 lists the key characteristics of two oncolytic adenovirus vectors: AdV-D24-ICOSL-CD40L and Ad5/3-D24-GM-CSF.

Table 1. Summary of the key features of AdV-D24-ICOSL-CD40L and Ad5/3-D24-GM-CSF.

Oncolytic Adenovirus	Tumor Selectivity	Transgenes	Studies	Major results
AdV5/3-D24-ICOSL-CD40L	Yes (E1A deletion)	ICOSL and CD40L for enhanced T-cell costimulation	Preclinical mesothelioma models (both in vitro and in vivo)	Safety profile, enhances efficacy, ICD, T-cell infiltration
Ad5/3-D24-GM-CSF (ONCOS-102)		GM-CSF for improved antigen presentation	Phase I/II in mesothelioma (NCT02963831)	T-cell infiltration, safety profile, improved efficacy

3. Preclinical and Clinical Insights from Mesothelioma Models

The AdV5/3-D24-ICOSL-CD40L vector has demonstrated promising preclinical efficacy in various in vitro and in vivo models of mesothelioma. In 2D and 3D spheroid cultures, the virus induced direct oncolysis and induced ICD. In immunodeficient and humanized mouse models bearing mesothelioma tumors, intratumoral injection of the vector resulted in significant tumor volume reduction—up to 60% in treated mice—especially when combined with anti-PD-1 therapy (Garofalo et al. 2023).

A phase I/II clinical trial with adenovirus Ad5/3-D24-GM-CSF in combination with pemetrexed and platinum-based chemotherapy in patients with unresectable MPM further underscored the clinical potential of this strategy (NCT02963831). This study revealed that treatment with the vector was well tolerated and promoted robust tumor immune activation. In total, 31 patients were enrolled. Anaemia (15.0% and 27.3%) and neutropenia (40.0% and 45.5%) were the most frequent grade ≥3 adverse events (AEs) in the Ad5/3-D24-GM-CSF (n=20) and chemotherapy-alone (n=11) cohorts. No patients discontinued Ad5/3-D24-GM-CSF due to AEs. An improvement in overall survival (30-month OS rate 34.1% vs 0; median OS 20.3 vs 13.5 months) with Ad5/3-D24-GM-CSF versus chemotherapy alone has been reported. Therapy with Ad5/3-D24-GM-CSF was associated with increased T-cell infiltration and immune-related gene expression, which was not observed in the cohort receiving only chemotherapy. Importantly, immune activation in the TME was associated with survival in a cohort treated with a virus (Ponce et al. 2023). Notably, increased infiltration of $CD4^+$, $CD8^+$, and granzyme B^+ T cells and upregulation of cytotoxicity gene signatures were observed. In contrast, these findings were not observed in patients treated with chemotherapy alone (Ponce et al. 2023).

4. Immune Profiling and Tumor Microenvironment Modulation

The combination therapy of adenovirus AdV5/3-D24-ICOSL-CD40L with PD-1 inhibition in preclinical studies led to elevated levels of $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and granzyme B^+ effector cells, indicating active cytotoxic engagement. However, the number of regulatory $FoxP3^+$ T cells was reduced, suggesting a shift toward a proinflammatory TME (Garofalo et al. 2023).

Chiaro et al. confirmed that intratumoral injection of peptide-coated adenoviruses (PeptiCRAd) in mice enhanced local immune responses, with significant increases in antigen-specific $CD8^+$ T cells and improved tumor control. Additionally, the study demonstrated that among strong MHC-binding peptides, only a subset is expressed on tumor cells, supporting the importance of immunopeptidomics for peptide target validation, especially in cancer vaccine approaches. These results support the concept that specific peptides, when delivered via adenoviral platforms (PeptiCRAd), can reprogram the TME, elicit systemic immunity and enhance antitumour effects (Chiaro et al. 2023).

Similarly, multiplex immunofluorescence analyses in the Ad5/3-D24-GM-CSF clinical trial on mesothelioma revealed significant increases in the $CD8^+$:Treg ratio and M1:M2 macrophage polarization. Transcriptomic analysis of tumor biopsies revealed elevated expression of T-cell markers (CD3E, CD4, and CD8A) and cytotoxicity-related genes in patients with the highest survival (Ponce et al. 2023).

Furthermore, gene expression analyses of the chemokines CXCL9 and CXCL10 revealed upregulation in tumours from combination-treated mice. In fact, these chemokines play critical roles in T-cell recruitment and retention, supporting the observation of increased numbers of tumor-infiltrating lymphocytes (TILs). The present findings underscore the role of the adenovirus vector as a potent immune primer capable of remodelling the TME toward an immune-permissive state (Garofalo et al. 2023).

5. Perspectives on Adenoviral Vector Design and Combinations

The shift toward armed oncolytic vectors expressing cytokines, chemokines, and costimulatory molecules reflects a broader effort to engage both innate and adaptive immunity, leading to increased anticancer effects. Recent studies with adenoviruses expressing TIMP2 and PADI1 have demonstrated antitumour efficacy in preclinical models of melanoma (Kuryk et al 2023). TIMP2, a tissue inhibitor of metalloproteinases, disrupts tumour–stroma interactions and inhibits angiogenesis, whereas PADI1 expression induces immunogenic cell death through citrullination of histones, potentially increasing tumour antigenicity (Wu et al. 2023). Arming oncolytic adenoviruses with nonimmunostimulatory transgenes offers a complementary approach to enhancing their efficacy through various mechanisms, such as the TME and metabolic remodelling. This multimodal mode of action enhances immune T-cell infiltration and improves treatment responsiveness when combined with ICIs. Importantly, such strategies strengthen immunostimulatory approaches by disrupting stromal barriers, modulating

tumor metabolism, and promoting immune cell infiltration (Wu et al. 2024).

The concept described by Chiaro et al. shows how immunopeptidomics analyses can be combined with PeptiCRAd, enhancing the specificity and potency of oncolytic immunotherapy vaccines (Chiaro et al. 2023). This opens new avenues for personalized multivalent vaccine design. Future designs may include neoantigens alongside traditional immune costimulatory ligands, enzymes, inhibitors, cytokines, and chemokines to synergize with ICIs and other immunomodulators.

These advancements suggest that vector design can be tailored to tumour-specific features. For mesothelioma, combining oncolytic adenoviruses with chemotherapy, ICIs, or other immune modulators may enhance clinical efficacy and improve patient quality of life.

6. Future directions and clinical translation

The successful translation of novel oncolytic adenoviruses into the clinic will rely on optimizing multiple aspects, such as dosing frequency, route of administration, vector design, patient immune fitness and genetic background. Refined delivery strategies, such as repeated intratumoral dosing, systemic administration with tumour-targeting modifications, or shielding via carrier cells, are actively being explored to increase biodistribution and reduce antiviral neutralization.

Combination therapies that combine oncolytic viruses with ICIs, cancer vaccines, and TME modulators are expected to further increase antitumour efficacy. Importantly, predictive biomarkers such as baseline TIL, IFN-related gene signatures, or CXCL9/10 expression may guide patient selection and therapeutic personalization in the near future.

Clinical findings from the Ad5/3-D24-GM-CSF phase II study in patients with unresectable MPM support the viability of this strategy. In this trial, patients who received the adenovirus in combination with chemotherapy reached a median OS of 20.3 months versus 13.5 months in the group treated with chemotherapy only. Furthermore, profound infiltration of $CD8^+$ T cells and upregulation of interferon-stimulated and cytotoxic gene signatures were observed in the combination group (Ponce et al. 2023).

Although the intratumoral administration of adenoviruses remains a standard approach, novel intravenous delivery systems are more commonly and frequently utilized to target distant malignancies, thus broadening the therapeutic reach of adenovirus-based immunotherapy.

7. Challenges and Limitations of Oncolytic Adenovirus-Based Immunotherapy

Despite encouraging results, several challenges limit oncolytic adenovirus-based therapies in the treatment of malignant mesothelioma. One major barrier is the immunosuppressive TME, which affects the infiltration of TILs, the functionality of effector T cells and tumor recognition (Garofalo et al. 2023).

Additionally, preexisting immunity to adenoviruses may reduce viral spread and replication, thus potentially reducing therapeutic efficacy in patients previously exposed to the virus (Li et al. 2015). Nevertheless, various strategies, such as the use of chimeric fibres or shielding via carrier cells, are being developed to overcome this challenge (Bessis et al. 2004).

Another limitation is the heterogeneity of antigen presentation, as mesothelioma has a low mutational burden and limited neoantigen diversity, limiting the immunogenic potential of peptide-based vaccines (Chiaro et al. 2023).

Importantly, intratumoral injection of adenoviruses may not be feasible for patients with inaccessible tumors, stressing the need for improved systemic delivery systems (Zheng et al. 2019). Furthermore, there is an unmet need for validated predictive biomarkers that can be incorporated into clinical diagnostic routines. Currently, markers such as T-cell infiltration density or interferon-response gene signatures are essential for guiding therapeutic decisions and tailoring treatment strategies, highlighting the importance of clinical immune monitoring in clinical studies.

8. Conclusion

Adenovirus-based oncolytic immunotherapy serves as a promising approach to resolve the unmet clinical need in mesothelioma therapy. Through direct tumor lysis, immune activation, and ICD, these vectors can convert cold tumors into hot tumors. Engineered adenovirus vectors have shown potential in both preclinical and early clinical studies, particularly when combined with checkpoint blockade or chemotherapy. Continued improvements in the use of viral vectors in combination with standard and emerging therapies may significantly enhance outcomes for mesothelioma patients.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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REVIEW
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ROLE OF *FUSOBACTERIUM NUCLEATUM* AND *PORPHYROMONAS GINGIVALIS* IN ORAL CANCER: A LITERATURE REVIEW

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Abstract: Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is a multifactorial disease influenced by genetic, environmental, and microbial factors. Among the emerging contributors to oral carcinogenesis, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, two Gram-negative anaerobic bacteria that reside in the human oral cavity as part of the normal microbiota, have gained attention for their pathogenic roles. Both species play key roles in periodontal disease and exhibit significant tumour-promoting activities through distinct and synergistic mechanisms. They modulate the tumor microenvironment by promoting the release of pro-inflammatory cytokines, facilitating immune evasion, and contributing to microbial dysbiosis. Clinical studies have identified their presence in OSCC tissues, correlating with advanced tumor stages, lymph node metastasis, and poor prognosis. Their involvement underscores the critical interplay between oral microbiota and host-pathogen interactions in cancer development. This review highlights the molecular mechanisms through which *F. nucleatum* and *P. gingivalis* contribute to oral carcinogenesis, emphasizing the need for further research to explore their diagnostic and therapeutic potential in oral cancer management. Addressing these microbial drivers could pave the way for innovative strategies in cancer prevention and treatment.

Keywords: carcinogenesis, *Fusobacterium nucleatum*, oral microbiota, oral squamous cell carcinoma (OSCC), *Porphyromonas gingivalis,*

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, accounting for over 90% of oral cancers (Li et al. 2024). OSCC ranks among the top 10 cancers worldwide, with an annual incidence exceeding 300,000 cases. Higher rates are observed in South Asia, parts of Africa, and the Middle East. It predominantly affects individuals over 40 years of age, with a higher prevalence in males. However, cases in younger populations, particularly women, are increasing. Five-year survival rates range from 50% to 60%, depending on the stage at diagnosis (Shao et al. 2021). OSCC arises from the squamous epithelium lining the oral cavity and is characterized by its aggressive growth, as well as a tendency for local invasion and metastasis. The anatomical distribution of OSCC includes the anterior two-thirds of the tongue, lower and upper gingiva, buccal mucosa, hard palate, floor of the mouth, retromolar triangle, and vermilion mucosa (Li et al. 2024). Some OSCCs may arise *de novo*, whilst others arise from oral potentially malignant

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disorders (OPMDs), such as leukoplakia and erythroplakia (Warnakulasuriya et al. 2020). Traditional risk factors, such as tobacco use, alcohol consumption, betel nut chewing, and human papillomavirus (HPV) infection, are well-recognized. Other factors, including infections resulting from poor oral hygiene, exposure to ionizing radiation, and environmental pollutants, may also play a significant role. Moreover, emerging evidence highlights the role of microbiota in carcinogenesis (Shao et al. 2021). However, around 15% of OSCC cases develop without any of these known risk factors (Singh et al. 2023). Some evidence suggests that the imbalance of oral microbiota plays a crucial role in the onset, progression, and prognosis of OSCC, thereby offering potential biomarkers for the disease. The oral microbiota can elicit chronic inflammation, generate inflammatory mediators and carcinogenic agents, and influence cell proliferation and apoptosis. Among oral pathogens, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* have garnered attention for their potential role in the development, progression, and metastasis of OSCC (Nie et al., 2024; Pang et al., 2024).

This review aims to enhance our understanding of the molecular mechanisms underlying the initiation and progression of OSCC to inform the development of more effective preventive measures. Furthermore, it seeks to expand our understanding of the intricate relationship between microorganisms and cancer development.

2. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* – characteristic features.

F. nucleatum is a non-motile, Gram-negative bacteria belonging to *the Fusobacteriaceae* family and is characterized by its spindle-shaped form (Bolstad et al. 1996) (Moraes et al. 2002). It is also known for its remarkable ability to co-aggregate with diverse bacterial species and adhere to host tissues. These traits enable *F. nucleatum* to serve as a "bridging organism" in biofilms, facilitating the integration of early and late colonizers in the oral microbial community (Chen et al. 2022). *F. nucleatum's* pathogenic potential is attributed to several virulence factors: adhesins - Fusobacterium adhesion A (FadA) and Fusobacterium outer membrane protein A (FomA). FadA promotes adhesion and invasion of epithelial and endothelial cells. FomA facilitates biofilm formation and microbial co-aggregation (Chen et al. 2022). The bacteria produce short-chain fatty acids (SCFAs) such as butyrate and propionate, which influence cell signalling and apoptosis (Dahlstrand Rudin et al. 2021). It also suppresses immune responses by inhibiting neutrophil activity and downregulating inflammatory pathways and induces the production of pro-inflammatory cytokines such as IL-6 and TNF-α, contributing to chronic inflammation (Wang et al. 2023). Recent research has linked *F. nucleatum* to various systemic conditions, underscoring its potential role as a pathobiont in colorectal cancer (CRC) (Galasso et al. 2025). It has also been linked to cardiovascular disease, adverse pregnancy outcomes and endometriosis (Fan et al. 2022; Selvaraj et al. 2024; Muraoka et al. 2023).

Porphyromonas gingivalis is a Gram-negative, non-motile, obligately anaerobic bacterium belonging to the Porphyromonadaceae family (Shah and Collins 1988; Hahnke et al. 2018). A notable feature of *P. gingivalis* is its black pigmentation, which results from the accumulation of iron-containing heme compounds on its cell surface, contributing to both its virulence and survival within the periodontal environment (Shah and Collins 1988; How et al. 2016). The pathogenic potential of *P. gingivalis* stems from its wide range of virulence factors, including fimbriae, lipopolysaccharides (LPS), gingipains and a capsule (Singh et al. 2011; Jia et al. 2019). Fimbriae play a critical role in adhesion to host epithelial cells, oral tissues, and other bacterial species, thereby promoting biofilm formation and persistence within the oral cavity (Horvat Aleksijević et al. 2022). The lipopolysaccharides (LPS) of *P. gingivalis* are structurally diverse, a feature that helps the bacterium evade immune surveillance and modulate inflammatory responses (Wang and Ohura 2002). Gingipains, a family of cysteine proteases, significantly contribute to pathogenicity by degrading host proteins, disrupting immune responses, and directly contributing to tissue destruction (How et al. 2016; Bi et al. 2023). A polysaccharide capsule protects the bacterium from phagocytosis and enhances its survival in the host. These factors allow *P. gingivalis* to invade gingival tissues, manipulate host immune pathways, and establish chronic infection. *P. gingivalis* is often described as a "keystone pathogen" because its small numbers can disproportionately influence the microbial ecosystem and disease progression (Li et al. 2024). It achieves this by interfering with the complement system, subverting neutrophil activity, and producing toxins that exacerbate tissue damage. Emerging research links *P. gingivalis* to systemic diseases beyond the oral cavity, for instance, cardiovascular disease (Xie et al. 2020), rheumatoid arthritis (Ahmadi et al. 2023) and Alzheimer's Disease (Kanagasingam et al. 2020). A comparison of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* is presented in Table 1.

Table I. Comparison of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

Feature	*Fusobacterium nucleatum*	*Porphyromonas gingivalis*
Cell Morphology	Long, spindle-shaped rod (Chen et al. 2022)	Short rod (coccobacillus) (Lamont and Jenkinson 2000)
Gram Stain	Gram-negative (Shao et al. 2021)	Gram-negative (Shah and Collins 1988; How et al. 2016)
Oxygen Requirements	Obligate anaerobe (Shao et al. 2021)	Obligate anaerobe (Shah and Collins 1988; How et al. 2016)
Motility	Non-motile (Bolstad et al. 1996)	Non-motile (Shah and Collins 1988; How et al. 2016)
Production of pathognomonic enzymes or chemoattractants	Produces short chain fatty acids (SCFAs), e.g. acetate and butyrate (Dahlstrand Rudin et al. 2021).	Produces proteases (gingipains) (Singh et al. 2011; Jia et al. 2019)
Key Virulence Factors	Adhesins (FadA) (Chen et al. 2022), endotoxin (LPS) (Shao et al. 2021)	Gingipains, LPS, fimbriae (Singh et al. 2011; Jia et al. 2019)
Role in Diseases	Periodontal disease (Chen et al. 2022), colorectal cancer (Galasso et al. 2025), preterm birth (Shao et al. 2021), endometriosis (Muraoka et al. 2023)	Periodontal disease (Shah and Collins 1988; How et al. 2016), cardiovascular disease (Xie et al. 2020), rheumatoid arthritis (ahmadi et al. 2023), Alzheimer's Disease (Kanagasingam et al. 2020)
Natural Habitat (Niche)	Human oral cavity (Chen et al. 2022), gut (Galasso et al. 2025), placenta (Wang et al. 2013; Chen et al. 2022)	Oral cavity, subgingival plaque (Horvat Aleksijević et al. 2022)
Clinical Significance	Involved in polymicrobial infections, biofilm formation (Chen et al. 2022; Horvat Aleksijević et al. 2022)	Major pathogen in periodontitis (Horvat Aleksijević et al. 2022), linked to systemic diseases (Xie et al. 2020; Horvat Aleksijević et al. 2022; ahmadi et al. 2023)
Drug Resistance	Some resistance to beta-lactams (presence of β-lactamase-producing strains) (Bolstad et al. 1996)	Limited resistance to beta-lactams, resistance to macrolides (Conrads et al. 2021)
Capsule	Occasionally has a present capsule (Bolstad et al. 1996)	Has a polysaccharide capsule (Singh et al. 2011)

3. Association between *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and oral cancer.

Approximately 15% of oral squamous cell carcinoma cases have no clear etiology but are linked to infectious agents, including viruses (e.g., human papillomavirus, Epstein–Barr virus), fungi (*Candida albicans*), and bacteria. Chronic inflammation caused by infections is a significant contributor to carcinogenesis, with periodontitis and its associated pathogens playing a notable role (Kuper et al. 2001). Multiple studies highlight a strong association between periodontitis and OSCC, with chronic periodontal disease increasing the risk of premalignant lesions and progression to OSCC (Tezal et al. 2009; Laprise et al. 2016). *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, two prevalent opportunistic bacteria present in the oral cavity, have garnered significant attention due to their abundance in periodontal plaque and their elevated levels in individuals with OSCC. Many publications indicate the presence of bacteria from the Fusobacterium genus (Nagy et al. 1998), particularly *F. nucleatum* (Al-Hebshi et al. 2017; Chang et al. 2018; Zhang et al. 2020; Su et al. 2021), as well as from the Porphyromonas genus (Nagy et al. 1998; Al-Hebshi et al. 2017; Zhang et al. 2020), especially *P. gingivalis* (Chang et al. 2018; Chen et al. 2021; Katz et al. 2011). An increase in the abundance of *Fusobacteria* may be more common in late-stage OSCC, as observed by Yang et al. They found that increased abundance occurs more often depending on OSCC staging, ranging from 2.98% in healthy controls to 7.92% in stage 4 OSCC (Yang et al. 2018). Similarly, when OSCC samples using immunostaining, it was found that all specimens were positive for *P. gingivalis*,

whereas adjacent healthy oral tissues tested negative. Among the cancer samples, 69.5% exhibited strong positivity, while 30.5% were weakly positive (Li et al. 2024).

Several studies have aimed to characterize the microbial species associated with OSCC tumor tissues compared to non-tumorous controls, utilizing various detection methods. Studies applying NGS analysis of both V1-V3 DNA regions (Al-hebshi et al. 2017) and V3-V4 (Chang et al. 2018) DNA regions of OSCC samples, as well as quantitative PCR (Chang et al. 2018) and immunohistochemical staining (Katz et al. 2011; Li et al. 2024) has shown this increase of the abundance of both bacteria in cancerous tissue. In addition, Park et al. reported that serum levels of *P. gingivalis* IgG were significantly elevated in OSCC patients compared to non-OSCC controls (Park et al. 2019). In OSCC samples with an increased abundance of the bacteria, the most commonly detected subspecies of *F. nucleatum* at the tumor site include *F. nucleatum* subspecies *polymorphum* (Al-hebshi et al. 2017), *F. nucleatum* subspecies *vincentii* (Pushalkar et al. 2012) and *F. nucleatum* subspecies *nucleatum* (Hooper et al. 2006). However, in the case of the last subspecies, *F. nucleatum* subspecies *nucleatum*, other studies have shown different results, finding *that F. nucleatum* subspecies *nucleatum* is predominantly present in non-tumorous regions (Pushalkar et al. 2012). Other changes in the microbiota of OSCC tissue were also observed, including a reduction in the *Streptococcus* genus (Su et al. 2021) and the *Actinobacteria* phylum (especially *Rothia*) (Schmidt et al. 2014), while *Prevotella* and *Alloprevotella* were enriched (Ganly et al. 2019). Interestingly, studies exploring the abundance of *P. gingivalis* and *F. nucleatum* in premalignant lesions, such as leukoplakia, have found no significant differences between affected patients and healthy controls (Shridhar et al. 2021).

F. nucleatum-high cases were significantly associated with non-white ethnicity and more infiltrative tumor lesions (Fernandes et al. 2024). In contrast, *P. gingivalis* was significantly associated with tobacco use, poor oral hygiene, and inadequate periodontal health. Moreover, it showed associations with larger tumor size, low tumor differentiation, advanced T stage and clinical stage, lymph node metastasis, and higher mortality rates (Li et al. 2024). In addition, other investigations have identified correlations between *P. gingivalis* infection and adverse clinical outcomes, including late-stage disease, poor tumor differentiation, lymph node metastasis, and reduced overall survival in OSCC patients (Wen et al. 2020; Xie et al. 2020). On the other hand, Neuzillet et al. found that *F. nucleatum*-positive tumors showed lower recurrence rates with fewer metastatic relapses compared to *F. nucleatum*-negative tumors. *F. nucleatum*-associated OSCC occurs more often in older, non-drinking patients and is linked to a favorable prognosis (Neuzillet et al. 2021). Similarly, Chen et al. demonstrated that *F. nucleatum* enrichment in head and neck squamous cell carcinoma (HNSCC) tissues correlated with improved cancer-specific survival, lower relapse rates, and lower tumour and nodal staging, highlighting its potential as a prognostic biomarker (Chen et al., 2020). These findings are unexpected, given the association of *F. nucleatum* with poor prognosis in other cancer types. However, despite these conflicting studies, there might be a potential role of *P. gingivalis* and *F. nucleatum* as biomarkers for disease progression and prognosis in OSCC.

4. Carcinogenesis mechanisms of *Fusobacterium nucleatum and Porphyromonas gingivalis*

The specific roles of *F. nucleatum* and *P. gingivalis* in inducing the carcinogenesis of oral cancer, as well as their underlying mechanisms remain incompletely understood, with only a few studies providing preliminary insights. The first experimental evidence suggesting that *F. nucleatum* and *P. gingivalis* could induce malignant transformation in the oral cavity was presented by Binder Gallimindi et al., who used a mouse model of periodontal infection-associated oral tumorigenesis. This study demonstrated the effects of *P. gingivalis* and *F. nucleatum* on human oral cavity SCC cells *in vitro*. These pathogens were shown to activate Toll-like receptor (TLR) signalling in both precancerous and cancerous oral epithelia, leading to the overexpression of epithelial-derived IL-6. Exposure to *P. gingivalis* or *F. nucleatum*, either individually or in combination, significantly increased IL-6 expression in epithelial-like cells isolated from the tongues of SCC patients, as well as in the SCC-25 and CAL27 cell lines. Notably, TLR2 inhibition markedly reduced pathogen-induced IL-6 expression, whereas TLR4 inhibition did not, emphasizing the predominant role of TLR2 in this response. *In vivo* infection with *P. gingivalis* and *F. nucleatum* induced nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signalling, a key pathway implicated in tumorigenesis (Gallimidi et al. 2015). *P. gingivalis* infection has also been shown to cause the increased production of other protumor molecules in OSCC cells, including suprabasin, IL-1R2, IL-18, and TGF-α (Liu et al., 2020). In addition, Groeger et al. reported that extracts from the *P. gingivalis* W83 membrane induced upregulation of oncogenesis-associated genes, including NFκBIA and TRAF5, in infected OSCC cells. These genes are known to play critical roles in inflammatory signalling pathways linked to cancer progression (Groeger et al. 2022).

5. Migration and Invasion

F. nucleatum has been implicated in enhancing the migration and invasion of OSCC cells through various mechanisms, often involving interactions with host signalling pathways. Both *F. nucleatum* and *P. gingivalis* can produce matrix metalloproteinases (MMPs), such as MMP-1 and MMP-9, which are crucial for extracellular matrix remodelling and cancer cell invasion (Gallimidi et al. 2015; Harrandah et al. 2020). Harrandah et al. demonstrated that oral cancer cells co-infected with multiple pathogens showed upregulation of MMP1, MMP9, and interleukin-8 (IL-8), along with elevated expression of survival markers such as MYC, Janus kinase 1 (JAK1), signal transducer and activator of transcription 3 (STAT3), and epithelial-mesenchymal transition (EMT) markers zinc finger E-Box binding homeobox 1 (ZEB1) and transforming growth factor beta (TGF-β). Notably, *the F. nucleatum* culture supernatant, containing LPS, was sufficient to induce IL-8 secretion, suggesting that direct bacterial contact with cancer cells is not required for these effects. A 4-nitroquinoline-1-oxide (4NQO)-induced oral tumor model showed that bacterial infections resulted in significantly larger and more numerous lesions compared to non-infected mice, supporting the role of *F. nucleatum* in promoting OSCC progression (Harrandah et al. 2020). Inaba et al. demonstrated that *P. gingivalis* infection activates proMMP-9 in highly invasive SAS cells through protease-activated receptor 2 (PAR2) signalling pathways. Additionally, *P. gingivalis* has been shown to activate multiple kinase pathways, including NF-κB, ERK1, and p38, leading to the overexpression of proMMP-9 (Inaba et al. 2013). Consistent findings were reported by Ha et al. and Woo et al., who observed overexpression of MMP-1, MMP-2, MMP-9, and MMP-10 in *P. gingivalis*-infected cells, accompanied by increased levels of IL-8. This IL-8 elevation was associated with the enhanced expression of MMPs, thereby further promoting cancer cell invasion and migration (Ha et al. 2015; Ha et al. 2016; Woo et al. 2017).

Another possible mechanism includes an increase of ZEB1 protein expression by gingival epithelial cells infected with *P. gingivalis*. ZEB1 protein, acting as a transcription factor, regulates multiple genes and has been implicated in activating metastasis in several types of cancer. Therefore, the elevated expression of ZEB1 after exposure to the bacterium was correlated with increased numbers of MMP-9 and vimentin proteins, resulting in increased cell migration (Sztukowska et al. 2016). ZFP36, also known as tristetraprolin (TTP), is a member of the zinc finger protein family that regulates numerous pro-inflammatory proteins, including itself. It is recognized as a regulator of the inflammatory response. Lu et al. demonstrated that persistent infection with *P. gingivalis* suppresses ZFP36 by forming ZFP36/CCAT1/MK2 complexes, thereby enhancing the tumorigenic potential of human-immortalized oral epithelial cells (HIOECs). Clinical samples from patients with periodontitis and OSCC exhibit reduced ZFP36 expression. Furthermore, CCAT1 functions as a molecular scaffold, promoting the assembly of the ZFP36/CCAT1/MK2 complex, which strengthens MK2-mediated inhibition of ZFP36 phosphorylation. Reduced ZFP36 expression diminishes its inhibitory effect on cancer-associated biological processes such as proliferation, cell cycle regulation, apoptosis, migration, and invasion, thereby enhancing the tumorigenic capability of HIOECs (Chang et al. 2018; Lu et al. 2024)

Further studies have highlighted the involvement of *F. nucleatu*m in EMT, a critical process for cancer metastasis. Cai et al. identified significant enrichment of *F. nucleatum* and other bacteria in OSCC, with *F. nucleatum* contributing to cellular invasion through interactions with the E-cadherin/β-catenin signalling pathway, the TNFα/NF-κB pathway, and matrix remodelling via the upregulation of the EMT transcription factor SNAI2 (Cai et al. 2024). Similarly, Zhang et al. found that infection with *F. nucleatum* increased cell migration and apoptosis while reducing E-cadherin expression. This was accompanied by elevated expression of lncRNA MIR4435-2HG and SNAI1, both of which are associated with OSCC progression (Zhang et al. 2020). Min et al. further showed that *F. nucleatum* activated EMT through upregulation of SNAI1 (Min et al. 2024).

Shao et al. revealed that both live and heat-inactivated *F. nucleatum* enhanced cancer cell invasiveness by upregulating pro-EMT genes, with the bacterial outer membrane proteins FadA and Fap2 playing significant roles in this process. LPS from *F. nucleatum* was also implicated in EMT induction through TLR signalling pathways (Shao et al. 2021). Uitto et al. observed that *F. nucleatum* stimulated the migration of epithelial cells, particularly at wound margins, and upregulated collagenase 3 expression via p38 MAPK activation. This highlights *F. nucleatum's* ability to activate multiple cell signalling systems, leading to enhanced invasion and survival of infected epithelial cells (Uitto et al. 2005). Inflammasome activation also plays a role in *F. nucleatum*-induced OSCC pathogenesis. Aral et al. demonstrated that *F. nucleatum* promotes interleukin-1β (IL-1β) production by upregulating AIM2 and downregulating POP1, thereby contributing to chronic inflammation that facilitates the progression of HNSCC (Aral et al. 2020). Similarly, Abdulkareem et al. reported that *F. nucleatum* may induce

EMT in OSCC cells by elevating TGF-β, TNF-α, and epidermal growth factor (EGF) signalling pathways. Despite these findings, many studies remain at the *in vitro* stage, and the molecular mechanisms by which *F. nucleatum* promotes OSCC progression have not yet been thoroughly investigated (Abdulkareem et al. 2017). Kamarajan et al. highlighted the tumor-promoting effects of periodontal pathogens, including *F. nucleatum*, in a mouse model. Pathogen-challenged OSCC cells exhibited greater tumor burden compared to controls. The study also demonstrated that *F. nucleatum* enhanced OSCC cell migration, invasion, and tumor sphere formation via integrin alpha V and focal adhesion kinase (FAK) activation. Blocking αV or FAK expression inhibited these effects, while nisin therapy, an antimicrobial agent, was shown to modulate these pathogen-mediated processes, presenting a potential therapeutic strategy. Overall, these data underscore the role of *F. nucleatum* and other periodontal pathogens in promoting a highly aggressive cancer phenotype through crosstalk between TLR/Myeloid differentiation primary response 88 (MyD88) and integrin/FAK signalling pathways (Kamarajan et al. 2020). Extended exposure to *F. nucleatum* has a significant impact on epithelial cell behavior. Nakano et al. observed that continuous stimulation of human tongue SCC cells by *F. nucleatum* for two to four weeks led to increased proliferation, invasion, and migration. The cells underwent EMT, as indicated by a time-dependent decrease in epithelial markers and an increase in mesenchymal markers. Morphological changes, including the formation of spindle-shaped cell structures and a loss of cell-to-cell contact, were also noted. These processes were associated with the upregulation of CD44, a marker of cancer stem cells. Interestingly, dexamethasone treatment inhibited *F. nucleatum*-induced EMT, suggesting that anti-inflammatory agents may mitigate bacterial-driven tumor progression (Nakano et al. 2024). Selvarai et al. further explored the strain-specific effects of *F. nucleatum subsp. polymorphum* on OSCC-derived keratinocytes (H357 and H376). They reported enhanced transcriptional and cytokine responses related to cell migration and angiogenesis, with significant upregulation of MMP9 in H376 cells. This was linked to increased invasive phenotypes and secretion of pro-angiogenic factors such as vascular endothelial growth factor A (VEGF-A). Inhibition of VEGF-A signalling using resveratrol significantly reduced the formation of capillary-like structures by endothelial cells, underscoring the role of angiogenesis in *F. nucleatum*-mediated tumour progression (Selvaraj et al. 2024).

There is also a potential role of outer membrane vesicles (OMVs) of *P. gingivalis* and *F. nucleatum* in modulating host cell behavior. OMVs contain packaged small RNAs (sRNAs) that have the potential to alert host mRNA function and/or stability. Liu et al. found that sRNA23392, one of the most commonly found sRNAs in OMVs of *P. gingivalis*, reduces the expression of desmocollin-2 (DSC2), a desmosomal cadherin family member, which results in the promotion of invasion and migration in OSCC cells (Liu et al., 2021). Chen et al. highlighted the role of *F. nucleatum* in promoting cancer metastasis. OMVs induced cancer cell invasion and migration both in vitro and in vivo by altering EMT-related protein expression. RNA sequencing revealed that OMVs activate intracellular autophagy pathways, and blocking autophagic flux with chloroquine significantly reduced the invasive capacity of cancer cells. This suggests that autophagy may play a crutial role in OMV-mediated tumor progression (Chen et al. 2024). Transcriptomic analysis by Zhang et al. revealed substantial dysregulation of mRNAs and lncRNAs in oral epithelial cells exposed to *F. nucleatum*. Functional analysis identified top hub genes (e.g., FYN, RAF1, ATM, VEGFA, JAK2) and lncRNA-hub gene co-expression networks involved in malignant transformation (Zhang et al. 2021).

6. Proliferation

Recent research highlights the role of *F. nucleatum* in promoting the proliferation of OSCC cells through various mechanisms. Li et al. demonstrated that *F. nucleatum* enhances OSCC cell proliferation both *in vitro* and *in vivo*. While the bacterium did not affect non-cancerous cells or alter E-cadherin CDH1 expression levels in CAL27 cells, it significantly increased tumor volume and Ki-67 proliferation indices in BALB/c nude mice. Interestingly, the overexpression of phosphorylated CDH1 in 293T cells did not influence β-catenin expression or the expression of cell cycle-related genes, suggesting that *F. nucleatum's* effects may bypass canonical CDH1 signalling (Li et al. 2024). Nie et al. proposed that *F. nucleatum* contributes to OSCC progression by fostering tumor cell proliferation, recruiting macrophages, and promoting macrophages' M2 polarization. The interaction between OSCC cells and macrophages mediated by chemokine (C-X-C motif) ligand 2 (CXCL2) was identified as a key driver of the pro-tumorigenic activity of *F. nucleatum*. These findings highlight the bacterium's ability to influence the tumor microenvironment in favor of cancer progression (Nie et al. 2024). Geng et al. highlighted additional mechanisms by which *F. nucleatum* promotes the proliferation of OSCC cells. The bacterium induces DNA damage in infected cells, leading to accelerated cell cycle progression. Furthermore, the downregulation of *Ku70* and *p53*, genes critical for DNA repair

and cell cycle inhibition, suggests a link between *F. nucleatum* infection and genomic instability (Geng et al. 2020). *F. nucleatum* was also found to produce hydrogen sulfide, which promotes OSCC cell proliferation via activation of COX2, AKT, and ERK1/2 signalling pathways in a dose-dependent manner (Zhang et al. 2016). This finding aligns with earlier findings by Ma et al., which demonstrated that hydrogen sulfide accelerates the cell cycle in OSCC cell lines (Ma et al. 2014). Uitto et al. reported that *F. nucleatum* upregulates cyclin-dependent kinases (CDKs) 7 and 9, enhancing the proliferation of human immortalized keratinocytes (Uitto et al. 2005). Together, these studies highlight the multifaceted role of *F. nucleatum* in promoting OSCC cell proliferation through mechanisms that involve direct effects on tumor cells, modulation of the tumor microenvironment, and metabolic alterations. A study by Ha et al. found that repeated infections by *P. gingivalis* alter the morphology of OSCC cells. Cytokeratin 13 was underexpressed, while N-cadherin and α-SMA were increased, suggesting EMT at the molecular level. EMT influences the acquisition of cancer stemness, which induces resistance to chemotherapeutic drugs and results in higher cancer aggressiveness (Ha et al. 2015). Furthermore, Groeger et al. observed that the *P. gingivalis* membrane upregulates the expression of genes involved in downstream TLR, NFκB and MAPK signalling pathways, associated with the pro-inflammatory immune response in primary and malignant oral epithelial cells, thus resulting in increased cancer proliferation (Groeger et al. 2017). OMVs may also play a role in accelerating cancer proliferation by upregulating the expression of PD-L1 protein in infected OSCC. This effect is sustained in cancer cells even after the bacterium has been eradicated. Elevated PD-L1 expression depends on receptor-interacting protein kinase 2 (RIP2) signalling. Activation of PD-L1 has been shown to protect cancer cells from host immune response (Groeger et al. 2020). Interestingly, a study by Cho et al. found that *P. gingivalis* infection might reduce cancer cell proliferation by inhibiting the cell cycle at the G1 phase. Infected OSCC cells exhibited an increase in the expression of p21, a kinase inhibitor that regulates cell cycle progression through the G1 phase. Conversely, the expression levels of cyclin D1 and cdk4, both critical for cell cycle progression, were decreased. However, these effects were transient, being prominent within the first 24 hours post-infection and diminishing by the 72-hour mark. Additionally, *P. gingivalis* infection was found to promote autophagy in OSCC cells, suggesting a possible survival mechanism by which the bacteria exploit support its persistence and potentially contribute to carcinogenesis (Cho et al. 2014).

Both *F. nucleatum* and *P. gingivalis* are pathogens associated with both inflammatory diseases and cancer. They share common mechanisms of action, which suggests a synergistic effect on cancer development. The common mechanisms are presented in Table 2.

7. Change in the Local Tumor Microenvironment

F. nucleatum has been implicated in modulating the immune microenvironment of tumors, facilitating immune evasion, and contributing to cancer progression and resistance to therapy. One of the key mechanisms involves the bacterial outer-surface protein Fap2, which binds to and activates inhibitory receptors TIGIT and CEACAM1 expressed on T and Natural Killer (NK) cells. This interaction suppresses anti-tumor immune responses by inhibiting the activity of these immune cells. Gur et al. demonstrated that this immune inhibition could be targeted using TIGIT and CEACAM1 inhibitors, suggesting a potential therapeutic strategy for tumors colonized by *F. nucleatum* (Gur et al. 2019). In addition to immune suppression, *F. nucleatum* has been associated with resistance to chemotherapy. Rui et al. reported that patients with a higher abundance of *F. nucleatum* exhibited reduced responsiveness to induction chemotherapy. Using 16S rRNA sequencing and metagenomic shotgun analysis, the study revealed that *F. nucleatum* was enriched in the nonresponsive group. Functional analyses highlighted its association with the platinum drug resistance pathway, microRNAs involved in cancer, and RNA degradation pathways, indicating a role in mediating therapy resistance (Rui et al. 2021). Further evidence of its contribution to chemoresistance comes from the work of Da et al., who explored the effects of *F. nucleatum* on cisplatin resistance and migration in OSCC. Their findings showed that *F. nucleatum* activates the Wnt/NFAT signalling pathway, leading to the downregulation of tumor suppressors, including p53 and E-cadherin. Pretreatment of CAL-27 and HSC-3 cells with *F. nucleatum* significantly increased cell survival rates following cisplatin exposure. The bacterium induced higher expression of the Wnt pathway gene *wnt5a* and NFATc3. Inhibition of NFATc3 using the peptide VIVIT reversed these effects, restoring p53 and E-cadherin expression (Da et al. 2021). He et al. demonstrated that *F. nucleatum* is enriched in the stromal regions of tumors, where CD31+ blood vessels and inflammatory cells, including CD45+ leukocytes and CD68+ macrophages, are densely distributed. Cyclin D1 and β-catenin were primarily expressed in tumor cells and interstitial vascular endothelial cells, respectively, whereas E-cadherin expression was localized to tumor cell membranes. Notably, NF-κB was highly expressed in the cytoplasm

Table II. The mechanisms promoting carcinogenesis of *F. nucleatum* and *P. gingivalis*.

Mechanism	Trigger factor	Explanation
Promotion of chronic inflammation	IL-6 (Gallimidi et al. 2015), IL-8 (Ha et al. 2016; Harrandah et al. 2020), TNF-α (Gallimidi et al. 2015; Liu et al. 2020)	Pro-inflammatory cytokines create a tumor-friendly microenvironment, promoting mutations and uncontrolled cell proliferation.
	P. gingivalis: IL-1R2, IL-18 (Liu et al. 2020)	
	F.nucleatum : IL-1β (Aral et al. 2020), TGF-β (Abdulkareem et al. 2017)	
Modulation of the immune response	Disruption of immune signaling (Wang and Ohura 2002; How et al. 2016; Bi et al. 2023)	Bacteria evade immune responses, leading to persistent infections and immune suppression.
Activation of the signal pathways associated with cancer development	NF-κB (Inaba et al. 2013; Gallimidi et al. 2015)	These pathways stimulate tumor growth, enhance survival and contribute to metastasis.
	F.nucleatum: Wnt/NFAT (Da et al. 2021), STAT3 (Harrandah et al. 2020)	
Increased invasion and metastasis	Epithelial-Mesenchymal Transition (EMT) (Ha et al. 2015; Harrandah et al. 2020), Matrix metalloproteinases (MMPs) (Gallimidi et al. 2015; Ha et al. 2016; Harrandah et al. 2020)	EMT enables cancer cells to detach and spread, while MMPs degrade the extracellular matrix, aiding metastasis.
Production of toxins and factors supporting tumor growth	Gingipains (P. gingivalis) (How et al. 2016; Bi et al. 2023), FadA (F. nucleatum) (Chen et al. 2022)	Gingipains disrupt cellular homeostasis, and FadA binds to E-cadherin, increasing epithelial permeability and promoting cancer progression.
Microbiota dysbiosis	Dysbiosis (Horvat Aleksijević et al. 2022; Cai et al. 2024)	Both bacteria contribute to changes in the microbiome , which promotes the proliferation of pathogenic microorganisms and increases the risk of developing cancer.

of tumor and stromal cells, while hypoxia-inducible factor 1-alpha (HIF-1α) was predominantly observed in the cytoplasm of stromal cells. HIF-1α expression was particularly high in areas with dense *F. nucleatum* distribution, suggesting that the bacterium exacerbates inflammation and hypoxia by interacting with NF-κB and HIF-1α signalling in OSCC tissues (He et al. 2023). These results underscore the role of *F. nucleatum* in promoting chemoresistance and enhancing cancer cell migration. Overall, *F. nucleatum* employs a multifaceted approach to evade immune surveillance and resist therapeutic interventions. By suppressing immune responses through TIGIT and CEACAM1 activation, modulating signalling pathways such as Wnt/NFAT, and contributing to drug resistance, *F. nucleatum* poses a significant challenge in the treatment of tumors it colonizes. Targeting these bacterial-mediated mechanisms presents a promising approach for enhancing cancer therapies (Da et al. 2021; Rui et al. 2021; He et al. 2023).

On the other hand, *Porphyromonas gingivalis* promotes OSCC progression by inducing the formation of neutrophil extracellular traps (NETs) within the tumor microenvironment (TME). These NETs enhance cancer cell migration, invasion, and colony formation. In vivo studies have further demonstrated that NETs play a crucial role in facilitating tumor metastasis (Guo et al., 2023). Furthermore, *P. gingivalis* promotes cancer progression by recruiting tumor-associated neutrophils through activation of the CXCL2/CXCR2 axis in the cancer microenvironment (Guo et al. 2022). Additionally, *P. gingivalis* inhibits macrophages from phagocytizing OSCC cells. Infection by a bacterium led to an increase in the polarization of M2 macrophages, which is tumor-promoting (Liu et al. 2020). Interestingly, Lan et al. investigated how *P. gingivalis* might suppress OSCC growth by downregulating MUC1 and CXCL17 expression, leading to the reversal of the immunosuppressive TME and thereby inhibiting OSCC progression (Lan et al. 2023).

8. Conclusions

The evidence presented in this review underscores the significant role of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in the pathogenesis of oral squamous cell carcinoma. These bacterial species contribute to tumor initiation and progression through shared mechanisms, including immune evasion, the upregulation of inflammatory mediators (e.g., IL-6, IL-8), and modulation of host cellular signalling pathways. In addition, they exhibit distinct but complementary virulence strategies, including the production of gingipains by *P. gingivalis* and FadA adhesin by *F. nucleatum*, which further support tumor development.

Their presence within the tumor microenvironment suggests potential diagnostic and prognostic relevance, particularly for *F. nucleatum*, which may serve as a biomarker of OSCC progression. Moreover, bacterial virulence factors offer promising targets for therapeutic intervention. However, further studies are required to elucidate the precise molecular mechanisms involved and to explore innovative antimicrobial and immunomodulatory strategies aimed at mitigating their oncogenic effects.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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NARLX TWO-COMPONENT SIGNALING SYSTEM AS A KEY MECHANISM OF BACTERIAL ADAPTATION

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Abstract: The two-component NarLX signaling system is a key mechanism for bacterial adaptation to changing environmental conditions. Composed of a histidine kinase (NarX) and a response regulator (NarL), it enables bacteria to accurately detect and respond to environmental changes, particularly in nitrate and nitrite concentrations.
Studies on various bacterial species, including *Escherichia coli*, *Salmonella enterica serovar* Typhimurium and *Pseudomonas aeruginosa*, have shown that the NarLX system plays a fundamental role in anaerobic metabolism, biofilm formation, colonization ability and virulence. In *E. coli*, the system activates 51 operons and inhibits 41 of them, while in other species it affects key survival processes.
In the context of increasing antibiotic resistance, the NarLX system represents a promising therapeutic target. Potential strategies include the development of small-molecule inhibitors that could precisely modulate bacterial behavior without destroying the bacterial flora.
The research points to the possibility of developing innovative methods to combat bacterial infections, offering an alternative to traditional antibiotics and minimizing the risk of increasing bacterial resistance.

Keywords: bacterial adaptation, NarLX signaling, nitrate sensing, therapeutic target, two-tomponent tystem

1.Introduction

Bacteria are among the oldest and most widespread forms of life on Earth, characterised by a remarkable ability to colonise almost any conceivable environment. Their diversity and adaptability extend to extremely diverse ecosystems - from the deep ocean hydrothermal vents with temperatures above 100°C, to Antarctic glaciers, to hypersaline lakes and environments with extreme pH (Rothschild and Mancinelli 2001; Pikuta et al. 2007; Cowan et al. 2010).

To do this, they have had to specialised, among other things, in a number of mechanisms that allow them to respond to changing conditions. The systems they evolved are broadly classified according to their molecular complexity, including one-component systems (OCS), which primarily respond to internal signals and two-component systems (TCS), which can detect and respond to extracellular signals (Parkinson 1993; Stock et al. 2000; Laub and Goulian 2007).

One- and two-component systems provide a fundamental mechanism for bacteria to track and respond to the slightest changes in the surrounding environment (Mitrophanov and Groisman 2008). Among these TCS are particularly significant due to their widespread presence in bacteria, their role in regulating critical processes such as virulence, biofilm formation, and stress responses, and their potential as targets for antimicrobial therapies. The classical two-component system consists of two components - a histidine kinase (HK) that monitors external stimuli and a response regulator (RR) (Fig.1). The former

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occurs as a transmembrane protein composed of a histidine kinase inside the cell and a differentiated sensing domain above the membrane. The second, on the other hand, usually being inside after receiving a signal, affects regulation of gene expression. HK switches between active and inactive states depending on the signal. In the activated state, histidine kinase transfers phosphate from ATP to histidine, its own amino acid, and then to the response regulator aspartate. TCS can respond to a number of different stimuli essential for bacterial processing. They can be activated by nutrients, pH changes, the occurrence of substances that

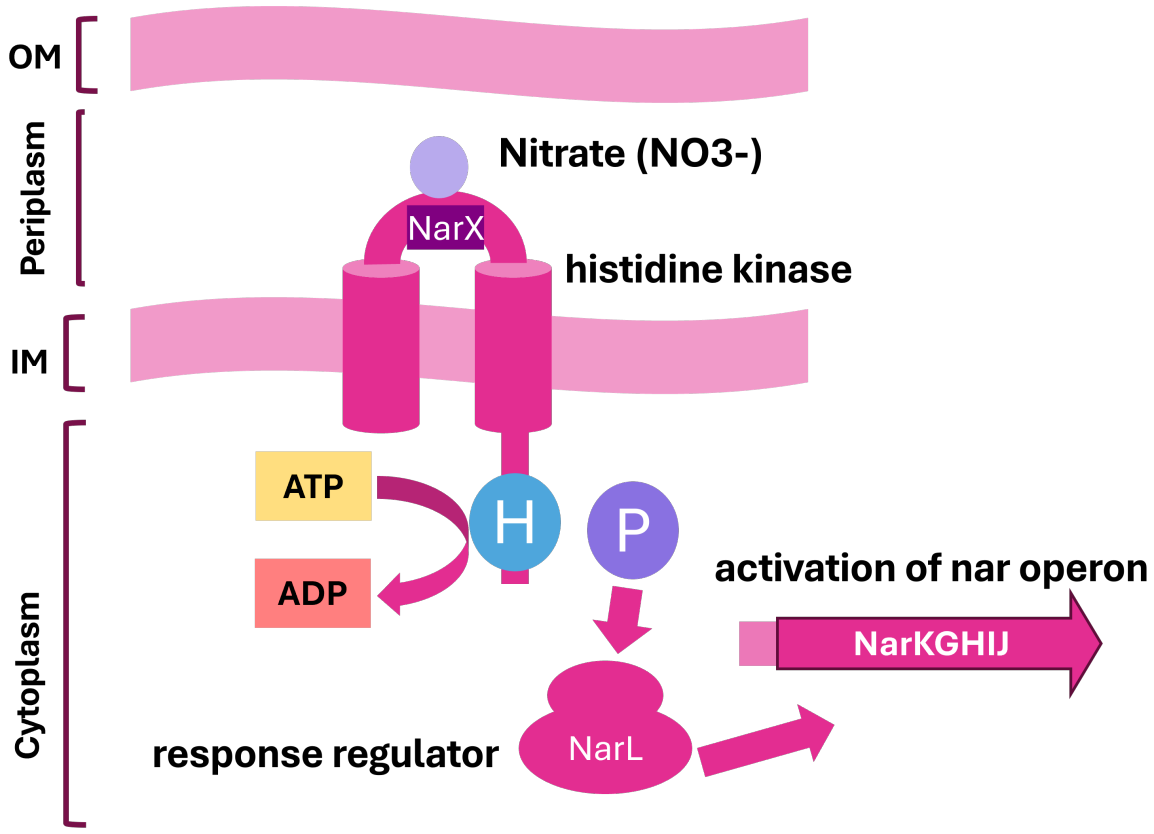

Figure 1. The general mechanism of action of two-component regulatory systems.

potentially threaten the integrity of the cell, and their own misfolded proteins (Burbulys et al. 1991; Kato et al. 1999; Skerker et al. 2005).

Many bacteria use nitrate and nitrite as electron acceptors in respiration in the absence of oxygen. For this metabolism to take place, the microorganism must first detect the presence of the proper conditions. Several two-component systems are responsible for detecting different forms of nitrogen. These do not occur in all bacteria in the same amount or activity. The focus of this study is to characterise NarXL, a specific two-component system associated with the detection and regulation of nitrate and nitrite metabolism, providing insights into their molecular mechanisms and their role in bacterial adaptation to anaerobic environments (Alvarez et al. 2016; Gao et al. 2019).

The NarXL system consists of the histidine kinase NarX and the response regulator NarL. The first component detects extracellular nitrate/nitrite, autophosphorylates and transfers the phosphate group to NarL, which then modulates gene expression. Using *Escherichia coli* as an example, their molecular complexity and evolutionary significance can be clearly traced (Cavicchioli et al. 1995). In these bacteria, NarXL systems play a fundamental role in nitrate metabolism, controlling the expression of genes responsible for nitrate and nitrite reduction under anaerobic conditions (Blattner et al. 1997; Constantinidou et al. 2006). The

NarL and NarX proteins form a highly precise molecular system that enables the bacteria not only to survive but also to efficiently utilise diverse nitrogen sources under varying environmental conditions (Unden and Schirawski 1997; Stewart 2003). In *E. coli,* it was discovered that NarL activation is responsible for the activation of 51 operons and the inhibiting 41 of them (Constantinidou et al. 2006).

One of the main targets of the response regulator is the activation of the *narKGHJI* operon, which encodes

Table I. NarXL-regulated operons in *E. coli* associated with anaerobic metabolism and response to the presence of nitrate.

Genes	Regulation	Function	Source
adhE	Repression	Bifunctional aldehyde-alcohol dehydrogenase	(Membrillo-Hernández and Lin 1999)
aspA	Repression	Aspartate ammonia-lyase	(Goh et al. 2005)
caiF	Repression	Transcriptional activatory protein	(Eichler et al. 1996)
dcuB-fumB	Repression	Anaerobic C4-dicarboxylate transporter/Fumarate hydratase class I, anaerobic	(Golby et al. 1998)
dcuSR	Repression	TCS responding to external C4-dicarboxylates	(Goh et al. 2005)
dmsABC	Repression	DMSO/TMAO reductase, anaerobic	(Bearson et al. 2002)
fdnGHI	Activation	Formate dehydrogenase-N	(Li et al. 1994)
focA-pflBA	Repression	Formate channel/pyruvate-formate lyase	(Kaiser and Sawers 1995)
frdABCD	Repression	Fumarate reductase	(Li et al. 1994)
hyaABCDEF	Repression	Hydrogenase 1	(Richard et al. 1999)
hybOABCDEFG	Repression	Hydrogenase-2	(Richard et al. 1999)
napFDAGHBC	Repression	Periplasmic nitrate reductase	(Stewart et al. 2003)
nirB	Activation	Nitrite reductase (NADH) large subunit	(Tyson et al. 1994)
nrfABCDEFG	Activation	Formate-dependent nitrate reductase	(Tyson et al. 1994)
nuoABCEFGH-IJKLMN	Activation	Respiratory complex I	(Bongaerts et al. 1995)
yeaR-yoaG	Activation	Unknown	(Lin et al. 2007)
ynfEFGHI	Repression	Putative dimethyl sulfoxide reductase chain	(Xu et al. 2009)

a membrane-bound antiporter and nitrate reductase. The operon includes five genes. NarK belongs to the major facilitator superfamily (MFS) of transmembrane transporters and serves as a nitrate/nitrite antiporter. Further genes in the operon encode NarG (catalytic subunit), NarH (iron-sulphur cluster subunit), NarJ (chaperone) and NarI (membrane anchor), thus forming the membrane-bound respiratory nitrate reductase (Nar) (Bertero et al. 2003). This system regulates dissimilatory nitrate reduction, an energy-generating process distinct from assimilatory pathways that focus on nitrogen attachment (Unden and Schirawski 1997) (Fig.2). Table I presents other operons regulated by NarXL system.

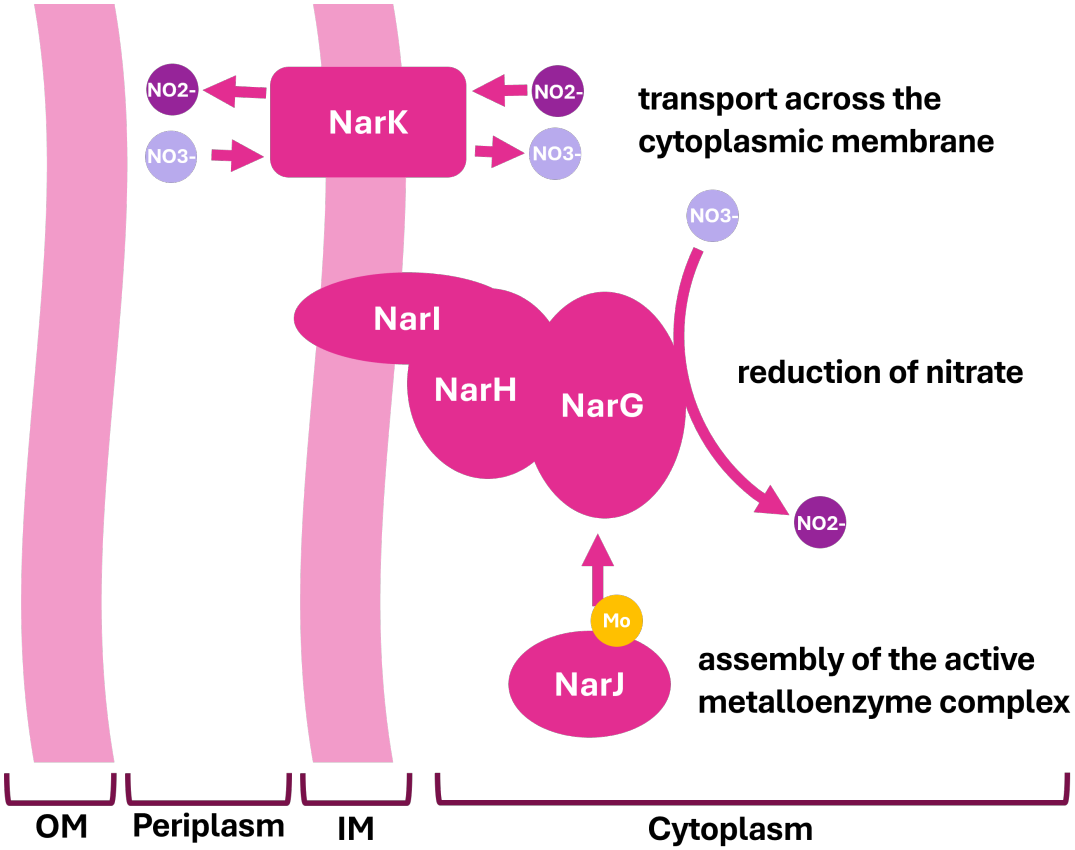

Figure 2. The general mechanism of action *nar* nitrate reductase and nitrate/nitrite antiporter NarK.

2. NarXL and NarQP interactions

Although the primary focus of this review is the importance of NarXL in nitrate metabolism and bacterial pathogenicity, however, studies on *E. coli* and *Salmonella enterica* have revealed the presence of an additional system, NarQP, which works alongside NarXL to detect and respond to changes in nitrate concentration. Although the two systems overlap functionally, they show differences in interaction specificity and regulatory strategies.

NarX binds to NarL more firmly than NarP, creating a high-affinity complex. The NarQ kinase, on the other hand, interacts with both NarL and NarP, allowing for a dual signal response (Chiang et al. 1997; Stewart 2003). The differences between the systems also relate to the kinetics of signal transduction. NarQ transfers a phosphate group to NarL at a rate ten times faster than NarX, facilitating rapid gene activation. However, after nitrate depletion NarX provides faster and more accurate signal termination, because it is more effective at dephosphorylating NarL. Notably, NarQ responds to both nitrate and nitrite, whereas NarX is selective for nitrate, allowing for differential regulation depending on environmental conditions. They regulate the expression of key operons such as the nitrate reductase operon (*narGHJI*), the fumarate reductase operon (*frdABCD*) and the nitrite reductase operon (*nrfABCDEFG*) (Rabin and Stewart 1993).

Analysis reveals significant differences in the genetic organisation of these systems. *NarX* genes are more closely related to nitrate reductase genes located in the *narGHJI* cluster, while the distribution of *narQ* and *narP* genes is characterised by greater variability (Schröder et al. 1994). The systems also differ in their DNA binding mechanisms. The phosphorylated NarL binds DNA as a stable dimer, recognising tandem inverted repeats with a sequence of 7-2-7 bp in the promoters of genes such as narGHJI and frdABCD. This structure enables cooperative binding and strong transcriptional activation of pathways crucial for energy acquisition. In contrast to NarL, the NarP protein binds DNA mainly as a monomer or briefly forming a dimer. This property limits its capacity to occupy high-affinity binding sites, narrowing NarP's regulatory role to low-affinity targets such as the *nrfABCDEFG* operon encoding the NrfA periplasmic formate-dependent nitrite reductase, which is responsible for re-

ducing nitrite to ammonium ions involved in nitrite detoxification. Structurally, both NarQ and NarX consist of seven domains, including a periplasmic sensor domain, transmembrane region, HAMP domain, signalling helix, GAF-like domain, DHp, and CA catalytic domain. These components mediate nitrate binding, signal transduction, and response regulation across the cell membrane, spanning more than 200 Å from the extracellular ligand-binding site to the cytoplasmic catalytic core. Interestingly, NarQ contains conserved cysteine residues in its CA domain that may form a disulfide bond responsive to the cellular redox environment, potentially linking nitrate sensing to the oxidative-stress response (Noriega et al. 2008, 2010; Godfrey et al. 2017; Gushchin et al. 2021).

3. Importance of nitrate metabolism in bacterial virulence

Nitrate metabolism plays a key role in the regulating functioning and virulence of bacteria. For example, study by Martín-Rodríguez et al. (2020) investigated how nitrate metabolism affects the biofilm formation and pathogenicity of uropathogenic *E. coli* (UPEC) strains. Mutations were made in genes encoding nitrate reductases (*narGHJI, narZYWV, napFDAGHBC*), creating single, double and triple mutants. These mutants were then used to study the effects of nitrate reduction on biofilm formation, the expression of the biofilm master regulator CsgD, and the efficiency of bacterial colonization in a rat model of urinary tract infection (UTI). The results showed that *narGHJI* genes, which encode a key membrane nitrate reductase, were essential for efficient nitrate reduction under experimental conditions. Nitrate availability significantly modulated biofilm structure, particularly through its effects on the biosynthesis of curli fimbriae and cellulose. These processes were tightly regulated by CsgD, the master regulator of biofilm formation. In this study NarL was found to directly or indirectly affect the expression of *csgD*, leading to changes in biofilm formation. In the absence of functional nitrate reductases or under nitrate-limiting conditions, CsgD expression increased, resulting in enhanced biofilm production. In an *in vivo* model, mutant strains lacking nitrate-reducing ability were strongly outclassed in competitive infections with the wild-type strain, indicating that nitrate metabolism plays a role as bacterial "fitness factor" in the host environment. The results suggest that nitrate-reducing ability, although not necessary, significantly enhances UPEC adaptability in host tissues (Martín-Rodríguez et al. 2020).

Nitrate metabolism not only supports anaerobic respiration but also acts as a critical environmental signal influencing biofilm regulation and pathogenicity via two-component systems (TCS). In *E. coli*, the ArcAB TCS senses respiratory stress and modulates biofilm responses to sub-inhibitory antibiotics. Deletion of *arcA* or *arcB* leads to constitutively elevated biofilm levels and eliminates further stimulation by antibiotics, while nitrate supplementation suppresses this effect by relieving oxidative stress. This suppression depends on active nitrate respiration, as shown by the inability of *narG* mutants to respond, linking nitrate metabolism to biofilm regulation through respiratory chain activity and ArcAB signalling (Yaeger et al. 2023).

The study on *Salmonella enterica serovar* Typhimurium revealed that the *narL* and *fnr* genes, which are responsible for anaerobic metabolism, exhibit synergy. These two systems regulate different but overlapping metabolic pathways. Deletion of both these genes prevents the use of nitrate as an electron acceptor, leading to decrease in nitrite production by 99%. Absence of the *narL* gene alone results in a 73% reduction in biofilm formation under anaerobic conditions, indicating a key role for this regulator in the formation of biofilm structures, which play an important role in bacterial colonization and survival. In terms of motility, the *ΔnarL* mutant showed a significant reduction in swimming (by 26%) and swarming (by 61%) abilities under anaerobic conditions, which can be attributed to dysregulation of genes related to flagella activity, such as *fliA*. In vitro analyses in a mouse model revealed that the *ΔnarL* mutant has a significant decrease in replication capacity inside macrophages and epithelial cells, indicating impaired adaptability and survival in host niches. Additionally, significant reduction in the number of *ΔnarL* mutant bacteria was observed in organs such as the spleen and liver, indicating a limited capacity for systemic dissemination and effective colonisation. Histopathological analysis revealed less intense inflammatory processes compared to the infection caused by the wild-type strain, highlighting the significant impairment of the *ΔnarL* mutant's ability to induce an inflammatory response (Priyadarsini et al. 2024). The natural inflammatory response in the host is produced during *Salmonella* infection drives the production of nitric oxide (NO), which is rapidly oxidised to nitrate (NO_3^-) via transient intermediates. Pathogens are able to use this inflammatory nitrate pool as an electron acceptor for anaerobic respiration, facilitating survival in hypoxic niches (Mian et al. 2013; Scales et al. 2016; Fang and Vázquez-Torres 2019).

Further expanding on this, recent research has shown that host-derived nitrate acts as a signalling

molecule influencing *Salmonella* lifestyle transitions. Nitrate exposure downregulates the production of a major biofilm component - curli fimbriae - through repression of *csgD* and reduction of intracellular cyclic-di-GMP levels. However, it simultaneously promote motility via flagellar activation. This shift from a biofilm-associated to a motile phenotype, mediated through the NarXL, facilitates epithelial invasion and systemic dissemination. Inhibition of nitrate production *in vivo* led to increased *csgD* expression, confirming nitrate's role in repressing biofilm formation during infection (Miller et al. 2022).

The NarXL mutants of *Burkholderia pseudomallei*, an endemic tropical bacterium is known as the etiological agent of melioidosis, show a couple of alterations, including a significant reduction in the expression of key biofilm matrix components and a significant reduction in the expression of genes responsible for the biosynthesis of secondary metabolites. These include non-ribosomally derived peptide synthetase (NRPS), polyketide synthases (PKS), and genes responsible for the biosynthesis of bacteriocins such as bactobolins, maleilactones, and syrbactins. The *narX* and *narL* mutants show key differences in their ability to replicate intracellularly. In the macrophage infection model, a significant reduction in the ability to multiply inside host cells altered gene expression patterns compared to the wild-type strain. Additionally, the disruption of immune response evasion mechanisms was observed (Mangalea and Borlee 2022).

In *Pseudomonas aeruginosa* the nitrate reductase system exhibits complex molecular interactions that determine its anaerobic metabolism. Notably, the *narQP* genes does not appear to be present in the genome of *P. aeruginosa*. In this bacterium, the NarL protein has a crucial function in the activation of *hemeA, nirQ* and *dnr* genes, which directly determine the metabolic transformation under anaerobic conditions (Vollack et al. 1998; Härtig et al. 1999; Schreiber et al. 2007). Further, Van Alst et al. (2007) analysed the role of the *narL* and *narX* genes, as well as the *narK1K2GHJI* and *napEFDABC* operons, in nitrate metabolism, motility and biofilm formation in *P. aeruginosa*. Experiments were conducted on mutants with regulatory (*narL, narX*) and structural (*narGH, napA*) genes deleted, evaluating their effects on mobility (swimming, crawling), biofilm formation and virulence in the nematode *Caenorhabditis elegans* infection model. The study showed that *narL* plays a fundamental role in regulating the balance between mobility and biofilm formation. Mutants lacking *narL* produced excessive amounts of rhamnolipids, which increased their crawling ability at the expense of stable biofilm formation. The *narX* gene was responsible for nitrate sensing; its absence led to impairing both mobility and biofilm formation. The *narK1K2GHJI* operon, which encodes membrane nitrate reductase, was essential for nitrate reduction and energy delivery during anaerobic circumstances. A *narGH*-deficient mutant not only lost the capacity to produce biofilm, but also became entirely avirulent in the nematode model, showing its critical involvement in pathogenesis (Van Alst et al. 2007).

Further, presence of assimilatory, dissimilatory nitrate reduction and complete denitrification pathway, likely provides *P. aeruginosa* with enhanced metabolic flexibility. Having all of this metabolic pathway is presumably advantageous for survival and competitiveness in dynamic or nutrient-limited environments.

Supporting this notion, recent findings suggest that nitrogen assimilation is not only crucial for metabolic homeostasis but also modulates host-pathogen interactions. For instance, *P. aeruginosa* has been shown to influence *C. elegans* chemotaxis and pathogenicity through the regulation of nitrogen assimilation, particularly via the production of volatile ammonia, a by-product of this pathway. The nitrogen assimilation mutants impaired both attraction and colonization of *C. elegans*, underscoring the significance of this pathway in infection dynamics (Marogi et al. 2024).

Moreover, a study by Kuang et al. (2021) demonstrated that inactivation of the nitrite-dependent nitric oxide (NO) biosynthesis pathway—part of the denitrification machinery—conferred increased resistance to the antibiotic cefoperazone-sulbactam in *P. aeruginosa*. This was associated with lower levels of NO due to impaired NADH-driven electron flow, linking metabolic shifts in nitrogen pathways to overlapping mechanisms of antibiotic resistance (Kuang et al. 2021).

A schematic comparison (Fig. 3) highlights the metabolic distinctions between *E. coli*, which possesses only a dissimilatory nitrate reduction pathway, and *P. aeruginosa*, which integrates assimilatory, dissimilatory, and complete denitrification routes—emphasising its greater ecological and pathogenic plasticity.

The diagram is structured around three major functional pathways: assimilatory nitrate reduction (purple), dissimilatory nitrate reduction (pink), and denitrification (blue). Substrates and products are shown as blue circles, and directional arrows denote the enzymatic flow from extracellular nitrate or nitrite towards terminal products, such as ammonia (NH_3) or dinitrogen gas (N_2).This schematic was reconstructed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps as a primary reference (Kanehisa et al. 2023), with organism-specific nitrogen metabolism data curated for *E. coli* and *P. aeruginosa*.

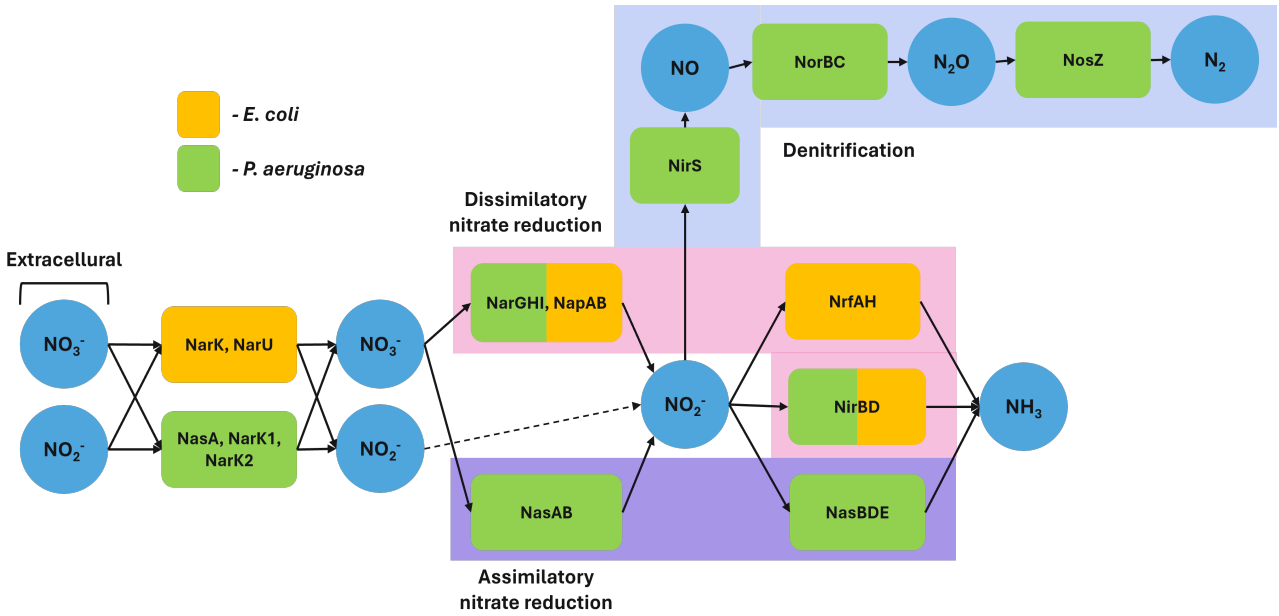

Figure 3. Comparative nitrogen metabolism pathways in *E. coli* and *P. aeruginosa*.
This schematic illustrates the assimilation and dissimilation of nitrogen pathways in *E. coli* and *P. aeruginosa.* The rectangles colour-coded according to the bacteria indicate the enzymes involved in the processes. Orange represents those present in *E. coli*, while green indicates enzymes found in *P. aeruginosa*. Those shared by both organisms are shown as split-coloured boxes.

Studies on BCG (Bacillus Calmette-Guérin) strain of *Mycobacterium bovis*, which is used as a TB vaccine, shed light on the processes of metabolic adaptation in anaerobic conditions. Deleting the *narG* gene drastically reduces the bacterium's capacity to thrive in important target organs. In mouse studies, a mutant lacking the *narG* gene was shown to exhibit significantly reduced colonization of the lungs, liver and kidneys. Of particular interest, the effect of lacking the nitrate reductase enzyme is highly selective - minimal changes in bacterial adaptive potential were observed in the spleen tissue (Fritz et al. 2002). Interestingly, while the BCG genome encodes the *narXL* two-component regulatory system involved in nitrate sensing and response, its components are dispersed across the genome, suggesting potential regulatory decoupling or modularity. Moreover, the genome lacks the *narQP* system altogether (Gomes et al. 2011).

Unlike many other bacteria, *Staphylococcus aureus* lacks the binary systems NarXL and NarQP. However, these bacteria make up for this with a network of alternative regulatory systems that can contribute to the regulation of nitrate reductase activity. One component of this network is the two-component system SrrAB. It responds to both nitrosative stress and hypoxia by modulating genes involved in electron transport, anaerobic metabolism. Although SrrAB does not directly replace NarXL or NarQP, it can influence pathways involved in nitrate respiration by indirectly detecting electron transport chain activity and redox status (Kinkel et al. 2013). Recent detailed studies indicate that *S. aureus* has as many as 17 different two-component systems, many of which share similar regulatory pathways, making it more complicated to control genes related to virulence and metabolism, including those for nitrate and nitrite processing (Ahator et al. 2024). This creates a more complex regulatory network than in organisms with dedicated nitrate systems and highlights the adaptive plasticity of the *S. aureus* regulatory architecture.

Studies on *S. aureus*, particularly in the context of methicillin-resistant strains (MRSA), have revealed an important role for the nitrate reductase NarGHJI in virulence regulation. The NarGHJI operon affects the expression of virulence genes such as *RNAIII*, *agrBDCA*, *hla*, *psmα* and *psmβ*, and its inactivation leads to their down-regulation and a reduction in haemolytic activity. Observations on mouse models and *Galleria mellonella* confirmed that *narG*-deficient strains show significantly reduced virulence. Expression of *narGHJI* is the highest in the early and mid-log phases, suggesting a major role for these phases in the regulation of *agr*, a global regulator of virulence genes. RNA-seq analysis showed that inactivation of *narGHJI* results in an increase in the expression of 63 genes and a decrease in 89, including *vraX*, which encodes a protein involved

in pathogenesis. NarGHJI can modulate virulence in an *agr*-dependent and independent manner, indicating a complex regulatory mechanism that requires further study. The results highlight the novel role of NarGHJI in the control of molecular determinants of virulence and provide a potential basis for developing strategies to combat *S. aureus* infections (Li et al. 2023).

4.Practical aspects of studying NarXL two-component system

The NarXL system is a key signalling mechanism for the survival and establishment of bacterial virulence in the host. Its role in bacterial virulence, particularly in the context of biofilm formation and the potential for urinary tract infection, makes it an extremely promising therapeutic target, especially for uropathogenic bacteria.

In the context of a growing global antibiotic threat, two-component systems (TCS) such as NarXL offer an alternative approach to combating bacterial infections. The World Health Organization (WHO) indicates that antibiotic resistance is one of the ten most serious global public health threats, with up to 10 million deaths predicted annually by 2050 and economic losses estimated at around $100 trillion (Jonas et al. 2017; Murray et al. 2022).

Studies to date point to a remarkable variety of bacterial resistance mechanisms that can be activated by two-component systems. These include but are not limited to modification of the cell surface, decreased drug uptake or increased drug removal, activation of antibiotic-degrading enzymes, and biofilm production (Beier and Gross 2006; Alvarez and Georgellis 2023). Further research on NarXL requires a deeper understanding of its mechanisms of action and the development of highly specific inhibitors. It will also be crucial to conduct thorough analyses to avoid potential side effects associated with possible interaction with host cells.

Identifying small-molecule inhibitors of the NarXL system would allow precise modulation of pathogenic bacterial behaviour, such as biofilm formation and expression of virulence factors. This type of approach could not only be used as an adjuvant therapy in combination with traditional antibiotics but could also minimise side effects of the treatment, such as destruction of the gut microbiota. In addition, the high structural similarity of signalling circuit elements in different bacterial species offers the possibility of developing broad-spectrum medicine (Barrett et al. 1998; Worthington et al. 2013).

A potential solution may be to search databases of chemical molecules using bioinformatics methods and molecular modelling. In this study authors used the ZINC22 database of 37 billion commercially available compounds (including 4.5 billion in 3D docking-ready form) to identify potential inhibitors of the NarL protein in Mycobacterium tuberculosis. After virtual screening, the researchers selected ZINC63191404 a nitrobenzeneaminopiperidine derivative. It was shown that it can potentially bind to the NarL phosphorylation site more stable than acetylphosphate, natural phosphate donor. In silico molecular dynamics analysis suggested that the selected compound forms stable hydrogen bonds with the protein and inhibits its activity at nanomolar concentrations. Despite encouraging theoretical results, the compound efficacy has not yet been confirmed experimentally. The authors emphasized the need for further *in vitro and in vivo* studies to assess its potential as an antimicrobial agent (Shivakumar et al. 2014; Tingle et al. 2023).

5. Conclusions

Two-component systems (TCS) are fundamental adaptive mechanisms in bacteria and are emerging as promising therapeutic targets due to their key roles in bacterial survival, virulence and antibiotic resistance. One of the process regulated by TCS is nitrate/nitrite metabolism, where NarXL proteins play important role.

Research on the NarXL system has significantly advanced our understanding of its molecular mechanisms. While additional interdisciplinary studies are needed to fully understand its functions, current evidence indicates that the NarXL system plays an essential role in regulating nitrate and nitrite metabolism, as well as mediating adaptive processes including biofilm formation and virulence factor expression. Therefore, these characteristics, combined with their specificity for bacterial cells, render the NarXL system a promising target for innovative therapeutic strategies, especially against uropathogenic bacteria.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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REVIEW
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THE IMPACT OF BACTERIAL DYSBIOSIS ON THE DEVELOPMENT OF PANCREATIC CANCER AND ITS TREATMENT IN HUMANS?

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Abstract: Pancreatic cancer is one of the deadliest types of cancer and is still difficult to treat, despite recent medical advances. Many studies show correlations between the human oral and intestinal microbiota and pancreatic cancer. The mechanism of action of microorganisms on the pancreatic microenvironment is still not fully understood. The aspect of immune response related to treatment and the microorganisms present has also been addressed. This review presents current evidence on the human microbiota, mainly focusing on its bacterial contribution and influence, and identifies the areas that may benefit most from earlier diagnosis and the potential for less invasive treatment of pancreatic cancer. A review of all the latest literature (n=86) in peer-reviewed English-language journals with an Impact Factor from PubMed (NCBI) and Google Scholar was conducted; most studies concerned the analysis of bacteria in the human microbiota or in a mouse model.

Keywords: microbiome, pancreas, tumor

1. Introduction

Pancreatic cancer (PC) is characterised by a high mortality rate and low survival rates. It is classified as the third most common cancer as a cause of cancer deaths, right after lung cancer and colon cancer. The estimated number of deaths in 2024 from pancreatic cancer in the United States for both sexes is 66.440 thousand, of which 51.750 thousand will be fatal cases, accounting for 77.89% of pancreatic cancer mortality (Siegel *et al.* 2023; Siegel *et al.* 2024). A study by Santucci *et al.* (2024) forecasts a continued decline in cancer mortality across the EU, with age-standardized rates expected to fall by 6.5% for men and 4.3% for women relative to 2018 levels. On the contrary, pancreatic cancer shows an unfavorable prediction in studies in the EU, and mortality from this cancer is increasing in both sexes. Mortality rates for both sexes (+1.6% in men and +4.0% in women) (Santucci *et al.* 2024).

Pancreatic cancer is non-specific and difficult to diagnose in its early stages. The anatomical location of the pancreas is an additional complication in the diagnosis of pancreatic cancer (Maitra *et al.* 2024). The cancer can develop in the exocrine cells of the pancreas, which are responsible for producing digestive juices, or in the endocrine cells of the pancreas, which produce hormones. However, pancreatic cancer starts to develop in the exocrine cells in approximately 95% of cases and belongs to the histological subtype of ductal adenocarcinoma, which is associated with a poor prognosis and a lack of effective therapies (Grant *et al.* 2016; PDQ Adult Treatment Editorial Board 2024). Pancreatic exocrine insufficiency, resulting from obstruction of the pancreatic duct, fibrosis, or tissue loss, contributes to malnutrition and weight loss in patients (Vujasinovic *et al.* 2017; de la Iglesia *et al.* 2020; Sabater *et al.* 2016).

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The etiological factors of PC include non-modifiable germline genetic factors as well as modifiable factors such as smoking, obesity, and diabetes (Stanciu *et al.* 2022; Gentiluomo *et al.* 2022). Approximately 5-10% of pancreatic cancer patients have a familial background, while the genetic contribution in the remaining cases is unknown, suggesting a role for the environment in the etiology of the disease (Goggins *et al.* 2020). Molecularly, epigenetic alterations and somatic mutations, especially activating mutations in the *KRAS* gene, play a central role in the pathogenesis of PC. *KRAS* mutations, though rarely inherited, are commonly acquired and act as a critical driver of tumor initiation and progression (Baylin and Jones 2016; Chen *et al.* 2021; Alonso-Curbelo *et al.* 2021).

The study and development of epigenetic mechanisms of metastasis in pancreatic duodenal carcinoma has the potential to facilitate effective therapeutic intervention(Wang *et al.* 2021; Chen *et al.* 2021).

Recently, the role of the microbiome in the context of pancreatic cancer has also received much attention. A correlation has been observed between the commensal microbiome (classified as an environmental factor) and certain gastrointestinal cancers, including those affecting patients with pancreatic cancer. However, the available data remain inconclusive (Sexton et al., 2022). Further study of the epigenetic mechanisms underlying metastasis in pancreatic duodenal carcinoma could facilitate the development of effective therapeutic interventions. The natural ecosystem of microorganisms is also susceptible to various environmental factors, which may contribute to the disruption of the organism's overall homeostasis. It has been shown that alterations of the microbiota in the pancreas-gut axis can cause chronic pancreatic inflammation, which is one of the risk factors for pancreatic cancer. It has been proven that fungal intestinal microbiota is also associated with carcinogenesis. Fungal dysbiosis with yeasts of the genus *Malassezia* has been implicated in the development of cancer at an early stage (Speth *et al.* 2022). The previous studies have shown the influence of fecal and oral microbiomes not only on pancreatic carcinogenesis but also on immune modulation in pancreatitis (Thomas and Jobin 2020). Microbiota can be a powerful source of biomarkers for identifying individuals with pancreatic ductal adenocarcinoma and their prognosis (Pourali *et al.* 2024).

This review specifically highlights research on the bacterial microbiome, which represents the most extensively studied microbial group in the context of pancreatic cancer. In this article, we present a general overview of what is known about the impact of the bacterial oral and gut microbiome on the incidence of pancreatic cancer and discuss the effectiveness of therapy by modulating the microbiome. A literature search was performed using databases such as PubMed and Google Scholar, with search terms including "Pancreatic Cancer," "Resistance," "Therapy" and "Microbiota". The analysis included 89 articles, which were primarily based on meta-analyses, as well as prospective, retrospective, clinical, metagenomic, and expectorant studies. The study population comprised individuals diagnosed with pancreatic cancer or pancreatic ductal adenocarcinoma, as well as a control group of healthy individuals. We also included pancreatitis as one of the most important etiological factors of pancreatic cancer.

2. The association between the oral microbiome and pancreatic cancer

To date, 619 bacterial taxa representing 13 phyla have been identified in the human oral microbiome (Dewhirst *et al.* 2010). It is a highly diverse and dynamically changing group of microorganisms that varies even in terms of where the sample is taken from, for example oral mucous membrane, tooth pockets, teeth, and tongue. Examples include saliva, tongue plaque, and mouthwash (Nearing *et al.* 2020). Saliva contains a broad spectrum of bacterial species, and the sampling method is convenient and relatively cost-effective. Studies show that using diagnostic models to characterize the oral microbiota, microbial biomarkers can provide a diagnostic tool for pancreatic cancer. The studies with 16S rRNA sequencing revealed there are significant differences in quantitative and qualitative species composition between the microorganisms found in the saliva of healthy individuals and those with pancreatic cancer or a pre-cancerous condition (Fan *et al.* 2018; Wei *et al.* 2020). A malignant condition such as pancreatic ductal adenocarcinoma, is a consequence of chronic inflammation and is driven by ongoing inflammation associated with immunosuppressive CD4+ T lymphocytes. It can be concluded that chronic inflammation of the pancreas induces carcinogenesis (Guerra *et al.* 2007).

In the study presented by Chen *et al.* (2023), a group of patients with pancreatic cancer and chronic pancreatitis was compared to healthy controls. Fecal and saliva samples analyzed by the 16S rRNA sequencing method and real-time qPCR revealed that oral pathogenic genera significantly predominated in pancreatic cancer, especially *Granulicatella*, *Peptostreptococcus*, *Alloprevotella*, *Veillonella, Bacteroidetes*, *Firmicutes,* and *Proteobacteria*, accounting on average for more than 80% of all observed species. In patients with chronic pancreatitis, the average relative abundance of the two phyla of *Firmicutes* and *Verrucomicrobia* was significantly higher than that in healthy controls. A marked

enrichment of pathogenic bacterial genera, including *Granulicatella, Peptostreptococcus, Alloprevotella, Veillonella, Solobacterium,* and *Streptococcus*, was detected in the oral microbiota of patients diagnosed with pancreatitis. These results were similar to those described by Farell *et al.* (2012). Other studies, using the same technology of sequencing, demonstrated that the relative abundance of *Porphyromonas*, *Haemophilus,* and *Paraprevotella* genera was significantly higher in the healthy tongue plaque microbiome, while in patients with chronic pancreatitis, *Leptotrichia*, *Fusobacterium*, *Actinomyces*, *Rothia*, *Solobacterium*, *Oribacterium*, *Campylobacter*, *Atopobium,* and *Parvimonas* families occurred significantly more often. The most notable differences between the microbiota of the tongue lining of patients with chronic pancreatitis and healthy controls were low levels of *Haemophilus* and *Porphyromonas* and high levels of *Leptotrichia* and *Fusobacterium* (Mitsuhashi *et al.* 2015; Lu *et al.* 2019).

Further extensive research included: 361 cases of pancreatic adenocarcinoma and 371 matched controls selected from two large prospective cohort studies: The American Cancer Society Cancer Prevention Study II (CPS-II) and the National Cancer Institute Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial. The pre-diagnostic oral wash samples collected from participants were also analyzed by 16S rRNA gene sequencing. It showed that carrying *Tannerella forsythia* and *Prevotella intermedia* in the mouth, in contrast to *Porphyromonas gingivalis,* was not associated with pancreatic cancer risk. This association was evident for both those with low bacterial abundance (below the median relative abundance) and those with high bacterial abundance (above the median relative abundance) (Fan *et al.* 2018). Another large European prospective cohort study – The European Prospective Investigation into Cancer and Nutrition (EPIC) – analyzed 405 patients with pancreatic cancer compared to 416 controls. Using Luminex-based immunoassays, it was noted that individuals with high levels (>200 ng/ml) of antibodies to the periodontal pathogen *Porphorymonas gingivalis* had twice the risk of pancreatic cancer compared to those with lower levels of these antibodies (≤200 ng/ml). It was found that people with consistently high levels of antibodies to common oral bacteria had a 45% lower risk of pancreatic cancer compared to those with lower levels of antibodies. The antibody levels were measured in blood samples taken up to 10 years before the cancer was diagnosed, which is likely to minimize changes in the immune response after the development of pancreatic cancer (Michaud *et al.* 2013). The association between an elevated level of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* and an increased risk of pancreatic cancer was also shown. A long-term study involving 59,000 African American women, with a follow-up period of 21 years, found that participants with poor dental health had a higher likelihood of developing pancreatic cancer. Periodontitis has been associated with at least a 50% increased risk of pancreatic cancer and can be considered a potential risk factor for this malignancy (Pietzner *et al.* 2021; Ungureanu *et al.* 2023). Kaci *et al.* (2014) also found that the most prominent feature of the oral microbiome for pancreatic ductal adenocarcinoma was a significant reduction in *Streptococcus salivarius*, a bacterium known for its anti-inflammatory properties. It has been demonstrated that metabolically active *Streptococcus salivarius* JIM8772 exerts significant anti-inflammatory effects in murine models of both moderate and severe colitis. Based on these findings, it can be hypothesized that high levels of *Streptococcus salivarius* colonization in the mouth and intestine may be associated with a reduced risk of pancreatic cancer, possibly due to its anti-inflammatory and immunomodulatory effects (Kaci *et al.* 2014). In another study, it was shown that Ganoderma *atrum* polysaccharide (PSG) and White Hyacinth Bean polysaccharide (WHBP) were used to investigate their effects on the microbiota of the oral cavity, gut, pancreas, and lungs in rats with type 2 diabetes. The results showed that oral microbiota was significantly similar to pancreatic microbiota, more than the gut microbiota. This suggests a potential role for saliva as an early biomarker of pancreatic changes. Treatment with PSG and WHBP not only improved pancreatic condition but also helped stabilize the oral microbiota, indicating a strong correlation between these two environments (Wu *et al.* 2022).

3. The association between gut microbiome and pancreatic cancer

It is well known that the human gut microbiota plays an important role in the functioning of the body, both by influencing metabolism and regulating the immune system. Disturbances in the bacterial balance can have negative effects on various organs, including the pancreas (Adolph *et al.* 2019; Zhou *et al.* 2020). Altered gut microbiota is influenced by factors related to inflammatory, metabolic, and malignant diseases (Montenegro *et al.* 2022; Maev *et al.* 2023). A study involving 1.795 volunteers who provided stool samples found that changes in gut microbiota composition were primarily associated with pancreatic stromal cell function. Ductal cell function appeared to play a less significant role (Frost *et al.* 2019). To detect bacteria in human pancreatic ductal adenocarcinoma (PDAC) samples, Geller *et al.* (2017) used real-time quantita-

tive polymerase chain reaction targeting the bacterial 16S rRNA gene and confirmed bacterial presence with non-PCR methods: ribosomal RNA fluorescence in situ hybridization (FISH) and immunohistochemistry using an anti-LPS antibody. Research has shown that the most frequently identified species in pancreatic ductal adenocarcinoma (approx. 52% of all reads) belong to the class *Gammaproteobacteria*, with the majority represented by the *Enterobacteriaceae* and *Pseudomonadaceae* families in the *Proteobacteria* cluster. *Proteobacteria* are abundant in the duodenum, into which the pancreatic duct opens. This suggests that retrograde migration of bacteria from the duodenum into the pancreas may be the source of bacteria associated with pancreatic ductal adenocarcinoma (Geller *et al.* 2017). In a study comparing the gut microbiota between pancreatic cancer patients and healthy controls, it was found that the composition of the gut microbiota was similar in both groups, among the bacterial phyla, *Firmicutes* and *Bacteroidetes* were dominant. However, it showed again that patients with pancreatic ductal adenocarcinoma had a significantly higher presence of *Proteobacteria*, *Synergistetes,* and *Euryarchaeota* phyla compared to the control group (Pushalkar *et al.* 2018).

The above study mentioned Chen *et al.* (2023) described an interesting study of the gut microbiome in a group of cancer patients compared to healthy participants. They found that *Prevotella* spp. was present at significantly higher levels in cancer patients. In the study, its percentage of the pancreatic microbiota was 17.64% (relative average) compared to the control group, in which *Prevotella* spp. accounted for only 6.4% of the tested microbiota (relative average). Andréasson *et al.* (2024) analyzed stool samples from rheumatoid arthritis (RA) patients (n=50) before and after treatment using a 16S rRNA-based GA-map Dysbiosis Test and qPCR for *Prevotella copri,* assessing microbiota changes in relation to disease activity (DAS28-CRP). They have shown that *Prevotella copri* was involved in the development of RA in mice through the Th17/IL-17 pathway. Similarly, isolated *P. copri* strains (n=13) from RA patients and healthy controls were whole-genome sequenced. To assess their arthritis-inducing potential, two mouse models were used: collagen-induced arthritis under specific-pathogen-free conditions and SKG mice monocolonized with *P. copri*. *In vitro* stimulation of bone marrow–derived dendritic cells (BMDCs) showed that *P. copri* induced stronger IL-17 and Th17-related cytokine responses (IL-6, IL-23), suggesting a higher pro-inflammatory potential (Nii *et al.* 2023). The same pathway can also accelerate the development of pancreatic precancerous conditions (PanIN), suggesting that *Prevotella copri* may play a role in promoting pancreatic cancer through Th17 activation.

Additionally, multicenter study revealed distinctive gut microbiota profiles for PDAC patients, including significant increases in *Streptococcus* spp. and *Veillonella* spp. and a reduction in *Faecalibacterium prausnitzii*. The study was based on shotgun metagenomic analysis of fecal and salivary samples collected from 47 treatment-naïve PDAC patients and 235 non-PDAC controls across Japan, Spain, and Germany (Nagata *et al.* 2022). Studies have also shown that a cancerous pancreas contains a significantly more diverse microbiome compared to a healthy pancreas. An increased number of specific types of bacteria have been observed in pancreatic ductal adenocarcinoma compared to their presence in the gut, and it has been shown that these bacteria can migrate through the pancreatic duct (Dickson 2018; Del Castillo *et al.* 2019). Studies investigating the presence of bacteria in pancreatic juice and bile from patients with pancreatic ductal adenocarcinoma, using PCR targeting the 16S rRNA gene, consistently identified *Enterococcus* spp. and *Enterobacter* spp. as predominant in bile samples, suggesting a potential route of pancreatic infection. This method was applied in two separate studies: one analyzing 36 samples (Maekawa *et al.* 2018) and another analyzing 101 samples (Stein-Thoeringer *et al.* 2025), both confirming the frequent presence of bacterial DNA. Additionally, pancreatic cancer patients showed significantly elevated serum antibody levels against *Enterococcus faecalis* envelope polysaccharide compared to healthy individuals.

Analysis of the gut microbial profile showed that 15 taxonomic groups were significantly enriched in pancreatic cancer patients, mainly including the genera *Prevotella*, *Veillonella*, *Klebsiella*, *Selenomonas*, *Hallella*, *Enterobacter,* and *Cronobacter*. On the contrary, 25 taxonomic groups, primarily *Gemmiger*, *Bifidobacterium*, *Coprococcus*, *Clostridium* cluster IV, *Blautia*, *Flavonifractor*, *Anaerostipes*, *Butyricicoccus,* and *Dorea* were significantly reduced in fecal samples from pancreatic cancer patients compared to healthy individuals. The results were obtained using linear discriminant analysis. These studies also highlight differences in microbial composition depending on the stage of pancreatic cancer. The genera *Lactobacillus*, *Haemophilus,* and *Streptococcus* were significantly more abundant in patients with stage II disease compared to those with stage I (Ren *et al.* 2017). Also, patients with a stomach infection due to pathophysiological colonization of *Helicobacter pylori*, may be at higher risk for increased pancreatic cancer (Maisonneuve and Lowenfels, 2015). Seropositivity of antibodies to *H. pylori* and its virulence protein for CagA-positive strains of *H. pylori* was determined using commercial IgG immunoassays.

The results of these studies suggest that colonization by Cag A-negative strains of *H. pylori* may affect the risk of pancreatic cancer. It is associated with changes in stomach acidity, Cag A-negative strain, and hyperchlorhydria modulate pancreatic carcinogens and increase the morbidity of pancreatic cancer (Risch *et al.* 2014). Gastric acidity stimulates bicarbonate and fluid secretion from pancreatic ductal cells. This mechanism allows *H. pylori* to reside in the stomach and interfere with the function of the pancreatic ductal epithelium. Thus, it can be suggested that the differential modification of chronic gastric acidity by CagA-negative strains of *H. pylori* may affect the risk of pancreatic cancer. However, further studies analyzing this relationship are needed to draw firm conclusions (Risch 2012; Hirabayashi *et al.* 2019), because the seropositivity of *H. pylor*i strains varies depending on the continent where the study was conducted, so the seropositivity test may show different results depending on the origin of the study group (Wang *et al.* 2014; Huang *et al.* 2017). There is a lot of evidence that *H. pylori* is significantly correlated with the occurrence of pancreatic cancer. Based on the results of a study of the Asian population, a correlation between *H. pylori* infection and the incidence of pancreatic cancer was proven (Hsu *et al.* 2014; Xu *et al.* 2022).

Literature data clearly indicate that differences in the composition of the gut microbiota may reflect or be associated with the presence of pancreatic cancer (Daley, 2022). This may become an important diagnostic marker. However, it should be remembered that patients with pancreatic cancer are often a heterogeneous group of patients with other comorbidities. Therefore, samples from cancer patients differ significantly in the microorganism's composition. Nevertheless, higher proportions of bacterial genera belonging to the phyla *Bacteroidetes* and *Firmicutes* have been observed in pancreatic cancer patients compared to healthy individuals (Half *et al.* 2015). The pancreas, which secretes substances outside the intestine, plays an important role in shaping the composition and stability of microorganisms found in the intestines of healthy people who do not suffer from any pancreatic diseases. Pancreatic lipase, trypsin, and glycoprotein 2 play an important role in shaping the composition and stability of microorganisms found in the intestines of healthy individuals. These enzymes also protect the intestines by inducing bacterial lysis and acting as antimicrobial agents (Nishiyama *et al.* 2018; Edogawa *et al.* 2020; Shin and Seeley, 2019). Figure 1 shows the types of microorganisms whose decreased or elevated levels in the mouth and intestines can increase the incidence of pancreatic cancer (Fig. 1).

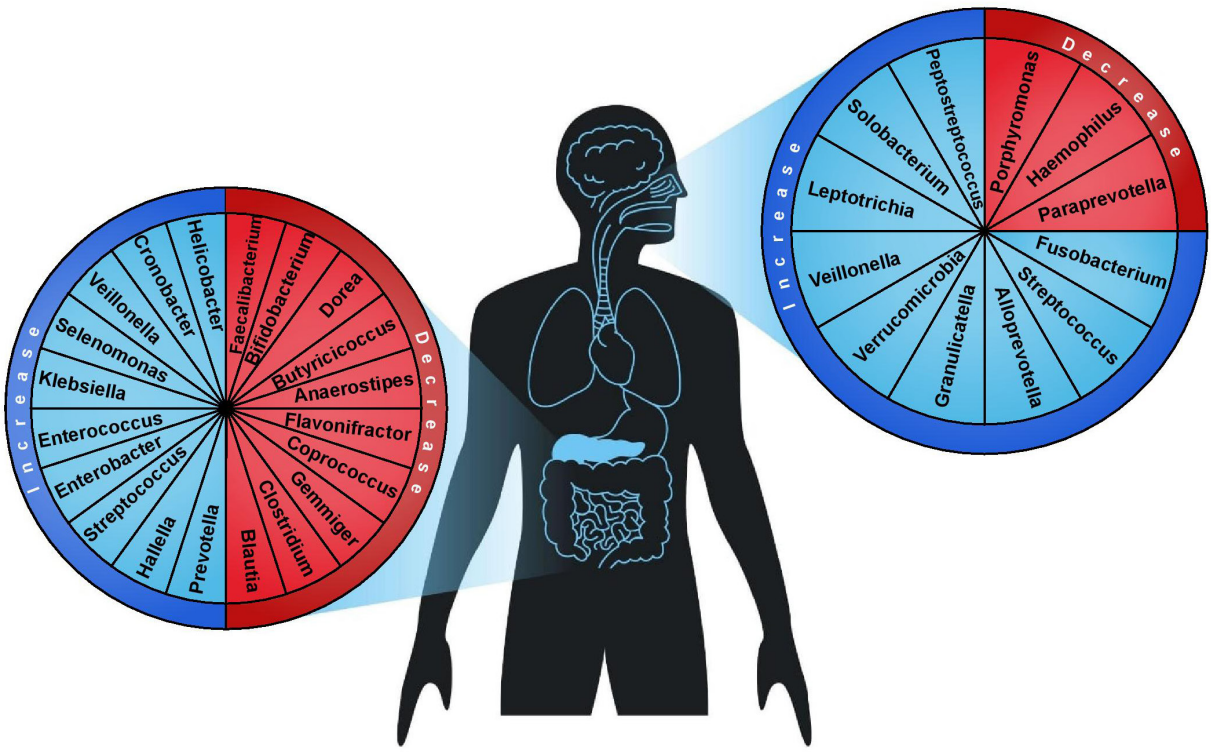

Figure 1. Occurrence of taxonomic groups of bacteria in the oral and intestinal microbiome of patients with pancreatic cancer compared to the population of healthy people. Red fields in the pie chart - a decrease in the number of bacteria, blue fields in the pie chart - an increase in the number of bacteria. Original graphic.

4. Known mechanisms of the association between microbiota and pancreatic cancer

Microbial dysbiosis as well as epithelial barrier dysfunction may affect tumor transformation through bacterial translocation (Plottel and Blaser, 2011). In most malignancies, the intestinal microbiome is in direct contact with or near the diseased organ. On the contrary, the anatomical location of the pancreas does not indicate that the gut microbiome can influence disease development (Meng *et al.* 2018). Changes in the microbiota may result from the movement of bacteria from the duodenal intestine to the pancreas through the opening of the pancreatic duct in the duodenal papilla, but this movement may not be a normal physiological process. However, studies suggest that there is a specific gut and pancreatic microbiome associated with pancreatic cancer that may accelerate the development of the disease by weakening the body's immune response (Pushalkar *et al.* 2018). Performing bacterial ablation leads to immunogenic reprogramming of the PDAC tumor microenvironment. It decreased the number of bone marrow-derived suppressor cells, and increased the differentiation of M1 macrophages, promoted the differentiation of CD4 Th1 T cells, and the activation of CD8 T cells. In addition, bacterial ablation enhanced the efficacy of checkpoint-targeted immunotherapy by upregulating PD-1 expression. Mechanically, the PDAC microbiome generated a tolerogenic immune program through differential activation of selected Toll-like receptors in monocyte cells (Seifert *et al.* 2016; Pushalkar *et al.* 2018). A continuous decrease in the number of short-chain fatty acid-producing bacteria, such as *Faecalibacterium prausnitzii, Eubacterium rectale,* and *Ruminococcus bicirculans,* was observed in the intestines of patients with pancreatic adenocarcinoma. This decline partially coincided with the characteristic changes in the gut microbiome found in various diseases. Short-chain fatty acids regulate intestinal immune function through G-protein-coupled receptors on the cell surface. Their absence leads to inflammation, which may promote the development of PDAC (Parada Venegas *et al.* 2019; Ubachs *et al.* 2021). In turn, oral pathogens such as *Porphyromonas gingivalis* cause mutations in the pro-tumorigenic genes *TP53* and *KRAS* by secreting peptidylarginine deaminase. On the other hand, *Fusobacterium nucleatum* promotes tumor formation and metastasis in several ways: it binds to host cells via FadA adhesion proteins, enabling their internalization and activation of NF-κB and IL-6 pathways, leading to pro-inflammatory cascades (Kostic *et al.* 2013; Gnanasekaran *et al.* 2020). Fecal microbiota transplantation is also beginning to play an increasingly important role. An experiment was performed in which fecal microbiota was transplanted from donors who were pancreatic cancer patients with either short- or long- term survival. As a result, scientists were able to influence tumor growth and immune infiltration of the tumor. This shows that the composition of the pancreatic microbiota of PDAC patients, communicating with the gut microbiota, influences the host immune response (Riquelme *et al.* 2019; Yang *et al.* 2022).

5. The impact of dysbiosis on the effectiveness of pancreatic cancer treatment

An increasing number of studies indicate that the intestinal microbiota may play a dual role in pancreatic cancer by contributing to its pathogenesis and modulating the efficacy and toxicity of anticancer therapies. These effects are mediated through alterations in drug pharmacokinetics, changes in the host metabolic milieu, and modulation of the tumor microenvironment composition. As a result, intestinal microbiota can affect the effectiveness of treatment, and its modulation can lead to different therapeutic effects. Microbes can be responsible for the resistance of cancer cells to various chemotherapeutic agents, for example, gemcitabine, 5-fluorouracil, or oxaliplatin. These drugs are used in pancreatic cancer treatment regimens, among others. While most of the studies on gut microbiota, and the efficacy of chemotherapy are based on colorectal cancers, it can be speculated that the same mechanisms occur in pancreatic cancer patients due to the use of a similar treatment regimen and the presence of similar microorganisms also in the pancreatic cancer microenvironment such as *Fusobacterium nucleatum* and bacteria belonging to *Gammaproteobacteria* class (Nejman *et al.* 2020; Sevcikova *et al.* 2022). The presence of these bacteria contributes to resistance to oxaliplatin and/or 5-fluorouracil therapies by activating autophagy in tumor cells (Yu *et al.* 2017) or increasing BIRC3 expression, which inhibits tumor cell apoptosis (Zhang *et al.* 2019).

Tests on human pancreatic ductal adenocarcinoma samples taken during surgery revealed the presence of an average of one bacterium per 146 human cells. These bacteria are part of the pancreatic tumor microenvironment. The most detected species belong to the class *Gammaproteobacteria*, mainly to the *Enterobacteriaceae* and *Pseudomonadaceae* families, which express CDDL. Gemcitabine is particularly important in the treatment of pancreatic cancer, and the presence of these bacteria may contribute to resistance to the drug (Geller *et al.* 2017). It can be eliminated after effective antibiotic therapy and contribute to clinical success (Ramanathan *et al.* 2016; Imai *et al.* 2019; Fulop *et al.*

2023). However, when the microenvironment of the pancreas is examined, fungi are found in addition to bacteria. Fungal ablation enhanced the effect of gemcitabine-based chemotherapy. It is also worth noting that fluconazole treatment had a protective effect. Moreover, consistent with the absence of increased fungal infiltration in pancreatitis, antifungal drugs did not alleviate mild pancreatitis. Thus, it can be concluded that fungal removal may have therapeutic effects only for pancreatic cancer patients (Aykut *et al.* 2019). There is a high degree of inter-individual variability, and an excess of different microbiota taxa and host-microbiota interactions are highly dynamic and complex. These include not only direct interactions between the microbiota and the tumor, but also indirect interactions through the immune system. All these mechanisms are not yet fully understood.

An increasingly successful therapeutic option is immune checkpoint therapy (O'Reilly *et al.* 2019). Drugs such as durvalumab and tremelimumab are the human monoclonal antibodies (mAbs) against programmed death 1 (anti-PD-L1) IgG class 1 and human anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) IgG class 2, respectively. In a study of PDAC patients, the two therapies were combined in the hope of an additive or synergistic effect of the drugs, but this did not have the desired effect. The use of anti-CTLA-4 blockade alone as part of the combination therapy shows a positive anti-tumor effect in patients participating in the clinical trial (Le *et al.* 2013). The modulation of the immune response by microbiota is significant and thus can have a huge impact on the efficacy of immunotherapy. The intestinal microbiome ablation performed showed protection against pre-invasive and invasive pancreatic ductal adenocarcinoma, while transfer of bacteria from pancreatic ductal adenocarcinoma hosts, but not from the control group, nullifies this anti-tumor protection. Bacterial ablation leads to immunogenic reprogramming of the PDAC tumor microenvironment, including reduction of bone marrow-derived suppressor cells and increased differentiation of M1 macrophages, which in turn promotes differentiation of CD4+ Th1 T cells and activation of CD8+ T cells. In addition, bacterial ablation enhances the efficacy of checkpoint-targeted immunotherapy by upregulating PD-1 expression (Pushalkar *et al.* 2018).

A complex from the NOD-like receptor family, the inflammasome containing pyrin domain 3 (NLRP3), is responsible for the initiation of innate inflammatory responses. During the study, NLRP3 levels were shown to be significantly increased in patients with pancreatic cancer in general, and it was demonstrated that selective ligation of NLRP3 could sustain this pro-tumorigenic pancreatic inflammation (Daley *et al.* 2017). Inhibition of dipeptidyl peptidase (DPP) may increase the efficacy of immunotherapy in pancreatic cancer. In mouse models, after oral administration and intraperitoneal injections of BXCL701, which is an oral small-molecule dipeptidyl peptidase inhibitor, increased NK and T cell immune infiltration and decreased tumor growth were observed. BXCL701 increases the movement of CXCR3+ NK and CD8+ T cells into tumors, which mediates tumor regression (Fitzgerald *et al.* 2021). Positively transforms the PDAC immune microenvironment, enhancing antitumor activity and improving the effectiveness of therapy. It should be noted here that the gut microbiota reduces the activity of tumor-infiltrating NK cells, which in effect promotes the development of pancreatic ductal adenocarcinoma. To improve patient survival, one possible solution may be to modulate microbiomes with antibiotic therapy, which may not be enough if patients have immune deficiencies. Reducing the number of NK cells with antibodies has been shown to lead to advanced cancers, even in the absence of microbiota (Shi *et al.* 2023; Yu *et al.* 2022).

With the development and plethora of immune therapies, the question arises as to how individual diversity, immunocompetence or lack thereof, and the individual microbiota of a pancreatic cancer patient affect the effectiveness of treatment.

6. Conclusion

The association between microbiome and pancreatic cancer is a rapidly evolving field that holds immense potential for early detection, risk assessment, and therapeutic intervention. It was proved that both the oral and gut microbiomes play significant roles in the pathogenesis of pancreatic cancer, with emerging evidence suggesting that microbial dysbiosis, imbalanced or altered microbial communities, may contribute to tumorigenesis through inflammatory pathways, immune modulation, and bacterial translocation to the pancreas. Specifically, bacteria like *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella* spp. have been implicated in promoting carcinogenesis via pro-inflammatory pathways, immune system manipulation, and direct interaction with cancerous cells.

Additionally, the composition of the microbiome in both the oral cavity and gut has been found to significantly differ in pancreatic cancer patients compared to healthy individuals. These differences in microbial composition could serve as diagnostic biomarkers or therapeutic targets in the future. More importantly, the role of microbiomes in modulating the efficacy of pancreatic cancer treatments, such as chemotherapy and immunotherapy, highlights the critical need for per-

sonalized therapeutic strategies that account for individual microbiome variations.

Furthermore, microbial interactions with the immune system, particularly through immune checkpoint inhibition and microbial ablation strategies, suggest that modulating the microbiome could enhance the effectiveness of current treatment options and offer a novel approach to improving patient outcomes. Although there is still much to learn about the complex associations between microbiota and pancreatic cancer, the growing body of research offers promising avenues for both early detection and innovative treatments that leverage microbiome modulation.

As we move forward, understanding the intricate role of the microbiome in pancreatic cancer will likely open doors to more effective, personalized therapies and could ultimately transform the way we approach both the diagnosis and treatment of this devastating disease. However, larger-scale studies and clinical trials are necessary to fully understand the underlying mechanisms and to validate microbiome-based diagnostic and therapeutic interventions.

Table 1. is a collection of studies analyzed in the article. It shows how the associations between the microbiome and the risk of pancreatic cancer were studied and what the conclusions of the studies were (Table 1).

Tab. 1 Summary of key research articles on the associations between the microbiome and pancreatic cancer.

Legend: CP - Chronic Pancreatitis, PC - Pancreatic Cancer, PDAC – Pancreatic Ductal Adenocarcinoma, NK – Natural Killer Cells, Th – T helper cells, PHC – Pancreatic Head Carcinoma, IM – Intestinal Microbiota, EPI – Exocrine Pancreatic Insufficiency, SI – surgical intervention, CPS – Chronic Pancreatitis Syndrome, IBS – Irritable Bowel Syndrome, PA – Pancreatic Adenocarcinoma, LTS – Long-Term Survival, PFS – progression-free survival, OS – overall survival, GI – gastrointestinal

References	Type of research	Materials and methods	Conclusions
Fan et al. 2016	Case- control study	Adenocarcinomaa of pancreas patients (n=361), 16S rRNA sequencing of mouth-wash samples	Carriage of oral pathogens, *P. gingivalis* and *A. actinomycetemcomitans* were associated with higher risk of pancreatic cancer
Wei et al. 2020	Prospective study	Pancreatic cancer patients (n=41), healthy individuals (n=69); 16S rRNA sequencing of saliva	Carriage of *Streptococcus* spp. and *Leptotrichia* spp. (z-score) was associated with a higher risk of PDAC
Chen et al. 2023	Multisite analysis	pancreatic cancer patients (n=40), chronic pancreatitis patients (n-15), healthy controls (n=39); 16S rRNA sequencing of saliva	The chronic pancreatitis group exhibited the lowest microbial diversity, while no significant difference was found between the pancreatic cancer and healthy controls groups
Lu et al. 2019	Clinical study / analysis	patients with pancreatic head carcinoma (n=30), healthy individuals (n=25); 16S rRNA sequencing of the tongue coating samples	The microbiota dysbiosis of the tongue coat in PHC patients and provide insight into the association between the human microbiome and pancreatic cancer
Pietzner et al. 2021	Study population / analysis	16S rRNA sequencing of fecal samples (n=2226)	The effect of exocrine pancreatic function on intestinal microbiota composition alters the availability of microbial-derived metabolites in the blood and thus directly contributes to the host metabolic changes associated with exocrine pancreatic dysfunction
Zhou et al. 2020	Correlation analysis	healthy controls (n=69), chronic pancreatitis (n=71); 16S rRNA sequencing of fecal samples	Patients with chronic pancreatitis have gut microbiota dysbiosis that is partly affected by pancreatic exocrine function
Maev et al.. 2023	Comparative analysis	patients (n=85) including pancreatitis without extrinsic pancreatic insufficiency (EPI, n=16), with chronic pancreatitis and mild EPI (n=11), with severe pancreatitis and EPI (n=17); 16S rRNA sequencing of fecal samples	The IM of all groups showed the dominance of phyla *Firmicutes* with the lowest representation in the severe EPI group, both with SI and CP, and the growth of the phyla of *Actinobacteria*, *Verrucomicrobiota* and *Fusobacteria*
Nii et al., 2023	Experimental study	Bacterial strains (n=13) isolated from the feces of patients and healthy controls; *in vitro* stimulation of bone marrow-derived dendritic cells, genome sequencing of *Prevotella copri*	Stimulation experiments have shown up-regulation of IL-17 and Th17-related cytokines (IL-6, IL-23) of *Prevotella copri*, which contributes to causing rheumatoid arthritis, the same pathway may contribute to pancreatic cancer through Th17 activation

Maekawa et al. 2018	Metagenomic analysis	16S rRNA sequencing of pancreatic juice from pancreatic cancer patients (n=20) and duodenal cancer patients (n=16)	*E. faecalis* was frequently detected in pancreatic tissue from patients with CP and PC, and antibody titers against *E. faecalis* capsular polysaccharide were elevated in *E. faecalis*-positive patients compared to healthy donors
Stein-Thoeringer et al. 2024	Prospective study	non-cancer participants (n=38), pancreatic cancers patients (n= 63); 16S rRNA sequencing of samples collected from various sites of the GI tract and surgical sites, microbial culturing	The presence of *Enterococcus* spp. in bile ducts of PDAC patients undergoing pancreatic surgery represents a significant risk factor for perioperative infections and, thereby, elevated postoperative and long-term mortality
Ren et al. 2017	Prospective study	pancreatic cancer patients (n=85), healthy controls (n=57); 16S rRNA sequencing of fecal samples	The gut microbial profile was unique in PC, providing a microbial marker for non-invasive PC diagnosis
Risch et al. 2014	Case-control study	pancreatic cancer patients (n=761), random controls (n=794); venipuncture specimens, antibody seropositivity for *H. pylori* and its virulence protein CagA was assessed using commercial enzyme-linked immunosorbent IgG assays.	*H. pylori* colonization may have diverse effects on cancer risk, depending on the organism strain type as well as on the cancer site.
Wang et al. 2014	Meta-analysis	cases of *H. pylori* infection on pancreatic cancer (n=2049), control group (n= 2861); databases analysis	Hp+ and CagA+ infections are associated with a decreased risk of pancreatic cancer in Eastern populations but have no significant associations in Western countries.
Xu et al. 2022	Meta-analysis	case-control studies (n=8), nested case-control studies (n=5), cohort studies (n=4); databases analysis	*H. pylori* infection can increase the incidence of pancreatic cancer in general. CagA/VacA-positive *H. pylori* infection is not associated with the incidence of pancreatic cancer
Half et al. 2019	Comparative study	pancreatic adenocarcinoma patients (n=30), pre-cancerous lesions patients (n=6), healthy individuals (n=13), non-alcoholic fatty liver disease patients (n=16); 16S rRNA sequencing of fecal samples	Find a distinct PC-associated gut microbiome signature in an Israeli cohort, manifesting primarily as an under-representation in several bacterial families prevalent in the healthy gut
Edogawa et al. 2020	Prospective study	patients with IBS (n=39), healthy volunteers (n=25), 16S rRNA sequencing of fecal samples	A subset of patients with IBS, especially in PI-IBS, has substantially high fecal PA, greater symptoms, impaired barrier and reduced microbial diversity.
Ubachs et al. 2021	Clinical study	Pancreatic cancer patients (n=107), household partners (n=76); 16S rRNA sequencing of fecal samples	There were no significant differences in the composition of the bacteriobiota, although cachexia prevalence was highest in pancreatic cancer (66.7%). Faecal calprotectin levels were positively correlated with the abundance of *Peptococcus*, *Enterobacteriaceae*, and *Veillonella*.
Riquelme et al. 2019	Clinical study	surgically resected PDAC tumor samples (n=68; 36 of LTS and 32 of STS); 16S rRNA sequencing of surgically resected PDAC tumors, PCR flow cytometry	The tumor microbiome unique to LTS may contribute towards shaping a favorable tumor microenvironment.
Yu et al. 2022	Cohort study	Mice model; NK cell depletion, bacterial manipulation	The gut microbiota mediates PDAC progression through NK cell modulation and that gut microbiota-derived supernatant can modulate anti-tumor NK cell activity

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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OVERVIEW OF *AGA GENES* AND THEIR ROLE IN UTILIZATION OF N-ACETYL-D-GALACTOSAMINE AND D-GALACTOSAMINE BY BACTERIA

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Abstract: Bacteria use a variety of mechanisms in order to successfully survive in hosts. To support proliferation sophisticated means of maintenance in host especially metabolism of carbon and nitrogen is needed. One metabolic mechanism is the metabolism of amino sugars which are being used simultaneously as a carbon and nitrogen source. D-galactosamine (GalN) and N-acetyl-D-galactosamine (GalNAc) and their derivatives are widely used by bacteria. For instance, they build parts of cell wall and LPS. The metabolism of both amino sugars is performed and controlled by proteins encoded by *aga* genes, present in a wide variety of *Proteobacteria*. The genetic mechanism underlying this metabolic pathway is, however not yet fully known. Despite this, there is a possibility of using this pathway as a target for therapy, especially in the times of ever-growing danger of bacterial drug resistance. The goal of this article was to present the current knowledge of *aga* genes and their importance in GalN and GalNAc metabolism.

Keywords: *aga genes*, Amino sugars, Antibacterial therapy, Metabolism regulation, Sugar metabolism

1. Introduction

Bacteria are known to be very versatile organisms, able to colonize a variety of environments. One such environment is the human organism, which they colonize and infect opportunistically or non-opportunistically, using their virulence factors, causing a variety of symptoms and diseases. This creates a number of challenges to overcome and treat such infections (Reizer et al. 1996).

However, these infections are possible for the bacteria only due to their mastery of utilizing metabolic pathways, allowing them to be flexible and adapt to the current conditions. Fortunately, we have managed to understand, harness, and apply these bacterial abilities, eg. to design safe and effective strains that generate a wide range of novel biological products. Unfortunately, this features also allows pathogenic bacteria to quickly gain an advantage over our therapy. The most noticeable advantage nowadays being growing bacterial drug resistance, and our relative inability to counter it in clinical conditions (Reizer et al. 1996).

One of the metabolic pathways responsible for creating substrates that are used by bacteria, to develop their virulence factors, is the use of amino sugars which are a variety of complex monosaccharides in which hydroxyl group is replaced by amine group (Reizer et al. 1996). Most of the research on bacterial metabolism of amino sugars comes from studies with *Escherichia coli* (Reizer et al. 1996), *Bacillus subtilis* (Freymond et al. 2006) and *Streptococci* (Afzal et al. 2016). Not enough, however, is known about processes and genetical mechanisms underlying amino sugar metabolism to potentially use them in our advantage.

Metabolism of amino sugars comes in different ways, differing between bacterial species or even strains (Sadler et al. 1979; Brinkkötter et al. 2000; Ray and Larson 2004; Freymond et al. 2006; Leyn et

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al. 2012). Hence, a vast number of molecular mechanisms controlled by different operon and genes had been developed in the course of evolution. Because of that, at the moment, we are unable to see the whole picture of bacterial amino sugar metabolism (Shen et al. 2025). However, *aga* genes operon responsible for catabolism of such sugars, namely D-galactosamine (GalN) (Fig.1A) and N-acety-D-galactosamine (GalNAc) (Fig.1B) might serve as a good model for future investigations. This operon is widespread, especially in pathogenic bacteria.

In many bacteria, such as *E. coli, Enterobacter* and *Shewanella* spp. an *aga* operon consists mostly of around twelve genes. This number, however, may vary due to other bacteria developing new genes, or adapting the genes responsible for other amino sugar catabolism (mainly Glucosamine) (Ray and Larson 2004; Leyn et al. 2012; Zhang et al. 2015). The characterization of *aga* genes as well as metabolic pathway controlled by proteins encoded by them are the subject of this article. We focused primarily on the mode of action, as well as the role of the *aga* genes in amino sugar metabolism. Finally, we rationalize why these pathways have a great potential as a target for therapy or diagnostics.

2. Role of N-acetyl-D-galactosamine and D-galactosamine in eukaryotic and prokaryotic cells

Both amino sugars are common components and build a variety of cell structures in both eukaryotic and prokaryotic domains. In bacteria, GalNAc is not only a component of the cell wall but is also found in lipopolysaccharide (LPS). For instance, *Pseudomonas aeruginosa* and *Campylobacter jejuni* both have GalNAc residuses in their LPS core, as well as require UDP-GalNAc in order to be able to build full length LPS molecule.(Masoud et al. 1995; Sadovskaya et al. 1998; Bernatchez et al. 2005). Some bacteria even use it to mimic our immune cells (De Jong et al. 2022). In mammals, it links carbohydrate chains in mucins (Carraway and Hull 1991). Both GalNAc and GalN are found in glycosylated proteins of both domains (Sadler et al. 1979; Abu-Qarn et al. 2008). Given these versatile roles we expect GalNAc/GalN metabolism to be closely linked to bacterial virulence (Zhang et al. 2015).

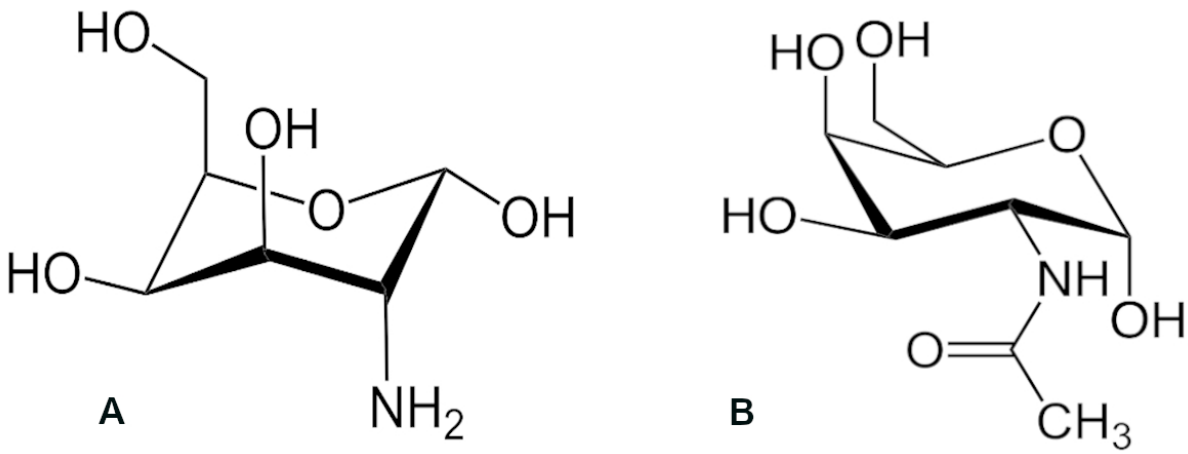

Fig. 1. The chemical structure of glycans. A-D-galactosamine (GalN), B- N-acety-D-galactosamine (GalNAc).

Such abundance and role of these amino sugars makes it no surprise that various mammalian pathogens use them to their advantage. For example, the amino sugars are known to be able to support the growth as carbon and nitrogen sources (Reizer et al. 1996). Brinkköter et al. presented in their study, that Wild-Type strains of *E. coli*, *Klebsiella pneumoniae* and *Salmonella enterica* Typhimurium could effectively use GalNAc and GalN as their main source of carbon and nitrogen. Compared to the laboratory strains, *E. coli* K-12, which only after some time developed mutations which allowed to use this metabolic pathway. These mutants had a phenotype suppressing the *gat* and *nag* genes responsible for galactitol and N-acetyl-D-glucosamine metabolism respectively. They could not however use GalN as their main source of carbon and nitrogen (Brinkkötter et al. 2000). This may suggest that an ability to utilize amino sugars is important for Wild-Type strains to maintain their virulence.

Another example of the usefulness of GalNAc for bacterial virulence may be the case of *Camplylobacter jejuni*, which can produce GalNAc-terminal Lipooligosacharides (LOS). These LOS can bind to the Macrophage Galactose-type lectin found on immature dendritic cells, which successfully mimics our immune system (De Jong et al. 2022).

3. The structure of the *aga* operon

The *aga* operon consists mainly of twelve genes encoding proteins serving various functions regarding GalNAc and GalN metabolism. However, as was already mentioned, there exist several orthologic groups of *aga* genes.

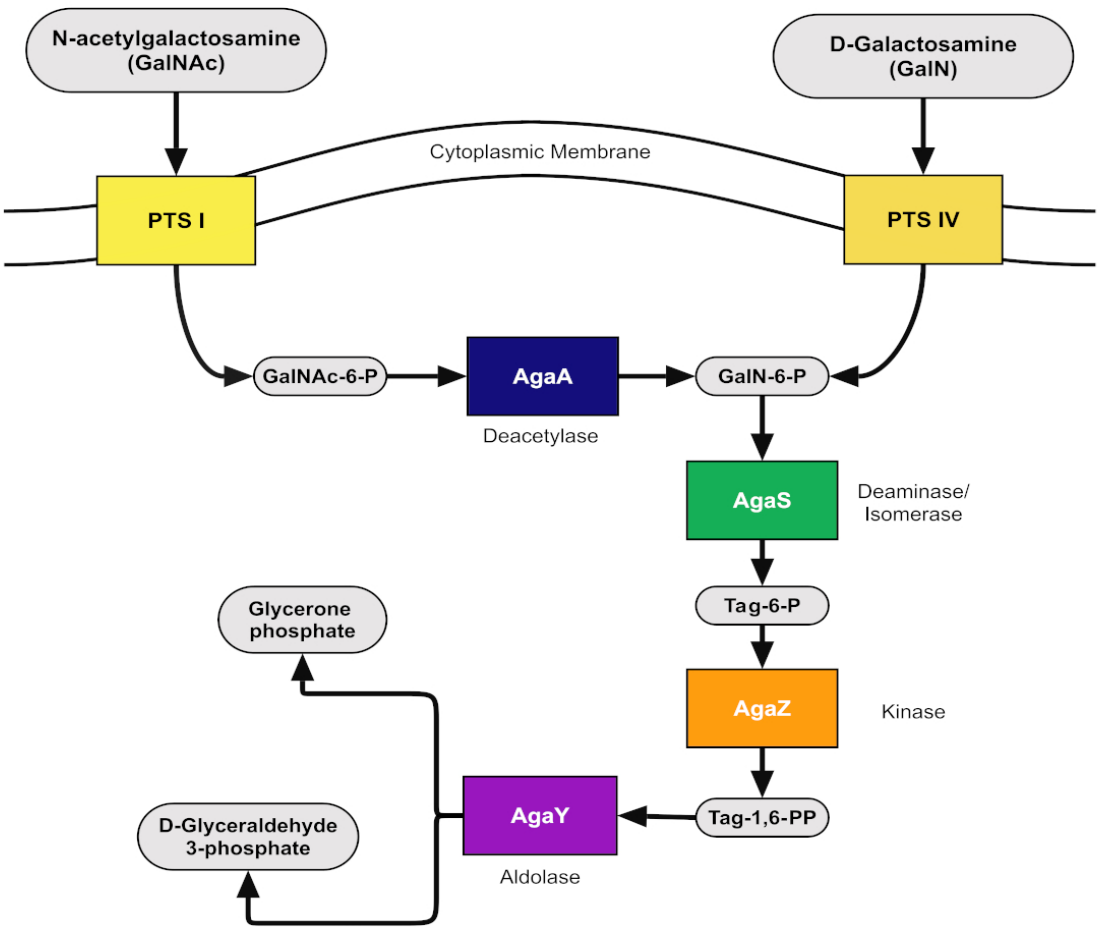

Fig. 2. The catabolic pathway that uses GalNAc and GalN.

The catabolic pathway that uses GalNAc and GalN consists of five steps, as presented in Figure 2. Transport (1) of the substrates is realized by two PTS systems encoded by *agaBCD* and *agaVWEF* genes. Succeeded by the following phosphorylation by AgaK kinase to GalNAc-6-P and GalN-6-P respectively. Subsequent deacetylization (2) of GalNAc-6-P to GalN-6-P catalyzed by AgaA. Deamination and isomerization (3) of GalN-6-P (galactosamine-6-phosphate) by di-functional AgaS enzyme to tagatose-6-phosphate (Tag-6-P) which then undergoes another phosphorylation (4) catalyzed by AgaZ to tagatose-1,6-diphosphate (Tag-1,6-PP). The final step of this GalNAc pathway is Tag-1,6-PP cleavage, catalyzed by AgaY aldolase which leads to the production of glyceraldehyde-3-phosphate and glycerone phosphate (PEP) (Ray and Larson 2004; Leyn et al. 2012).

Interestingly, comparative genomics study by Leyn et al. suggested a vast diversity regarding the utilization of GalNAc/GalN pathway by *Proteobacteria*, such as *Shewanella*. This concerned especially the two first steps, with the latter three being more conserved (Leyn et al. 2012).

The *aga* genes in *Proteobacteria* are regulated by AgaR transcriptomal regulator from DeoR family of transcriptional factors, which recognizes specific sequences located in *agaRZS* promoter regions. Not only does it serve as an autoregulator but also negatively controls the expression of *agaZ* and *agaS* genes, binding to the specific palindromes preceding these genes (Ray and Larson 2004).

4. AgaR – transcriptional regulator of the operon

AgaR is a transcriptional regulator belonging to the DeoR family. Members of this family can be found in a variety of species, ranging from Gram-positive (*Lactococcus lactis*, *Streptococcus mutans*) to Gram-negative bacteria (*E. coli*, *K. pneumoniae, P. aeruginosa*), being present in around 58% of species of *Prokaryota* (Pérez-Rueda and Collado-Vides 2000; Ray and Larson 2004). *E. coli* family of DeoR regulators contains at least fourteen members, which usually function as repressors in sugar metabolism (Elgrably-Weiss et al. 2006). Examples being GutR (glucitol metabolism), LacR (lactose metabolism) FucR and FruR responsible for fucose and fructose respectively, as well as other (Pérez-Rueda and Collado-Vides 2000). Proteins in this family exhibit several common features with high degree of size conservation ranging from 240 to 260 amino acids and highly conserved regions, such as the second helix, known as recognition helix of the helix-turnhelix DNA-binding motif in the N-terminus. The first helix of the DNA-binding domain, however, presents little conservation(Pérez-Rueda and Collado-Vides 2000).

Recent studies by Zhang et al. and Leyn et al. show that there is at least five different orthologs of AgaR regulator characterized by different DNA motifs, discovered throughout 21 species of *Proteobacteria*, some of which even carrying two copies of *agaR* genes. Leyn et al. also observed strong tendencies for *agaR* genes to cluster on the chromosome with GalNAc utilization genes, which suggests conservation of AgaR function. All of the AgaR binding motifs, investigated by comparative genomics approach, shared a common CTTTC pattern with a common consensus of a direct repeat of this sequence. Groups (I), (II) and (III) shared a common CTTTC-5nt-CTTTC consensus, with the copy number and orientation differing between species. In contrast to that, group (IV) had an inverted repeat with the consensus CTTTC-15nt-GAAAG, while group (V) had a common structure with two inverted repeats GAAAG-(16-18)nt-GAAAG (Leyn et al. 2012).

Zhang et al., however, discovered only two, distantly relative, groups of AgaR (R1 and R2), sharing only 30% sequence similarity, as well as proposed different binding site motifs for these two orthologs. In most genomes analyzed using comparative genomics approach, they discovered that *agaR1* had a tendency to cluster on a chromosome close with genes for glycoside hydrolase and PTS, while *agaR2* has a similar tendency for co-localization with the genes encoding deacetylase, isomerase, and aldolase (Zhang et al. 2015). The candidate motifs for binding both regulators also shared a similar palindrome structure. AgaR1 binding sites had a length of 20 bp, whereas AgaR2 binding sites had 18bp. In addition, both AgaR binding motifs had an AT-rich central part with AgaR2-binding motif being more conserved and GC-rich in general (Zhang et al. 2015).

Typically, DeoR binding site is formed by the residues of several amino acids belonging to peripheral subdomains labeled P1 and P2 as well as a double Rossmann fold subdomain at their interface. Although the total sequence identity between different DeoR family regulators may differ even up to 80% difference, the three subdomains forming the binding site have a conservative structure, as well as position and ligand binding orientation (Škerlová et al. 2014). The effector molecule forms a strong covalent linkage with binding sites amino groups, deeply burring itself in the effector-binding site (Škerlová et al. 2014). Škerlová et al. also discovered that C-DeoR was the first discovered bacterial transcriptional regulator, that had its effector module covalently bound (Škerlová et al. 2014), suggesting there can be more DeoR family members expressing this characteristic.

During the study by Ray et al., two promoters for AgaR binding were discovered within *agaR-agaZ* region in *E. coli* K-12, as well as the third one in *agaS-agaA* intergenic region (Ray and Larson 2004). They also discovered that AgaR negatively regulates transcription from each of the three promoters. The absence of this repressor led to growthrate on galactosamine similar to that on glucose without the repressor. In addition to that, *agaZ* promoter was discovered to be a subject not only to repression by AgaR but also catabolite repression. Its expression was discovered to be 10 times higher in cells grown on casamino acids and GalN than on glucose without the functional AgaR. *agaZ* promoter region contained a sequence similar to that recognized by the cAMP-cAMP receptor protein (CRP) complex-(Zhang and Ebright 1990). In mutants with or without the functional *agaR* gene addition of cAMP resulted in 10-fold upregulation (Ray and Larson 2004).

5. AgaS – isomerase right in the heart of the pathway

The most conserved enzyme in GalNAc/GalN pathway discovered by Leyn et al. was AgaS, a hypothetical sugar phosphate isomerase from the SIS family, that was present in all analyzed genomes (Leyn et al. 2012). Enzymes belonging to this family are essential for bacterial proliferation, being often responsible for sugar catabolism, thus regulating general bacterial metabolism, allowing for biogenesis of virulence factors such as LPS, and modifying those factors (for instance altering the O-antigen sugar composition of LPS) as a mean of drug resistance (Gourlay et al. 2010).

The enzymes contain Sugar ISomerase (SIS) domain, responsible for sugar isomerization or sugar binding activities, yet differing with regards to their overall structure and sequences, sometimes even presenting less than 20% sequence similarity (Sommaruga et al. 2009; Gourlay et al. 2010).

Usually, SIS domain in enzymes has a catalytic function as isomerase and binds to phosphorylated sugars (Bateman 1999), in case of AgaS being GalN-6-P. The domain is also present in a family of bacterial transcriptional regulators, such as the ribose-phosphate-isomerase regulator RpiR, which regulates the *rpiB* gene (Sørensen and Hove-Jensen 1996). However, there are no reports of SIS domain regulators demonstrating catabolic activity yet (Bateman 1999).

The deaminase/isomerase activity of AgaS was evaluated by Leyn et al. based on the enzyme overexpressed in *Shewanella*. Its activity with GalN-6-P was approximately 27-fold higher than with GlcN-6-P (glucosamine-6-phosphate) (9,48 μmol/mg x min to 0,35 μmol/mg x min respectively). Their comparative genomic analysis also suggested that *agaS* is a universal catabolic gene in *aga* operon for all *Proteobacteria*. Unfortunately, at the moment of writing there is not much known about *in vitro* enzymatic activities of AgaS derived from other bacteria. Considering AgaS high conservatism, its high specificity towards galactosamine derivatives compared to other amino sugars, and the lack of *in vitro* research of this enzyme, it would be fair to state that, at the moment, enzymatic activity of AgaS isolated from *Shewanella* is representative of AgaS isomerases as a whole. In addition to that *ΔagaS* knockout mutants derived from *Shewanella* completely lost their ability to use GlcNAc as a sole carbon and nitrogen source in minimal medium. This deletion also potentially prevented the transcription of the following *agaY* gene. Therefore Leyn et al. confirmed AgaS to be essential in GlcNac/GalN catabolic pathway (Leyn et al. 2012), possibly inhibiting bacterial virulence.

6. AgaA – deacetylating and merging the processes

AgaA performs the role of GalNAc-6-P deacetylase in this catabolic pathway. This enzyme functions as a catalyst for a bridge reaction of transforming N-acetyl-D-galactosamine-6-phosphate to D-galactosamine-6-phosphate. *aga*A gene is usually closely clustered with other *aga* genes. During their study Leyn et al. found two variants of AgaA enzyme, AgaA^{II} being distinct, and characteristic only for *Shewanella* spp. A number 11 other species of *Proteobacteria* in which *aga*A gene has been found, had been functionally and genetically identical to that of *E. coli*. In *Shewanella* spp. AgaA^{II} shared 50% of similarity to NagA, responsible for N-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P) deacetylation (Leyn et al. 2012).

Both variants of AgaA, as well as NagA belong to COG1820 protein family of amidohydrolases. Additional phylogenetic analysis by Leyn et al. has confirmed that AgaA^{II} is a close paralog of NagA, which suggested that its development might have been a result of recent gene duplication event. Interestingly, in six *Proteobacteria* species analyzed by them, GalNAc-6-P deacetylases of both types were missing, suggesting that these organisms had the ability to utilize GalN, but not GalNAc (Leyn et al. 2012).

Thus, the presence or absence of *aga*A gene is crucial for the bacteria to utilize GalNAc. Leyn et al. as well as Zhang et al. have suggested that the presence of *aga*A, thus would also determine PTS transporters specificity (*aga*A lacking bacteria would have GalN-specific PTS), as was the case for *Shewanella* spp. as well as some other species (Leyn et al. 2012). As the result of their comparative genomics study Zhang et al. discovered one PTS previously described as GalN-specific in *Lactobacillaceae*. However, its co-occurrence and co-localization of its genes with *aga*A in most of the studied organisms was contrary to the previous suggestion (Zhang et al. 2015).

Additionally, Leyn et al. discovered the expression of *aga*A^{II} as well as *agaK* and *agaS* in *Shewanella* grown on GalNAc to be elevated over 100-fold compared to cells growing on GlcNAc (Leyn et al. 2012).

Enzymatic activity of AgaA^{II} was also evaluated in the aforementioned study. The enzyme exhibited significantly higher deacetylase activity with GalNAc-6-P than with GlcNAc-6-P (7,98 μmol/mg x min to 0,76 μmol/mg x min respectively) (Leyn et al. 2012).

Further, *E. coli* K-12 strain had a large deletion of several *aga* genes as well as truncation of *aga*A, resulting in GalNAc-negative phenotype (Leyn et al. 2012).

7. AgaZ – the weak link

AgaZ, which functions as Tag-6-P kinase, was present in most genomes studied by Leyn et al. in their comparative genomics study. Contrary to that, Zhang et al. discovered this enzyme and a gene encoding it throughout *Lactobacillaceae*. No paralogs of this enzyme were also found in genomes lacking them. However, it was suggested that this function is compensated by other enzymes, PfkA (phosphofructokinase I) and LacC, both having tagatose-6-phosphate activity (Leyn et al. 2012; Zhang et al. 2015). Brinkkötter et al. even suggesting that AgaZ functions as a non-catalytic subunit of AgaY [9,8].

Despite that, Leyn et al. observed in their study, that patterns of distribution of *agaZ* and *agaY* genes

are different. This observation, as well as an upregulation of *agaZ* in GalNAc environment (see AgaR) (Ray and Larson 2004), however, does not support the previous hypothesis, and suggests that AgaZ has its essential role in GalNAc catabolism independent of AgaY, which has not been discovered. The Catabolic activity of AgaZ has not yet been studied and remains unknown.

8. AgaY – final stitches

AgaY is a tagatose-1,6-diphosphate aldolase, a final enzyme in GalNAc catabolic pathway, catalyzing Tag-1,6-PP breakdown to glycerone phosphate and D-glyceraldehyde-3-phosphate. It was found by Leyn et al. and Zhang et al. to be present in almost every member *Enterobacterales* and *Vibrionales* orders but missing in *Shewanella* and several other *Proteobacteria* (Leyn et al. 2012; Zhang et al. 2015). However, in *Shewanella* its activity is compensated by non-committed enzymes such as class II fructose-bisphosphate aldolase (Fba), present in all its species. Interestingly Fba in *Shewanella* spp. is more similar to AgaY than Fba in *E. coli* (50% to 35% sequence similarity) (Leyn et al. 2012). Moreover, *Haemophilus parasuis*, pathogenic bacteria which cause Glasser disease in pigs, had a unique variant of GalNAc utilization genes including: a different *agaY*^{II} type, encoding an alternative version of tagatose-1,6-biphosphate aldolase belonging to LacC family (Rosey et al. 1991), as well as a unique type V PTS (Leyn et al. 2012).

It is worth mentioning that during their study in *Shewanella* spp. Leyn et al. discovered that the deletion of *agaS* gene prevented *agaY* from expression, thus resulting in a mutant's inability to use GalNAc. Complementation of this effect via restoring both *agaS* and *agaY* resulted in restoration of this ability, which was not the case when only one of them was complemented (Leyn et al. 2012).

9. PTS systems (AgaBCD/VWEF) – not only the transport

agaBCD and *agaVWEF* from the AgaR regulon encode two types of phosphotransferase (PTS) systems. Complex enzyme systems widely used by bacteria for the detection, transport and phosphorylation of various sugar substrates, including monosaccharides, disaccharides, amino sugars, polyols, and other sugar derivatives (Sørensen and Hove-Jensen 1996; Leyn et al. 2012). PTS systems catalyze the uptake of carbohydrates as well as their conversion to their respective phosphoesters during transport. The source of energy for these systems is phosphoenolpyruvate or PEP. The PTS systems have two general components: Histidine Phosphocarrier protein (HPr) with enzyme I and membrane bound sugar specific permeases (Enzymes II). Each enzyme II consists of one or two hydrophobic and two hydrophilic domains. They can exist as distinct proteins, as well as a single multidomain protein. Catalysis of the uptake of sugars and their conversion to phosphoestre is performed by four successive phosphoryl transfers. Initial phosphorylation of enzyme I using PEP as a substrate, transfer of the phosphoryl group from enzyme I to HPr, the self-phosphoryl transfer in HPr catalyzed by enzyme II, after which the phosphoryl group is transferred to histidine or cysteine residues of enzyme II. The sugar is transported through the membrane-bound enzyme II and undergoes phosphorylation (Meadow et al. 1990; Hassan et al. 2014).

PTS^{IIGalNAc} (*agaBCD*) being GalN-specific and GalNAc-specific PTS^{IIGalN} (*agaVWEF*) (Reizer et al. 1996; Ray and Larson 2004; Leyn et al. 2012). Both systems belong to the mannose-sorbose family (Leyn et al. 2012). PTS^{II} contains three domains (IIB, IIC and IID) encoded by the three *aga* genes, which are fused to one peptide. *aga*F however, encodes IIA PTS protein, that functions in the transport of both amino sugars (Ray and Larson 2004). During their study Leyn et al. discovered four types of PTS in *Proteobacteria* and distinguished two separate clades of PTS based on the components that are encoded by gene loci containing the adjacent *aga*A deacetylase gene. This may hint that these two clades (labeled PTS^{I} and PTS^{III}) are GalNAc-specific, whereas PTS components not encoded in close vicinity to *aga*A (PTS^{II} and PTS^{IV}), are GalN-specific (Leyn et al. 2012). Zhang et al. discovered a separate uncharacterized PTS^{V} type system which is found in some *Proteobacteria* (Zhang et al., 2015).

Amino sugar specific PTS play a crucial role of defining bacterial ability of their utilizations. Bacteria without them, for example, *E. coli* K-12 strain which lacks GalNAc-specific PTS, cannot use this sugar as a sole carbon, nor nitrogen source like other *Proteobacteria* (Brinkkötter et al. 2000; Mukherjee et al. 2008; Leyn et al. 2012). Some bacteria, however, without this specific PTS systems, for example *Shewanella*, developed unique sets of GalNAc- and GalN-specific permeases (AgaP) and kinases (AgaK) (Leyn et al. 2012).

10. AgaP and AgaK – new additions to the family?

AgaP is an amino sugar transporter protein, which is commonly accompanied by other amino sugar related transporters belonging to Ton-B-dependent receptors. *Shewanella spp.*, as well as *Burkholderia*

cenocepacia and *Caulobacter spp*., which lacks typical GalNAc-specific transporters utilizes sugar uptake through the outer membrane using a TonB-dependent Omp^{Aga} transporter. Transport through the inner membrane is done by AgaP permease. The subsequent phosphorylation of amino sugar is performed by AgaK kinase (Leyn et al. 2012).

AgaP was found to be a close paralog (50% similarity) to NagP, a GlcNAc permease belonging to GGP sugar transporter family (Rodionov et al. 2010; Leyn et al. 2012). AgaK was found by Leyn et al. to be most similar to the *Shewanella spp.* Glk^{II} glucokinase belonging to ROK family (35% similarity) (Rodionov et al. 2010; Leyn et al. 2012).

The *aga* cluster genes in *Burkholderia cenocepacia* and *Caulobacter* encoded different $AgaP^{II}$ and $AgaK^{II}$ variants (belonging to EamA and BcrAD_BadFG families respectively). With the absence of GalNAc-6-P deacetylase, and GalNAc-, GalN-specific PTS in their genomes, it was suggested that these enzymes are involved in GalN uptake and phosphorylation (Leyn et al. 2012).

This hints at the importance of GalNAc utilization for bacteria, which develop alternative routes of its uptake if the classic *aga*-controlled pathway is unavailable.

11. Aga operon as potential target for therapy

Another important aspect of amino sugar metabolism is its practical use by pathogenic bacteria that can be turned to our advantage. As was mentioned earlier, both amino sugars are directly or indirectly connected to bacterial virulence factors, examples being: LPS, murein and protein glycosylation and in some cases molecular mimicry. At the moment, however, there is not much knowledge available, with the exception of a few case and genomic studies, about how GalNAc and GalN utilization influences bacterial virulence.

The example of a case study is one conducted by Mukherjee et al. on several *E. coli* O157:H7 strain isolates derived from 2006 spinach outbreak compared to other *E. coli* strain notably K-12. They discovered that most of the spinach-associated O157:H7 isolates presented similar to K-12 strain $GalNAc^-$ phenotype unlike most of the other available O157:H7 representatives. It was suggested that this change was a result of a neutral mutation in *agaF* gene responsible for encoding Enzyme II of GalNAc-specific PTS. The aforementioned mutation has had a tendency to spread among *E. coli* O157:H7 as an adaptation useful in colonizing new niches (Mukherjee et al. 2008). This hints at the fact that one of the reasons *E. coli* K-12, (in case of the other strains, a widely spread multi-niche pathogen) is unable to secure a successful infection to be a result of its inability to use amino sugars, represented by GalNAc, as a carbon and nitrogen source highly present in animal and human organisms. Knowing this may help us understand strain-specific pathogenicity and perhaps even combat it.

Confirming the previous statement, Zhang et al. in their study conducted several Proteobacterial invasion assays based on animal models, and discovered that in case of *Streptococcus suis*, mostly a cattle-based pathogen with a limited ability to transfer and cause infection in humans, GalNAc/GalN utilization regulated by AgaR2 played a crucial role in its virulence (Zhang et al., 2015).

In the same study authors went as far as to suggest that both amino sugars took part in bacterial recognition and crosstalk, thus through maintenance and regulation of their utilization pathway allowing for successful infections (Zhang et al. 2015).

On the other hand, for *Proteus mirabilis*, a mostly human pathogen responsible for Urinary Tract Infections (mostly catheter-associated urinary tract infections, CAUTIs), but sometimes appearing in veterinary conditions, usage of amino sugars via *aga* genes also seemed to be important. As a result of a genome-wide transposon mutagenesis of Armbruster et al. AgaR regulator was listed as one of the fitness factors for human kidney colonization in this uropathogenic bacteria (Armbruster et al. 2017). However, the exact role and extent of GalN/GalNAc metabolisms influence on uropathogenicity is yet to be investigated.

How will this knowledge allow us to specifically combat pathogen strains which base their pathogenicity on the ability to use amino sugars? This would be possible through targeting key pathway elements in their metabolism, or bacterial constructs using such sugars similarly to as discovered by Gill et al. during their study. According to them GalNAc-linked post-translational modifications were used by pathogenic bacteria to modify host GTPases, through toxins, with α-GalNAc-O-Tyr in order to promote their virulence (Gill et al. 2011). Based on this information Behren et al. prepared vaccine constructs as well as polyclonal antibodies specific to this modification. They suggested that GalNAc protein residues might become a vital target in specific glycoproteomal detection, as well as vaccines and therapy in the future (Behren et al. 2023).

12. Conclusion

Not much is still known about amino sugars, represented by N-acetyl-d-galactosamine and D-galactosamine, role in bacterial pathogenicity. Recent studies, however, show that human and animal pathogens,

able to successfully secure infections, developed highly sophisticated and specific processes of their utilization, especially in comparison to non-pathogens who often lack the ability (Shen et al. 2025). Amino sugars utilization often appears alongside other, already known to be important, virulence factors is sure to be directly or indirectly involved in developing bacterial pathogenicity. Hence, it is not to our surprise that there are more and more real possibilities for these amino sugars, their utilization pathways as well as the *aga* operon responsible for it, to be used as targets in bacterial classification, pathogen detection and possibly even therapy start to appear. To achieve this, however, more studies regarding GalNAc/GalN utilization pathway in bacteria should be conducted in the future.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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REVIEW
ADVANCEMENTS OF MICROBIOLOGY
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PHAGE THERAPY AS AN ALTERNATIVE TO ANTIBIOTICS: HARNESSING PHAGES IN ORAL MEDICINE

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Abstract: This narrative review aims to discuss the potential of phage therapy as an alternative to antibiotic therapy, with a particular emphasis on its potential applications in dentistry.
Despite historical fluctuations, phage therapy is gaining increasing acceptance and becoming more widespread in the treatment of multidrug-resistant (MDR) infections. Contemporarily, phage therapy has become a prevalent treatment modality for bacterial infections of the digestive system in countries such as Georgia. In clinical trials, it has been used to treat wounds, bone and blood infections. In theory, it can be used in any instance of bacterial infection, including within the oral cavity. There are numerous methods of administration, and the therapy itself is regarded as both safe and effective, particularly in cases where antibiotics are ineffective, such as in the development of biofilms or infections by MDR bacteria.
Despite the lack of ample clinical studies evaluating the effectiveness of this therapy in dentistry, a growing body of evidence suggests its potential use in restorative dentistry, endodontics, periodontology, as well as oral and maxillofacial surgery.
To determine whether phage therapy can be considered a viable alternative or to assess its effectiveness when used in combination with antibiotic therapy, further regulations and research are warranted.

Keywords: antibiotic resistance; bacteriophage; biofilm; dentistry; phage therapy

1.Introduction

Bacteriophages are a group of viruses that can adhere to bacteria, inject their genome into the bacterial cell, and eventually induce bacterial lysis (Naureen et al. 2020). Their discovery dates to the beginning of the 20th century, and their clinical implementation in the treatment of bacterial infections was tested shortly after. However, due to the introduction and development of antibiotic therapy, phage therapy was greatly abandoned by Western science (Marongiu et al. 2022).

Nowadays, humanity stands on the verge of the post-antibiotic era (Kwon and Powderly 2021), with a desperate need to find alternatives to treat infection of multidrug-resistant (MDR) bacteria, mostly belonging to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.). Contemporary scientific data indicate that the emergence of antibiotic resistance in bacterial pathogens is a growing concern for global health (Ventola 2015; Frieri et al. 2017; Hofstee et al. 2020; Man-

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cuso et al. 2021). There is a risk that bacteria causing infections that have been treatable with antibiotics will become resistant to these drugs within the next few decades. This imminent threat has prompted a response from the scientific community in diverse fields, with researchers undertaking efforts to identify novel, more efficacious antibiotics (Miethke et al. 2021).

There is a need to find alternative solutions that could mitigate the impact of this looming crisis. One of these is bacteriophage therapy, also known as phage therapy (PT). This approach traces its origins to the early 20th century, yet it has only recently garnered significant attention (Figure 1). The concept involves the use of carefully selected bacteriophages to combat pathogenic bacteria (Sawa et al. 2024).

This narrative review examines the potential of phage therapy, which can be utilized to both prevent and treat bacterial infections, particularly in dental settings. It revises forms of its administration, analyses the results of published clinical trials, and discusses its safety and potential risks.

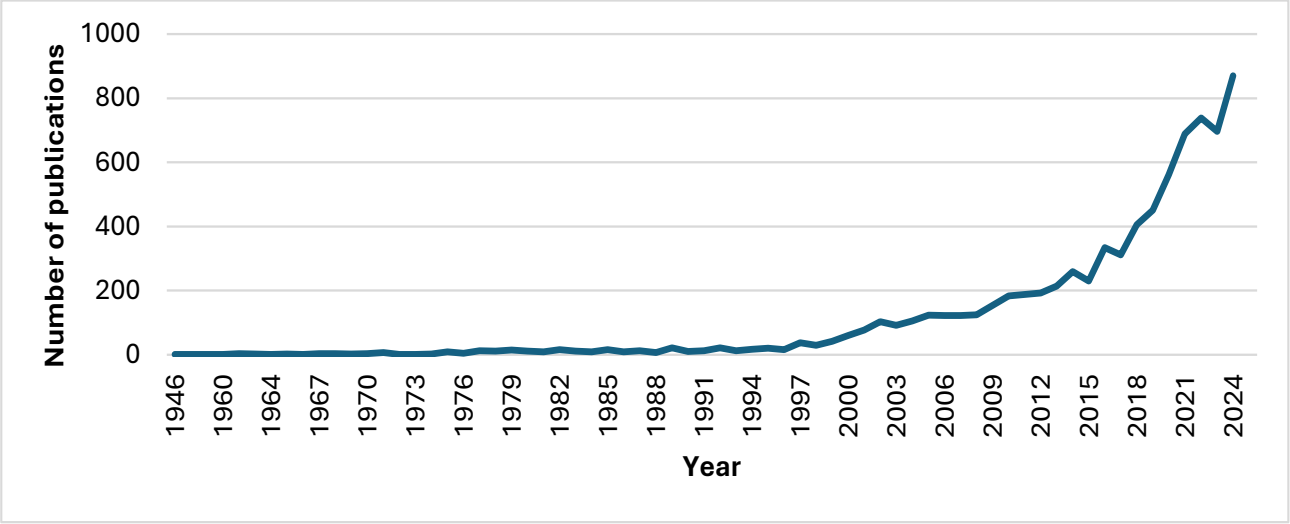

Figure 1. The number of papers retrieved from PubMed using the keyword 'phage therapy' that were published between 1946 and 2024 shows a significant increase in research in recent years.

2. Discovery and development of PT

Many scientists have observed bacteriophage activity since the late 19th century. However, the first to understand that they were dealing with a new organism, rather than, for example, an enzyme, was the French Canadian microbiologist Félix d'Hérelle (Marongiu et al. 2022). In 1915, during World War I, he investigated the outbreak of severe haemorrhagic dysentery among French troops. He made filtrates of faeces that were free from bacteria. Then, he spread them on the agar cultures of *Shigella* spp strains collected from sick soldiers. He observed blank spots that he later named *plaques*. Two years later, in 1917, he presented his findings during a meeting of the Academy of Sciences, and he proposed the name "bacteriophage" from bacteria and the Greek word *phage*, which translates as "to eat" (Chanishvili 2012). He realized the importance of his discovery, so after conducting small animal trials and testing phages for safety on himself, he proceeded to clinical trials on humans, initiating what is now known as PT (Marongiu et al. 2022).

In 1919, three young brothers with symptoms of dysentery were admitted to the hospital where Félix d'Hérelle worked. Their sister had died of this disease a day before. One day after the introduction of phage therapy, they fully recovered. He later treated in a similar way cholera with positive results, which encouraged other researchers to test the therapy. For example, British Lieutenant Colonel John Morison used phage therapy as a prophylaxis for cholera in the Indian region of Naogaon (now Bangladesh). He allegedly administered phages to 530,000 people, which resulted in zero cases of cholera in that region between 1925 and 1935. In contrast, during the same period in the neighbouring region, 1,500 people died of the disease (Summers 1993).

Despite many clinical successes, there were voices in the scientific community that d'Hérelle and his colleagues conducted experiments that did not meet

scientific standards and, therefore, did not provide a factual basis for using this form of therapy. Notably, he was accused of lacking controlled conditions and statistical significance in his results. In connection with the above, the scientific community at the time did not accept PT as an effective treatment method and continued to rely on preventive measures, such as improved hygiene and vaccinations (Summers 1993).

In 1930, two American physicians, E. D. Crutchfield and B. F. Stout, conducted a study on 57 patients with staphylococcal wound infections using phage therapy, achieving a success rate of over 90% (Crutchfield and Stout 1930). This result encouraged d'Hérelle to bring his discovery to the market and start mass production. However, over time, it became apparent that the effectiveness of these phage preparations was significantly lower than initially reported in preliminary studies, which discouraged pharmaceutical companies, patients, and scientists alike. The decrease in efficacy was caused by several factors, including poor storage conditions of the preparations, their short shelf life, the use of mercury as a preservative, the failure to inactivate them by gastrointestinal substances, and the implementation of phage therapy to treat viral infections (Ho 2001; Marongiu et al. 2022).

The subsequent introduction of the first antibiotics (penicillin and sulfanilamide), which was then considered a breakthrough in contrast to bacteriophages where each phage affected only one type of bacterium, led to a further decline in the popularity of PT in the West (Marongiu et al. 2022).

During d'Hérelle work at the Institute Pasteur in Paris, he met Georgian bacteriologist Georgiy Eliava. Eliava was fascinated by d'Hérelle's discoveries, and in 1923, he founded the Institute of Bacteriology in Tbilisi, the capital of Georgia. It received massive support from the authorities of the Soviet Union, who wanted to catch up with the West, also in the field of bacteriology. Unfortunately, in 1937, during the Great Purge, Eliava was declared an enemy of the people and then shot. This fact led the USSR to lose a scientist recognized in the West (Marongiu et al. 2022). Despite this, the Soviets continued to develop and use phage therapy (Summers 2001). The Iron Curtain after World War II hindered the flow of scientific knowledge and strengthened the belief of phages as a communist medicine among Western scientists (Summers 2012).

Further controversy was also caused by d'Herell's views, which some scientists even described as heresy. He claimed that phages, which naturally exist in the human microbiota, are responsible for the body's immunity and that phages can spread just like pathogens they are supposed to kill, so one should not pay too much attention to personal hygiene. He argued that contamination with faeces can paradoxically contribute to the improvement of the patient's condition because, in this way, one receives new portions of phages (Fruciano and Bourne 2007).

In 1946, at St. Mary's Hospital in London, the same hospital where penicillin was discovered, F. Himmelweit presented the concept of therapy based on the joint administration of phages and penicillin. The assumption was that this would lead to a reduction in the emergence of penicillin-resistant strains. The preliminary results of the research were auspicious, which was probably due to the different effects of penicillin and phages on bacteria. Unfortunately, this principle was not widely accepted in the scientific community (MacNeal et al. 1946; Fruciano and Bourne 2007).

It is estimated that in the process of finding new antibiotics, only five of the 5,000 to 10,000 molecules tested make it through to the first stage of testing, but only one of these is approved for use in humans. Given the enormous costs and complexity of developing new antibiotics, as well as the increasing multi-drug resistance of bacteria, the scientific world is seeking alternatives. Today, more than 100 years after d'Herell's discovery, PT is being rediscovered and increasingly tested; however, scientists agree that it needs to be standardized, and more clinical studies are warranted (Gordillo Altamirano and Barr 2019; Suh et al. 2022).

3. The characterization of phages

Phages, as representatives of viruses, are unable to function and replicate outside the host organism. They are the most abundant organisms on Earth, and in addition to bacteria, it has been proven that they can occur inside archaea and even eukaryotes (Lehti et al. 2017; Naureen et al. 2020). The reproductive cycles of bacteria and phages are closely linked; however, each cycle produces between 100 and 200 new phages but only two daughter bacterial cells. This phenomenon suggests that there would be a significant disproportion in the number of bacteria or even their disappearance, but this does not occur. To understand this, it is essential to have a comprehensive understanding of

phage biology and its impact on bacteria (Naureen et al. 2020).

Bacteriophages have a complex structure, particularly when compared to simpler viruses, such as those with only a capsid and genetic material. It consists of DNA or RNA, single or double-stranded, enclosed in a protein capsid that occurs in three forms: head with tail, head without tail, and filamentous form (Naureen et al. 2020). Tailed phages (collected in the *Caudoviricetes* class) are the most common group, previously represented as the families *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Figure 2). However, the predominant feature they exhibited was morphology; presently, the emphasis is on classification based on the genome. Despite this, attention is drawn to the importance of morphological similarities. Hence, the terms myovirus, siphovirus and podovirus remain in use (Turner et al. 2023).

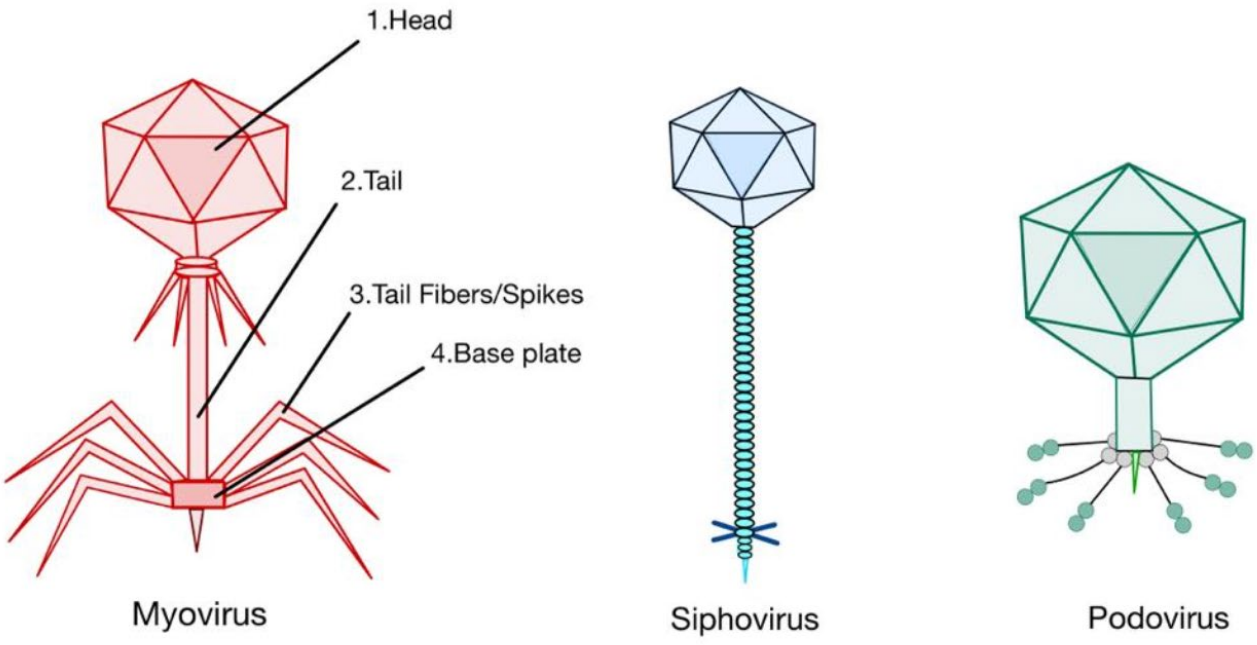

Figure 2. The diagram illustrates a simplified representation of the structural composition of different types of tailed phages class (*Caudoviricetes*): 1. Head - a protein structure, often icosahedral in shape, containing the phage's genetic material (single or double strand of DNA or RNA); 2. Tail - a tubular structure used by the phage to inject its genetic material into the host cell; 3. Tail fibres or spikes - enable the phage to recognize and attach to the appropriate receptors on the surface of the bacterial cell and determine the specificity of the bacteriophage to a particular bacterium; 4. Base plate - anchors the tail fibres and binds them to the surface of the bacterium, enabling the infection to begin. Graphic design was based on Nobrega et al. (2018).

There are four different phage life cycles: lytic, lysogenic, pseudo-lysogenic and chronic. Only lytic phages are currently considered the most suitable for treating humans (Figure 3) (Lin et al. 2017). First, the phage must attach to the bacterial cell wall by recognizing its specific receptor on the bacterial surface. Some phages possess the ability to produce specific enzymes, such as hydrolases, which can degrade the polysaccharide envelope of a bacterium, thereby exposing the appropriate receptors (Wittebole et al. 2014). After attaching to the cell wall, the phage creates a hole in it through which it injects its genetic material into a bacterium while the protein capsid remains outside. In the case of lytic phages, the bacterial ability to reproduce viral proteins and genetic material is exploited downstream. Phage assembly is followed by lysis of the bacterial cell and the release of new viral particles. The amount of viruses released depends on several factors, including the type of virus, the type of bacterium, and environmental conditions (Wittebole et al. 2014; Naureen et al. 2020).

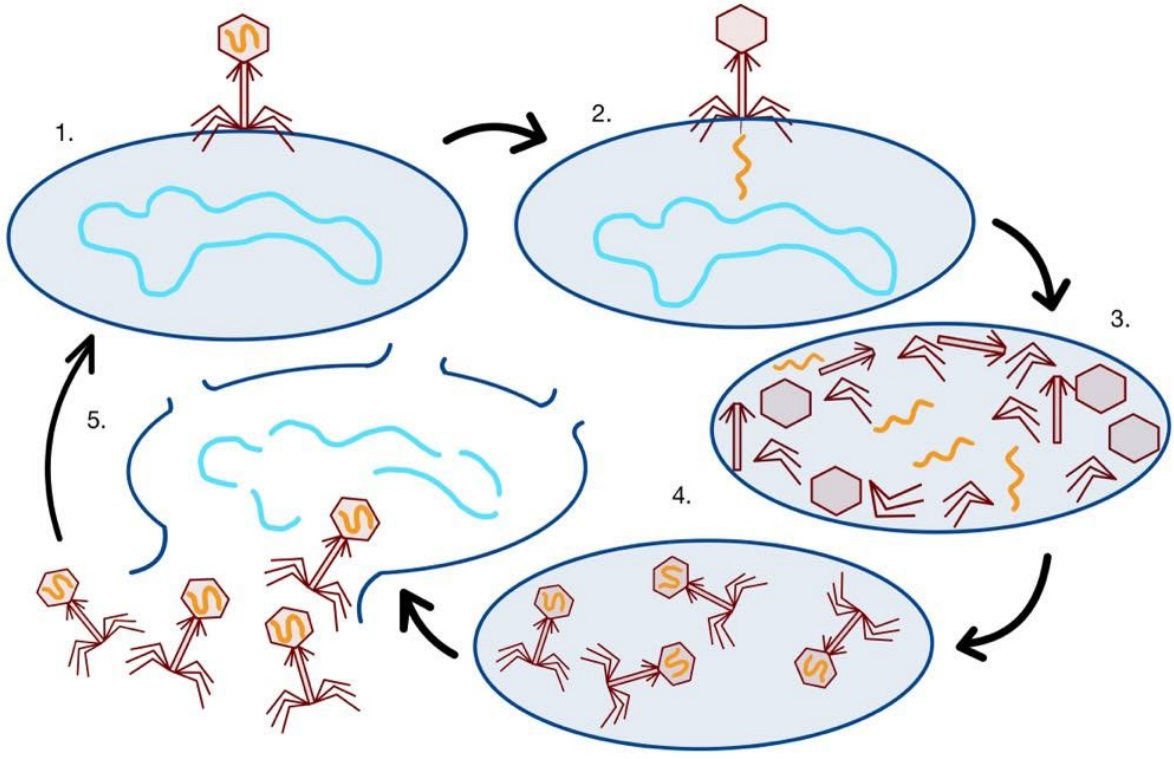

Figure 3. Diagram illustrating the lytic cycle of a bacteriophage, highlighting its mechanism of bacterial cell destruction – a key process in phage therapy: 1. Attachment; 2. Penetration; 3. Biosynthesis; 4. Replication and maturation; 5. Lysis. Graphic design based on Adesanya et al. (2020).

4. Methods of phage administration

The specificity of phage therapy predicated on the necessity to administer a particular phage to combat a specific bacterium and the brief duration of phage activity within the body necessitate the development of novel methodologies for phage administration to patients. In addition to oral or intravenous administration, there are also inhalation, implantation of biomaterials, intraperitoneal, intramuscular, subcutaneous, intranasal, endotracheal, rectal, intrauterine, vaginal or transdermal routes of administration (Dąbrowska 2019; Rotman et al. 2020). It had been suspected that phages might be rendered ineffective by the presence of low gastric acid pH, which required their administration in conjunction with acid-neutralizing medications. However, subsequent research has demonstrated that the co-administration of these drugs with phages results in no alteration to their efficacy (Dąbrowska 2019). The oral route of administration is efficacious in treating gastrointestinal diseases (Qadir et al. 2018; Dąbrowska 2019). For instance, a study was conducted on the treatment of *Clostridioides difficile* infection using phage therapy in an animal model, resulting in a reduction in *C. difficile* colonization and a delay in the onset of symptoms (Nale et al. 2016). However, the oral route of administration is the least effective for systemic penetration of phages. It is essential to note that increasing the administered dose of phages results in enhanced systemic penetration. A systematic analysis has demonstrated the superiority of parenteral administration, whether intravenous (IV), intramuscular (IM), or intraperitoneal (IP) over oral dosing (Dąbrowska 2019).

Regarding IV administration, Speck and Smithyman, in their work on the safety of intravenous phage therapy, have described the use of highly specific phage cocktails in treating rhinosinusitis caused by *Staphylococcus aureus*. Furthermore, they discussed a potential application of phage therapy in the management of acute infective endocarditis (Speck and Smithyman 2016). This condition may be caused by bacteraemia following oral surgery (e.g. tooth extraction) in a high-risk patient.

Recent studies on the intramuscular (IM) administration of phages are lacking; however, a significant number of such studies have been documented in the Soviet scientific literature. For instance, in the treat-

ment of typhoid fever, patients were divided into three groups: intramuscular (IM), oral, and combined intramuscular and oral administration. The most unfavourable outcomes were observed for oral administration, while significantly superior outcomes were demonstrated in other groups (Chanishvili 2012).

To date, no studies on IP administration have been conducted in humans. However, animal research demonstrated the efficacy of this route of administration, surpassing the effectiveness of phage inhalation, for instance, in the treatment of pneumonia caused by *Burkholderia cenocepacia* (Carmody et al. 2010).

Following systemic administration, phages can reach virtually all tissues and organs of the body, including, but not limited to, skeletal muscle, heart, bone marrow, salivary glands, kidneys, and even the brain and bones (Dąbrowska 2019). However, oral administration may be ineffective due to the poor penetration of phages into the circulation, and intravenous administration appears to be significantly more efficacious (Dąbrowska 2019; Vila et al. 2024).

Regarding oral diseases, the most efficacious treatment may be rinsing the affected area with a phage solution or the local application of a slow-releasing hydrogel, which enables the appropriate concentration of phages within the oral cavity.

4.1 Phage clearance

The primary organs responsible for the uptake of bacteriophages from the bloodstream are the liver and spleen, with the macrophages present in these organs playing a specific role in this process. Animal research has demonstrated that phages are filtered and reach their highest concentrations in the spleen; however, the fastest inactivation occurs in the liver via Kupffer cells (Inchley 1969). The renal excretion of phages in the urine is minimal, likely attributable to their morphology, as they are too large to be filtered by the kidneys. The dosage of phages, as well as their type (size) and route of administration, seem to be all significant factors. No renal clearance was observed with subcutaneous administration as opposed to intraperitoneal injection (Dąbrowska 2019).

There are several ways to delay phage clearance, such as increasing the dose or inactivating the complement system; however, the most promising approach appears to be encapsulation of the phage particles, as seen in the case of phages against *Klebsiella pneumoniae*, which have been enclosed in a liposomal shell. It has been demonstrated that this procedure results in a prolonged retention of phages within the body, even in the absence of the targeted bacteria (Singla et al. 2015).

Numerous technological methodologies have been developed to extend the shelf life of phages and delay their removal from the body through stabilization and encapsulation techniques. The most widely employed techniques are those involving freeze drying, spray freeze drying and spray drying. Freeze-drying, also known as lyophilization, is a process that can be categorized into two distinct stages. The liquid containing the phages is subjected to a freezing process at a very low temperature, after which it is dried and ground to yield a powder which may be used for inhalation. Spray freeze drying is based on a similar principle. The process involves exposing a spray containing phages to liquid nitrogen, resulting in the production of a porous phage powder. This method has been shown to induce a lower degree of thermal stress in the phages compared to the spray drying process, which involves spraying a suspension containing phages into a chamber filled with hot, dry gas. The water evaporates rapidly, leaving the phages in a powdered state. It should be noted, however, that numerous alternative methods are available (Malik et al. 2017). In terms of phage storage, maintaining a temperature of 4°C has been demonstrated to be an efficient method for prolonging shelf life (Xu et al. 2023).

5. Safety of phage therapy

It is widely accepted that PT is safe and has no severe side effects (Uyttebroek et al. 2022; Kim et al. 2024; Palma and Qi 2024). The safety of phage therapy stems from the specificity of the phage, which can target a particular bacterial cell exclusively. This selectivity distinguishes it from antibiotics, which can affect the host microbiota. The specificity of phage action also means that pathological bacteria acquire phage resistance much less frequently than with antibiotics. While it is acknowledged that phage administration can elicit an immune response, to date, there is no evidence that phages are capable of directly attacking human cells. In the presence of compatible bacterial cells, phages can replicate; otherwise, they tend to self-regulate (Uyttebroek et al. 2022).

A substantial body of scientific research (Uyttebroek et al. 2022; Kim et al. 2024; Palma and Qi 2024) has demonstrated the safety of phage therapy in treating patients. No severe adverse reactions have been reported; however, some authors have noted inadequate standardization of safety studies (Chung et al. 2023). However, it is acknowledged that there exists a risk of toxic shock precipitated by the release of bacterial endotoxins following phage-induced bacterial lysis (de Tejada et al. 2015; Aslam et al. 2019). A 2017

study provides a notable example. It documented the case of a 2-year-old child infected with multidrug-resistant *Pseudomonas aeruginosa*, complicated by sepsis. The patient exhibited a chronic medical condition characterized by DiGeorge syndrome, a complex congenital heart defect involving an interrupted type B aortic arch, and an allergy to antibiotics, among other co-morbidities. The presence of both allergies and infection with MDR *P. aeruginosa* led researchers to consider bacteriophage therapy as a potential treatment. The child was administered two bacteriophages, resulting in the resolution of the sepsis. However, this intervention led to an exacerbation of the underlying heart failure, most likely attributable to the release of endotoxins following bacterial degradation (Duplessis et al. 2018).

Despite extensive research conducted over several decades on phage administration and its potential adverse effects, no documented cases of anaphylactic shock have been reported in patients receiving phage therapy (Speck and Smithyman 2016; Doub et al. 2020; Chung et al. 2023).

The safety of PT in children has been well-documented (Howard-Jones et al. 2022). To the best of our knowledge, there are no reports on the safety of phage therapy in pregnancy.

5.1 Safety comparison of phages and antibiotics

It is now widely acknowledged that the gut microbiota plays a pivotal role in the optimal functioning of the human body (Khalil et al. 2024). The use of antibiotics can result in a substantial reduction of beneficial microflora within the gastrointestinal tract, thereby promoting the proliferation of multidrug-resistant (MDR) *Clostridioides difficile* or the yeast *Candida albicans*. Recent studies have shown that phages have a minimal impact on the intestinal microflora, mainly due to their specificity (Chung et al. 2023).

In addition to the adverse effects of antibiotics on the human microbiota, their use can cause many, often severe, complications like pseudomembranous colitis. Other serious complications may also arise, including ototoxic effects of macrolides and aminoglycosides, liver damage, encephalopathy, or anaphylactic shock, among numerous others (Cunha 2001). In contrast, most studies on PT report no adverse effects. However, some authors do note the occurrence of some minor side effects, including redness, inflammation, fever and hypotension. To provide an accurate assessment of possible side effects, further research is warranted, along with the systematization and regulation of PT in its entirety.

6. The legal regulation of phage therapy

The issue of the absence of appropriate legal regulations is raised in this work and numerous other studies on phages. As stated in the historical introduction, PT was utilized on a broader scale in the Union of Soviet Socialist Republics (USSR) following World War II, with a particular emphasis on Georgia Republic. It was also used in Poland. Following Poland's accession to the European Union in 2004, this therapy has become more comprehensive and systematic. Nowadays, it is considered an experimental treatment based on the Act on the Professions of Physician and Dentist, the Constitution of the Republic of Poland, as well as EU regulations, such as Directive 2005/28/EC, and the European Medicines Agency. PT is exclusively administered by the Phage Therapy Unit, a constituent of the Hirszfeld Institute of Immunology and Experimental Therapy (Yang et al. 2023).

In contrast, in countries such as Georgia and Russia, the regulatory landscape is significantly different, with phage preparations, including 'Intestiphage' and 'Pyophage', available for purchase without a prescription. Regarding Georgia, the prescription-only provision for personalized phage medicines is permitted in designated pharmacies. However, these products have not been recognized by Western regulatory authorities (Yang et al. 2023).

7. Biofilm penetration by phages

Many species of bacteria form a biofilm, a complex environment composed of polysaccharides, proteins, and lipids that are designed to protect the bacteria from external factors. In practical terms, biofilms can effectively block antibiotic access to bacterial cells, making the treatment of infections significantly more challenging (Yuan et al. 2019).

There are numerous reports of bacteriophages' effectiveness in penetrating the bacterial biofilms of bacteria such as *P. aeruginosa* or *Streptococcus suis*. However, these studies indicate the need for further trials and the potential possibility of antibiotic-phage combination therapy (Hanlon et al. 2001; Meng et al. 2011; Yuan et al. 2019).

Bacterial biofilms, which pose a serious clinical challenge due to their ability to block access to antibiotics, are present in many parts of the body, such as the oral cavity (Rath et al. 2021), intestines (Jandl et al. 2024), and the female reproductive tract (Obuobi and Škalko-Basnet, 2024). A mixture of aerobic and anaerobic bacterial strains generally constitutes it. As previously outlined, the capacity of phages to penetrate

deeply into biofilms offers a promising prospect for the effective management of the bacteria that form these structures, potentially in conjunction with or as an alternative to antibiotics (Figure 4).

Although phages isolated from the oral cavity have not yet been used clinically, they have shown promising results *in vitro*. The SMHBZ8 phage is a prime example, as it has been demonstrated to be efficacious in combating both planktonic and biofilm cultures of *Streptococcus mutans*. This phage has the potential for practical use in future clinical trials (Zhu et al. 2025).

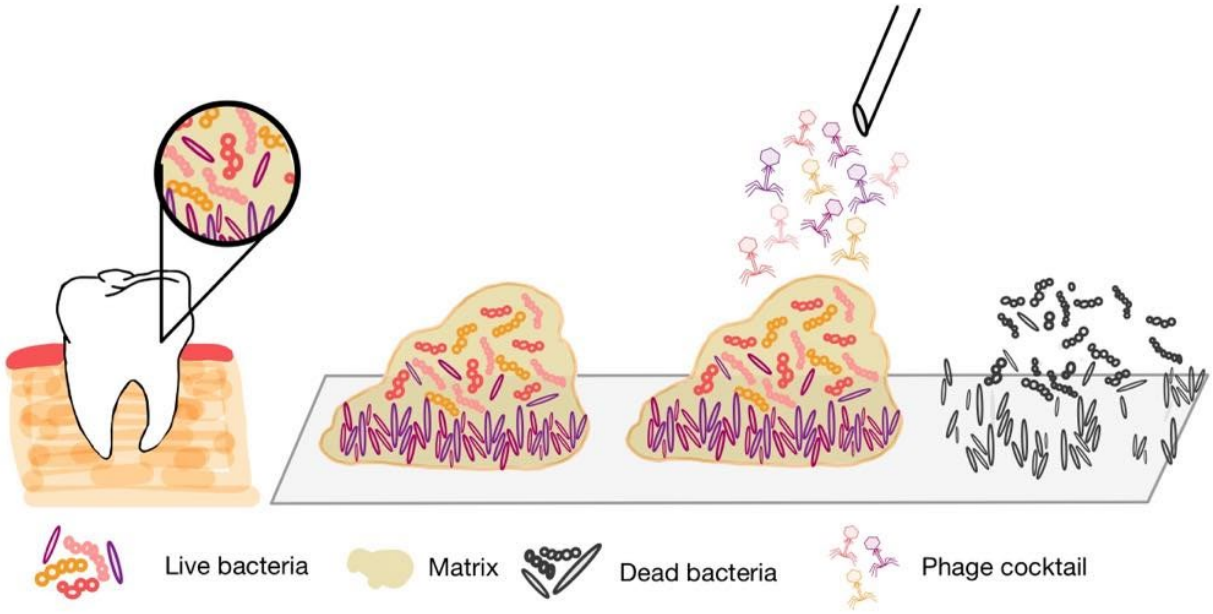

Figure 4. The biofilm in the oral cavity can lead to oral infections such as dental caries, gingivitis, or periodontal disease, and a carefully selected phage cocktail may have a potential effect on biofilm degradation.

8. Examples of phage prophylaxis and the concept of phage prophylaxis in oral surgery

For PT to be considered a complete alternative to antibiotic therapy, it would also need to be used as part of the prevention of bacterial infections. Broad-spectrum antibiotics, such as amoxicillin, are often administered to patients to prevent infection. Preventive action is challenging with phages due to their specificity; however, it is possible to create specific cocktails consisting of multiple different phages that are suitable for the groups of bacteria most likely to cause infections in a given area. The finding that encapsulated phages exhibit prolonged persistence within the human body (Singla et al. 2015; Malik et al. 2017) may provide a rationale for exploring the potential of phages in preventing bacterial infections. However, further research is necessary to test this hypothesis. A promising example of the prophylactic use of phage therapy is the study by Yu-Huai Ho et al., in which phage against *A. baumannii* carbapenem-resistant (CRAB) was aerosolized in intensive care units. This study demonstrated a reduction in the incidence of CRAB from 8.57 per 1,000 patient days in the hospital to 5.11 per 1,000 patient days (Ho et al. 2016). Another similar study was conducted by Chun-Chieh Tseng et al. in a unit with patients undergoing extracorporeal membrane oxygenation (ECMO). As before, an aerosol was used, this time containing a cocktail of four phages against the bacteria most commonly causing nosocomial infections in ECMO patients: A. baumannii, *K. pneumoniae*, *P. aeruginosa* and *Stenotrophomonas maltophilia*. As a result, no ECMO patient whose environment was decontaminated with phage aerosol became infected with any of the four target bacteria (Tseng et al. 2025).

The utilization of phages for prophylactic purposes in the domains of oral and general surgery is a matter of considerable complexity. The term "antibiotic prophylaxis" may refer to the administration of a broad-spectrum antibiotic to a patient at high risk of infective endocarditis prior to a dental procedure, such as tooth extraction. However, it is worth noting that phages require the presence of bacteria to be active and to carry out their life cycles. Consequently, these measures should be administered during or even after the procedure, in which case there is no question of prophylaxis. Advancements in phage encapsulation methodologies have the potential to extend the duration of phage presence in the body and delay clearance, thereby enabling the prophylactic use of phages prior to scheduled surgical interventions (Singla et al. 2015).

9. PT in clinical trials

Currently, one of the most popular methods of phage therapy (PT) is what can be described as profiled or even personalized PT. It is based on sampling the causative strain and selecting the appropriate phages for clinical administration. This method is most suitable for patients with intractable or recurrent infections caused by antibiotic-resistant bacteria. The following are examples of such use.

A patient with a spinal abscess caused by multi-drug-resistant *P. aeruginosa* underwent surgery during which the abscess was drained and a personalized cocktail of three bacteriophages, specifically prepared for the procedure, was administered into the abscess cavity along with a bicarbonate solution. At the same time, an antibiotic (cefiderocol) was administered intravenously. The pre-existing back pain disappeared after the operation, but there was diarrhoea associated with *C. difficile*. For the second surgery, related to the screw-in osteosynthesis implants, phages and antibiotics were again administered. In the months that followed, the patient experienced no pain or adverse effects (Ferry et al. 2022).

Another promising example of the use of personalized phage therapy is that of a 30-year-old female victim of the 2016 Brussels airport terrorist attack. The patient suffered severe injuries, including to her left thigh. The left thigh wound was found to be infected with pan-drug resistant (PDR) *K. pneumoniae*. Antibiotic treatment was implemented, which led to numerous side effects without eradicating the infection. After exhausting other treatment options, PT was administered 702 days after the accident. Phages were administered locally through a catheter left over from a previous procedure. They were given for 6 days along with a general course of antibiotics. There was a dramatic improvement in the patient's condition, with the permanent eradication of the *K. pneumoniae* infection (Eskenazi et al. 2022).

A retrospective observational analysis of 100 cases of personalized PT for difficult-to-treat infections showed that this therapy is most effective when combined with antibiotics. Without co-administration of antibiotics, bacterial eradication by phages alone was 70% less likely to occur. These examples imply that, in some cases, antibiotics and phage therapy can be synergistic (Pirnay et al. 2024).

The potential of PT to treat bacterial infections in any organ or tissue in the body is a promising avenue for further research (Figure 5).

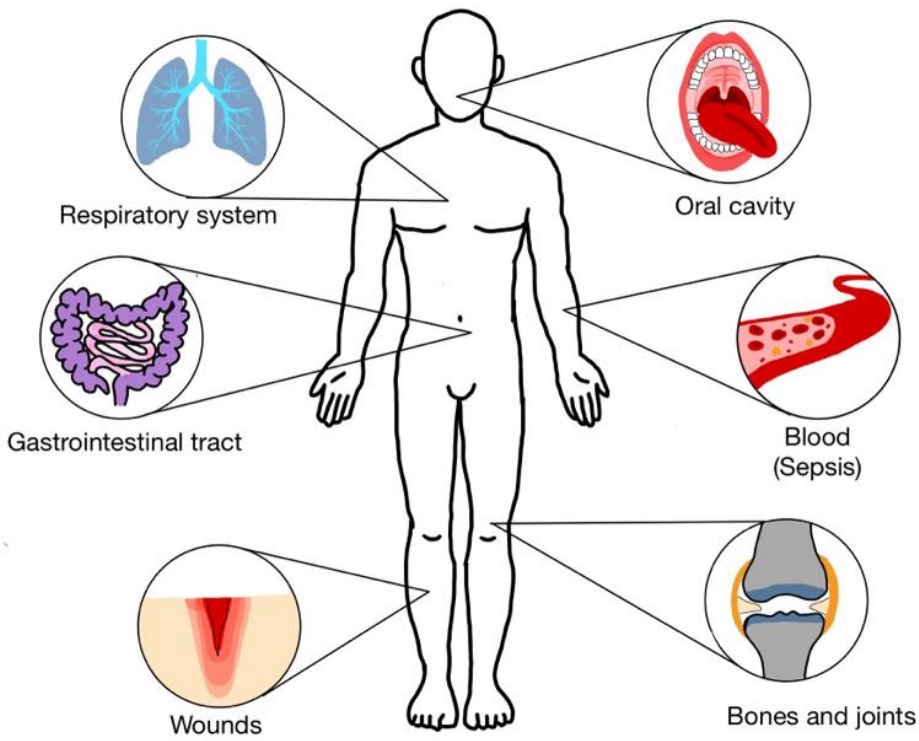

Figure 5. The diagram indicating the possible areas of PT use: oral cavity (Khalifa et al. 2016; Guo et al. 2024), blood (Górski et al. 2017), bones and joints (Peng et al. 2024), wounds (Jault et al. 2019), gastrointestinal tract (Nale et al. 2016) and respiratory system (Abedon 2015).

10. The use of phage therapy in dentistry and oral surgery

In the oral cavity, bacteria pose a significant challenge, given their role in the development of various conditions, including dental caries, pulp inflammation, periodontal disease, abscesses, peri-implantitis, and bone necrosis. This comprehensive list illustrates the diverse array of oral issues that can arise from bacterial infection (Figure 6). Antibiotics are frequently employed in the treatment of these; however, PT has the potential to either complement or replace them.

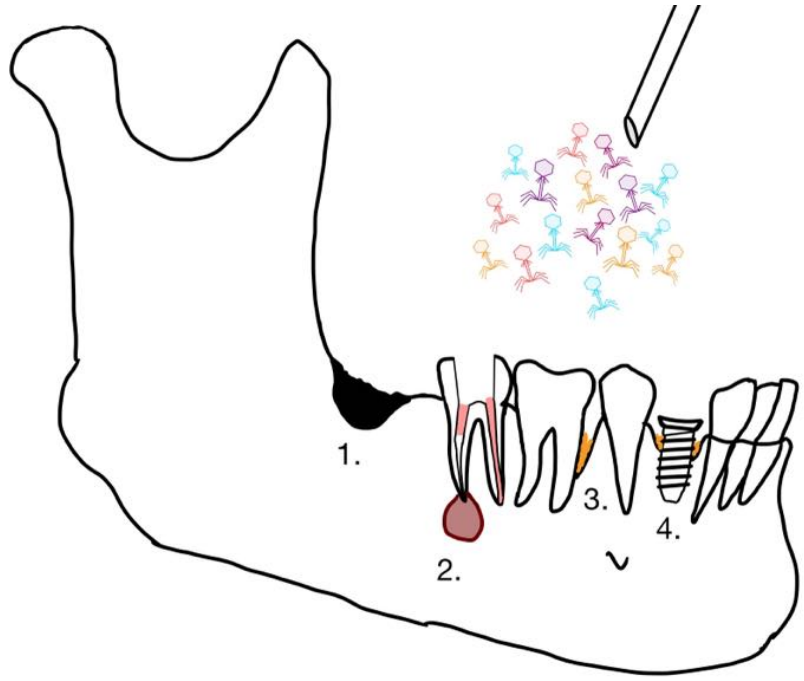

Figure 6. Examples of potential applications of PT in dentistry: 1. Osteonecrosis; 2. Necessity of endodontic re-treatment; 3. Periodontal treatment; 4. Periimplantitis.

10.1 Phage therapy in restorative dentistry and endodontics

The potential of phages to penetrate biofilms and tissues, their specificity of action, and the increasing antibiotic resistance of bacteria have led to the emergence of phage therapy (PT) as a promising tool in dentistry (Zhu et al. 2025).

Regarding restorative dentistry and caries treatment, the objective is to prevent or inhibit tooth decay by limiting the growth of pathogens responsible, such as *S. mutans*. *In vitro* and *in vivo* animal studies have demonstrated the efficacy of SMHBZ8 phage against the specified bacterium. This finding suggests that the phage could be utilized in future human treatments (Wolfoviz-Zilberman et al. 2021)). Concerning the endodontic treatment, particularly in cases of re-treatment, a frequently detected and profoundly problematic *Enterococcus faecalis* has been identified. *In vitro* studies on extracted human teeth have demonstrated the efficacy of phages in eradicating *E. faecalis* from root canals, particularly when combined with 0.5% sodium hypochlorite (NaOCl) rinsing, resulting in an 84% reduction in biofilm mass. This result is comparable to that obtained with the use of NaOCl at a concentration of 5.25%, which is potentially toxic to periapical and surrounding tissues. That result suggests that a lower concentration of NaOCl could be equally effective. Further research, including human trials, is necessary (Khalifa et al. 2016; Tinoco et al. 2017; Basak Erol et al. 2024).

10.2 Phage therapy in periodontics

Socransky's seminal work (Socransky et al. 1998) described the bacteria present in the oral cavity in the form of complexes, among them a red complex containing the bacteria most virulent in the periodontium, namely *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. In contrast to the latter

two, to date, no bacteriophage with lysogenic activity against P. gingivalis has been identified. The identification and description of phages active against other bacteria responsible for periodontitis has been successfully achieved. Among these are *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* (the discovered phage is specific only against one serotype of this strain), *Actinomyces naeslundii* and *E. faecalis* (often present in deep periodontal pockets). Further research is needed to determine the potential application of phages in the treatment of periodontitis in humans (Pazhouhnia et al. 2022; Guo et al. 2024).

Although no lytic phage has yet been identified against highly problematic *P. gingivalis*, it has been demonstrated that there is a relationship between *P. gingivalis* and *Streptococcus gordonii*, where the latter, as the pioneer colonizer, must create the appropriate conditions for *P. gingivalis* to attach to by creating specific binding sites and later to form its biofilm. One study implementing the phage ΦSG005 targeted against *S. gordonii* revealed a promising significant reduction in *P. gingivalis* abundance of over 99% (Wu et al. 2024). Another in vitro study revealed phage peptides capable of stimulating non-neoplastic proliferation of mucosal epithelial cells. This approach has the potential to be developed into a future treatment for oral mucosal healing, similar to the use of phages to heal skin wounds (Li et al. 2013).

However, it is important to note that phages may not necessarily have an exclusively positive effect on the periodontium. In patients diagnosed with periodontitis, the Siphoviridae_29632 phage was isolated, with a significantly higher prevalence observed in patients with periodontal disease compared to healthy individuals. This observation raises the suspicion that the virus may create favourable conditions for the development of periodontal disease; however, there is a lack of compelling evidence to substantiate this hypothesis. Further research is warranted to ascertain the potential correlation between this phage and the development of periodontal disease (Zhang et al. 2019).

10.3 Phage therapy in oral and maxillofacial surgery

There is great potential for the use of PT in oral and maxillofacial surgery. Phages have been demonstrated to be a safe and effective treatment option for bone and joint infections. Their ability to penetrate bacterial biofilms makes them an ideal choice for treating chronic infections. The effectiveness of treating bone infections using phage therapy (PT) has been demonstrated through both the administration of phage cocktails and individualized therapy. There are high hopes for their development (Clarke et al. 2020).

To date, no research has been conducted on the use of phage therapy in the treatment of periimplantitis. Recent systematic reviews and meta-analyses have demonstrated that *Staphylococcus epidermidis*, *F. nucleatum*, *T. denticola*, *T. forsythia*, *P. intermedia* and *P. gingivalis* are predominantly implicated in the development of peri-implantitis (Săndulescu et al. 2023). The efficacy of phage therapies has been demonstrated against *F. nucleatum*, *T. denticola*, *T. forsythia* and *S. epidermidis* (Štrancar et al. 2023; Guo et al. 2024). Regarding P. gingivalis, a reduction in titre has been observed (Wu et al. 2024), but no effective phage against this bacterium has yet been identified. Moreover, there are reports of the capacity of phages to adhere to zirconia, which engenders considerable optimism for the prospective treatment of periimplantitis with these microbes (Hashimoto et al. 2011). Consequently, the prospect of formulating a phage cocktail that is efficacious in treating periimplantitis is conceivable; however, conducting clinical trials is imperative to validate this hypothesis.

It has been demonstrated that bacteria such as *P. gingivalis*, *F. nucleatum*, and *T. denticola* can influence the development of oral squamous cell carcinoma (OSCC) by stimulating epithelial cell proliferation, inhibiting apoptosis, and modulating the inflammatory microenvironment. This dysbiosis is considered to represent a specific link between periodontal disease and OSCC. This may necessitate the implementation of PT against the aforementioned bacteria (Guo et al. 2024).

Animal studies also demonstrated that oral immunization with phage MS2-L2 VLPs can protect against infection with highly oncogenic types of HPV associated with head and neck cancers (mostly against HPV-16, -35, -39 and -58). This may offer the possibility of creating phage vaccines against oral malignancy (Zhai et al. 2019).

It is also evident that phages have the potential to be utilized in cancer therapy in various ways. It has been demonstrated that suitably modified phages can function as specific transporters of imaging agents or even drugs directly into cancer cells, thereby facilitating rapid diagnosis and treatment (Easwaran et al. 2024; Cao et al. 2025).

PT has been extensively employed in the treatment of osteomyelitis (Kishor et al. 2016; Onsea et al. 2019; Cobb et al. 2020; Simner et al. 2022). This condition is typically caused by a Staphylococcus aureus infection, which can take place via three primary routes. Haematogenous (most common in children), through infected adjacent tissues, but also trauma (including surgical) as well as vascular or neurological insufficiency (e.g. in the course of diabetes) (Birt et al. 2016;

Hofstee et al. 2020). A particular instance that poses a unique challenge in oral surgery is medication-related osteonecrosis of the jaw. Antiresorptive drugs, such as bisphosphonates or denosumab, used in the treatment of osteoporosis, have been shown to inhibit osteoclast activity and, consequently, bone remodelling. In cases involving surgical oral intervention in patients treated with antiresorptive drugs, a potential risk of osteonecrosis exists, which is often further complicated by the occurrence of bacterial infections such as with *Actinomyces* spp. (Ibrahim et al. 2022). Consequently, these conditions could be treated with phage therapy. However, to date, there have been no documented cases of phage therapy utilization in the field of oral and maxillofacial surgery for the management of this infectious inflammation. *Actinomyces* spp. bacteria occur naturally in the oral cavity but can become pathogenic bacteria under favourable conditions. Bacteria of this genus are found in oral abscesses, often infect necrotic bone tissue and are responsible for actinomycosis (mainly *A. israeli*) (Ibrahim et al. 2022). To date, phages have been developed that mainly target *A. naeslundii*. Thus, further research is needed (Szafrański et al. 2017).

To date, no studies have been conducted which describe the use of phages in the treatment of acute osteomyelitis. The process of identifying bacteria and finding a specific phage is often time-consuming, and bacterial biofilms in acute osteomyelitis are less developed than in chronic disease. This supports the greater likelihood of success with antibiotic treatment. However, PT may also be considered, although this method is typically used to treat chronic inflammations that demonstrate resistance to conventional treatment methods (Suh et al., 2023). This observation does not imply the inefficiency of PT in addressing acute inflammations; instead, it highlights the absence of studies specifically designed to investigate its effectiveness in such contexts. Consequently, there is a need for further research on this topic in the future.

Tuberculosis and actinomycosis are among the bacterial diseases that pose clinical challenges to oral surgeons. In the case of tuberculosis, treatment with phage therapy is complicated by the fact that the pathogen lives intracellularly in macrophages, which significantly reduces the availability of phages to the bacteria. In the later stages of the disease, significant amounts of bacteria are present in the extracellular environment, allowing phages to be effective, as demonstrated *in vitro*. Interestingly, studies have shown that some phages attack a wide range of *Mycobacterium tuberculosis* isolates, an unusual observation among phages (Guerrero-Bustamante et al. 2021).

11. Bacterial phage resistance? - the arms race between phage and bacteria

Bacteria and phages have coexisted for centuries, and we know that bacteria developed mechanisms that allow them to avoid phages. Bacterial defence against phages occurs at several stages of the phage reproductive cycle. These include modification of the bacterial surface to prevent phage adhesion, prevention of phage DNA entry and replication, cleavage of the phage genome, CRISPR-Cas systems, or abortive infection (Abi system), which involves the suicidal death of a bacterium attacked by a phage before its duplication occurs. Comprehension of these mechanisms may play a crucial role in counteracting the acquisition of phage resistance by bacteria (Hampton et al. 2020; Safari et al. 2020; Teklemariam et al. 2023).

However, unlike antibiotics, phages do not remain passive in the face of increasing bacterial resistance; instead, they produce systems that allow them to continue attacking bacteria. They adapt to new receptors on the bacterial surface, create anti-restriction modification systems, produce mutations that bypass CRISPR-Cas, or even create genes that counter the CRISPR-Cas system (Hampton et al. 2020; Safari et al. 2020; Teklemariam et al. 2023).

From a clinical perspective, it is noteworthy that phage administration in the form of a phage cocktail, rather than phage monotherapy, appears to be an effective method for preventing the development of bacterial resistance (Chan et al. 2013).

12. Combined antibiotic and phage therapy

As demonstrated by the following case report, the combination of antibiotic and phage therapy has been successfully implemented in several instances. The report details the treatment of a patient who, following a motorcycle accident, developed an infection of the tibia caused by multidrug-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*. In the absence of effective antibiotic treatment, the patient was at risk of losing his limb. However, combined phage and antibiotic therapy resulted in rapid healing and complete eradication of the bacteria from the infected site. No adverse effects of the therapy were observed (Nir-Paz et al. 2019).

Combined antibiotic and phage therapy has been demonstrated to yield significant and interesting results. The available research suggests that combining both forms of therapy increases and/or prolongs the effective action of the antibiotic. It has been hypothesized that phage infection exerts selective pressure

on bacteria, causing them to mutate and reduce the expression of factors related to toxicity, antibiotic resistance, and growth inhibition. Consequently, even if a bacterial strain develops resistance to phages, it becomes less toxic and more susceptible to antibiotics (Li et al. 2021; Diallo and Dublanchet 2022).

Further research is needed into this phenomenon. In addition, assigning specific phages to specific antibiotics and developing a sequence for administering them to patients must be undertaken, so that treatment is as effective and safe as possible (Diallo and Dublanchet 2022).

13. Conclusions

According to the World Health Organization, by 2050, approximately 10 million people worldwide may die annually due to the multidrug-resistant bacterial infections (de Kraker et al. 2016). This predicament underscores the need for the development of novel antibiotics or alternative therapeutic modalities.

Considering the studies presented in this review, it seems probable that PT may become a viable alternative to antibiotic therapy in the future, or at least a support for it.

In principle, its application is feasible in any medical discipline where bacterial infections are a problem, including dentistry, where it can potentially be used in caries prevention, re-endodontic treatment, peri-implantitis, periodontitis or even osteomyelitis.

PT has been demonstrated to have considerable potential; however, numerous challenges must be overcome before it can be implemented on a large scale clinically. These include the necessity for additional clinical trials, long-term observational studies on efficacy and safety, and the establishment of appropriate and uniform regulations, including phage administration regimens similar to those employed for other drugs. It is also necessary to reduce production costs and extend their shelf life.

The renaissance of phage therapy, or its rediscovery, evokes optimism for the future and the ongoing effective combat against bacterial diseases.

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