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Review

Application of Invasive Bacteria for the Delivery of Anti-Cancer Therapeutics

Rasaq Akinsola [1,2,*] and Kumaran Narayanan [3,*]

[1] Department of Medicine, Division of Hematology and Cellular Therapy, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
[2] Samuel Oschin Comprehensive Cancer Institute, Cedars Sinai Medical Center, Los Angeles, CA 90048, USA
[3] Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor Darul Ehsan, Malaysia
* Correspondence: rasaq.akinsola@cshs.org (R.A.); kumaran.narayanan@monash.edu (K.N.)

Abstract: Bacterial vectors for biomolecule delivery to targeted organelles, facilitating temporary or continuous protein production, have emerged as a promising approach for treating acquired and inherited diseases. This method offers a selective cancer eradication and targeting strategy with minimal side effects. Bacterial vectors provide an alternative to viral gene delivery, given their capacity to deliver large genetic materials while inducing minimal immunogenicity and cytotoxicity. Bacteria such as *Bifidobacterium*, *Salmonella*, *Clostridium*, and *Streptococcus* have demonstrated potential for tumor-targeted biomolecule delivery or serve as oncolytic bacteria. These vectors have also been used to transfer and amplify genes encoding biomolecules such as pro-drug-converting enzymes, toxins, angiogenesis inhibitors, and cytokines. The microenvironment of necrotic tumors offers a unique opportunity for targeted therapy with the non-pathogenic anaerobic bacterium. For example, *Clostridium sporogenes* can germinate selectively in the necrotic regions upon injection as endospores, which helps to enhance the specificity of *Clostridium sporogenes*, resulting in tumor-specific colonization. Also, *E. coli* and *Salmonella* sp. can be capacitated with a hypoxic sensing promotor gene for specificity delivery into the core region of solid tumors. The uniqueness of the tumor microenvironment, including hypoxia, immunosuppression, metabolite deficiency or enrichment, and necrosis, selectively enables bacteria in the tumor. Combining traditional cancer therapy with bacterial therapy will significantly complement and cover the limitations of other treatments. This review provides an overview of the use of the bacteria vector in cancer therapy, discussing strategies to maximize delivery efficiency and address potential challenges. In this review, we discuss the potential of bacteria vectors as anti-cancer therapeutics while focusing on therapeutic delivery strategies. We highlight the complementary use of bacteria therapy with other cancer therapies and the mechanism of bacteria cancer immunotherapy with limitations and perspectives for future use.

Keywords: bacteria vector; cancer therapy; invasive *E. coli*; gene therapy; protein; cargo; vaccination

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1. Introduction

Bacterial vectors for biomolecule delivery to targeted organelles, facilitating temporary or continuous protein production, have emerged as a promising approach for treating acquired and inherited diseases [1]. These vectors can be used to deliver controlled and sustained anti-cancer agents into tumor microenvironments singly or combined with other forms of therapy [2]. Bacteria vectors offer an attractive alternative to viral vectors due to their ability to deliver sizeable genetic material, proteins, and other forms of nucleic acids [3]. Bacteria vectors induce minimal immunogenicity and cytotoxicity along with high gene transfer efficiency, a broad host range, rare re-arrangements in the delivered DNA, and safety, since for gene transfer to occur, the donor bacteria have to die [4,5]. The tumor microenvironment provides an enabling environment for the survival and thriving of these bacteria vectors for selective targeting [6].

Although the efficiency and selectivity of these bacteria vectors for cargo delivery need to be optimized, bacteria such as *Bifidobacterium* [7], *Salmonella* [8], *Clostridium* [9] *Streptococcus* sp. [10], and *E. coli* have demonstrated the potential for tumor-targeting by preferentially replicating within solid tumors when injected from a distal site [11,12]. These vectors have also been used to transfer and amplify genes encoding biomolecules such as pro-drug-converting enzymes, toxins, angiogenesis inhibitors, and cytokines [13–15]. Attenuated *S. Typhimurium* vectors represent the most widely applied bacterial vectors at the clinical trial level due to their ability to selectively colonize tumors to inhibit their growth and prolong survival after systemic infection [16].

Cancer is a many-sided disease, and combining other therapies with bacteria therapy can help achieve synergistic effects by improving penetration limitations and maximizing the activities of other therapies while reducing systemic toxicity to the host [17]. Using clostridial species (*Clostridium histolyticum*), spores for targeted tumor killing by direct injection into the transplanted sarcomas of mice and subsequent vegetative growth of the organism, resulted in significant tumor lysis, and for those mice that were co-treated with penicillin and antitoxin, an extended survival rate was observed compared to tumor-bearing mice that were not injected with clostridial spores [18].

Furthermore, the microenvironment of necrotic tumors offers a unique opportunity for targeted therapy with the non-pathogenic anaerobic bacterium *Clostridium sporogenes*, enabling it to germinate selectively in necrotic regions upon injection as endospores, which helps to enhance the specificity of *Clostridium sporogenes*, resulting in tumor-specific colonization [19]. Some of these delivery vectors are engineered to invade mammalian cells and can bind specifically with β_1 integrin receptors, abundantly expressed in cancer cells [20,21]. For example, *E. coli* DH10B is incorporated with genes from the invasin gene from *Yersinia Pseudotuberculosis* and the *Listeriolysin O* gene from *Listeria monocytogenes*, which facilitate the cellular entry and endosomal escape, respectively, before the cytosolic release of cargoes [22]. This bacteria vector is a diaminopimelic acid auxotroph, resulting in the bacteria's suicidal tendency, which helps reduce the risk of systemic infection or organ colonization after the delivery of the therapeutic cargo [23]. The invasive bacteria vector will offer an exciting alternative to eliminating cancer cells, especially the cells in the core region of the tumor that are resistant to other forms of therapy [24,25].

The uniqueness of the tumor microenvironment, including hypoxia, immunosuppression, metabolite deficiency or enrichment, and necrosis, selectively enables bacteria to thrive in the tumor. Here, we discuss the potential of bacteria vectors as anti-cancer therapeutics while focusing on therapeutic delivery strategies. We highlight the complementary use of bacteria therapy with other cancer therapies and the mechanism of bacteria cancer immunotherapy with limitations and perspectives for future use.

2. Engineered Bacteria Studies

Our laboratory and others have established the quantification of internalized *E. coli* vectors, optimized the time the vector can be wholly internalized into eukaryotic cells, and described its trafficking through the lysosomal and autophagy pathways [5,22,23,26]. *E. coli* invasion into eukaryotic cells is a pre-requisite to determining the molecular mechanisms of how it functions to obtain insights for improving its efficiency with the potential to be used in cancer gene therapy [27].

Our previous experiments showed high cellular infection of up to 70.47%, 27.4%, and 26.2% in MCF-7, A549, and HEK-293 cells, respectively. To drastically improve *E. coli* payload delivery efficiency, we have combined it with other commercially available chemical vectors. For example, lipofectamine increases *E. coli* vector gene delivery efficiency up to 2.8 folds in HeLa cells [28]. Further, since endosomal escape is one of the crucial barriers that need to be overcome by an integrin-mediated vector, we demonstrated in one of our works that the inhibition of lysosomal V-ATPase enhances *E. coli* bactofection by 6.9, 3.2, 5.0, 2.8, and 4.5-fold in HeLa, HEK-293, A549, HT1080, and MCF-7 respectively, compared to untreated cells [29]. Recent studies improved the *E. coli* vector gene delivery capabilities

by non-covalent coupling with cell-penetrating peptides and elucidating the interaction to form a hybrid vector using atomic force microscopy (unpublished) and combining the sophistication and real-time possibility of imaging flow cytometry for *E. coli* tracking and gene delivery in cancer cells. The imaging flow cytometry combines the features of fluorescent microscopy and laser scanning cytometry, enabling the acquisition and identification of a heterogeneous population of cells [30,31]. However, extensive studies are required on the *E. coli* therapeutics cargo delivery into cancer cells both in vitro and in vivo for *E. coli* for it to make it to clinical trials like its counterparts, such as *Salmonella typhimurium* [32–34], *Listeria monocytogenes* and *Clostridium* sp.

On the other hand, genetically modified bacteria such as *Salmonella typhimurium* serovar VNP20009 and *Clostridium butyricum* M55 can selectively colonize tumors and have been used to deliver cargo into mice models without a severe immune response or significant side effects [10,35]. *Salmonella* spp. has been reported to be attracted by serine, aspartate, and ribose and can thrive in the presence of nutrients derived from dying tumor cells, as seen in animal models [36]. Further, attenuated *S. Typhimurium* with a natural affinity for solid tumors delivered shRNA directly into tumor cells. Mechanistically, this shRNA constitutively activated signal transducer and activator of transcription 3 (Stat3), a crucial transcription factor involved in both hepatocellular carcinoma (HCC) growth and metastasis [37]. Thus, this induces remarkably delayed and reduced HCC in many mouse populations. In a similar experiment, using attenuated *Salmonella typhimurium* serovar carrying a plasmid that co-expresses endostatin, an inhibitor of tumor neo-vasculogenesis, and a shRNA that targets Stat3 to suppress prostate cancer growth [38]. *Salmonella*-delivered pEndo-Si-Stat3 decreased Stat3 levels with increased endostatin expression in mouse tumors, significantly suppressing tumor growth by knocking down the expression of Stat3, resulting in the over-expression of endostatin, which synergistically inhibited prostate cancer growth. *Clostridium* is targeted selectively to the tumor microenvironment and is the most reported for use in cancer therapy, including *Clostridium novyi* and *Clostridium sporogenes.* However, the more clinically advanced of the *Clostridium* species used for cancer therapy is *C. novyi*-NT [39,40]. This strain is an attenuated variant with the lethal α-toxin gene removed. In previous in vivo experiments (CT26) and rabbit (VX2) models, intravenous injection with the endospores of *C. novyi*-NT germinated in necrotic tumor areas produced complete responses in up to 30% of treated animals. This resulted in an anti-tumor immune-mediated cellular immunity to the original tumors. A subsequent dose-escalation study in dogs with spontaneously occurring tumors and intravenous injection of *C. novyi*-NT endospores resulted in colonizing naturally occurring tumors in dogs [41]. The outcome of this study provides invaluable insight into the design of clinical trials in human cancer patients.

A human study (NCT01924689) using *C. novyi*-NT enrolled 24 patients with injectable, treatment-refractory solid tumors to receive a single intratumoral injection of *C. novyi*-NT at various doses, which led to bacterial spores' germination and the resultant lysis of injected tumor [42]. Out of the 22 patients evaluated, nine (41%) had a decrease in the size of the injected tumor, which accounts for about 41%, and 19 (86%) had stable disease as the best overall response in injected and non-injected lesions combined. *C. novyi*-NT injection triggered a transient systemic cytokine response and enhanced systemic tumor-specific T-cell responses. In this study, the *C. novyi*-NT toxicities were significant but manageable. Further, a phase 1 clinical trial is ongoing to investigate the intratumoral injection of *Clostridium novyi*-NT in combination with pembrolizumab [43]. Pembrolizumab is a novel anti-program death 1 (PD-1) monoclonal antibody used to treat different forms of cancer, including melanoma and carcinoma, either singly or in combination with another form of therapy [44–47]. Some preclinical and clinical studies have been highlighted in Table 1.

Table 1. Preclinical and clinical trial of the use of bacterial vector in cancer therapy.

Bacterial Strain	Cancer Type	Treatment Strategy and Approach	Outcome	Ref
Clostridium novyi-NT	Preclinical: Endogenous neoplasia in dogs Clinical trial: Solid tumor in humans	Spores Attenuated strain of *C. novyi*	Resulted in increased TNF-α production, LTA-induced IL-10 production, and NK cell-like function, suggesting *C novyi-NT* spores induce longer-term immune cell function changes. *C. novyi*-NT injection elicited a transient systemic cytokine response and enhanced systemic tumor-specific T-cell responses.	[42,48]
Salmonella typhirium (VNP20009) *Salmonella typhirium (VNP20009)* *Salmonella typhirium*	Clinical trial: Metastatic renal cell carcinoma Preclinical: B16F10 subcutaneous xenograft model Preclinical: Hepatocellular carcinoma	Attenuated by chromosomal deletion of the *purI* and *msbB* genes Plasmid-expressed IFNb (VNP-IFNb) DNA vector delivered by attenuated *S. typhimurium*	Induced a dose-related increase in the circulation of proinflammatory cytokines, such as interleukin IL-1β, TNF-α, IL-6, and IL-12. No tumor regression observed. Compared with VNP, VNP-IFNb recruited more NEs and macrophages (M4s) with antitumor phenotypes in lung metastases and activated dendritic cells (DCs) differentiation, which activated antitumor immune responses of CD4+ T cells, and ultimately, inhibited melanoma progression. Treatment resulted in significant alteration of Stat3 and endostatin levels and levels of the downstream gene VEGF, decreased cell proliferation, induced cell apoptosis, and inhibited angiogenesis.	[49–51]
Salmonella typhi	Clinical trial: Advanced pancreatic cancer	Live-attenuated *Salmonella typhi* carrying an expression plasmid encoding VEGFR2	At least 3-fold increase in VEGFR2-specific T-cell response over baseline levels.	[52]
Listeria monocytogenes	Preclinical: Metastatic breast cancer Clinical: Metastatic pancreatic adenocarcinoma	Attenuated bacterium *Listeria monocytogenes* Live-attenuated *Listeria monocytogenes*–expressing mesothelin	There was a significant reduction of the population of myeloid tumor suppressor cells in blood and primary tumors and conversion of a remaining subpopulation of into an immune-stimulating phenotype producing IL-12, in correlation with significantly improved T-cell and NK cell responses. Enhanced mesothelin-specific CD8 T-cell responses that were associated with longer overall survival, regardless of treatment arm.	[53,54]
Escherichia coli BW25133	Preclinical: Mammary tumors	Strain capable of expressing cardiac peptides and GFP signaling protein	Suppressed tumor growth rate and expression of MMP-9, VEGFR2, CD31, and Ki67 biomarkers. It significantly reduces concentrations of IL-1β, IL-6, GC-SF, IL-12, and TNF-α proinflammatory cytokines. Reduces IL-10, IL-17A, and INF-γ cytokines.	[55]
E. coli Nissle 1917	Preclinical: Melanoma, lymphoma, mammary carcinoma, and colon carcinoma	Engineered bacterial strain that targets STING-activation	Targets STING-activation to phagocytic antigen-presenting cells in the tumor and activates complementary innate immune pathways.	[56]
E. coli	Preclinical: Colorectal, adenocacarcinoma, melanoma, and breast cancer	Outer membrane vesicles (OMV)	Accumulates in the tumor tissue, and induces the production of antitumor cytokines CXCL10 and INF-γ.	[57]

APC, Antigen presenting cell. IL-12, interleukin 12. IL-17A, interleukin 17A, IL-1β, interleukin 1 beta. TNF-α, tumor necrotic factor alpha. CXCL10, C-X-C motif chemokine ligand 10. CD8, cluster of differentiation 8. MMP-9, metalloproteinases 9. GC-SF, INF-γ, interferon-γ. CD31, cluster of differentiation. GFP, green fluorescent protein. STING, stimulator of interferon (IFN) genes. Ki67, Kiel-67.

Using bacteria vectors, either as a monotherapy or complementarily with other therapies, will significantly help to address the specific limitations of traditional treatments. However, more studies are needed to understand how all these therapies can be combined to alleviate cancer. Also, understanding the change in the gut microbiota signature upon delivery of anti-cancer therapeutics and immune response will be pertinent, as the host's resident flora possibly affects the host tumor's susceptibility to cancer therapies.

3. Gut Microbiome and Anti-Cancer Payload Delivery

Over the last decade, evidence has linked microbiota with the physiology and function of the host. Human microbiomes consist of complex communities of bacteria, yeast, fungi, protozoa, archaea, and viruses that inhabit the surface barriers of the human body [58,59]. Gut microbiome dysfunction can lead to many diseases, such as inflammation, cancer, and neurodegenerative disease [60–62]. Overwhelming evidence has suggested the link between commensal microbiota and cancer. Gastrointestinal microbiota plays a pivotal role in modulating responses to cancer immunotherapy, and some data demonstrates that the microbial community within the tumor microenvironment can contribute to therapeutic efficacy [63–65]. Therefore, it is pertinent to understand how the gut microbiome would respond to therapeutic cargo delivery into cancer cells and how both can be used synergically to alleviate cancer. Previous reports have shown that gut microbiota may shape responses to forms of cancer therapy through an effect on multiple metabolic pathways [66]. Modulating the gut microbiome in preclinical models has improved the host response to disease treatment, including cancer. Cancer treatment with microbial agents or their products can potentially shrink tumors [67].

The emergence of the metagenomics and transcriptomics sequencing of diverse cell populations to quantify the microbial community has helped shed more light on the gut microbiome composition, function, and dynamics [68]. However, the basic mechanistic understanding of the individual genetic factor that drives the overall function of the gut microbiota needs to be adequately understood. These will give us better insights into harnessing the gut microbiome for anti-cancer benefits. There are still questions about whether targeting the gut and tumor microbiotas will be a better option by delivering therapeutic cargo to remove or populate specific beneficial or harmful bacteria within host-associated microbial communities (Figure 1).

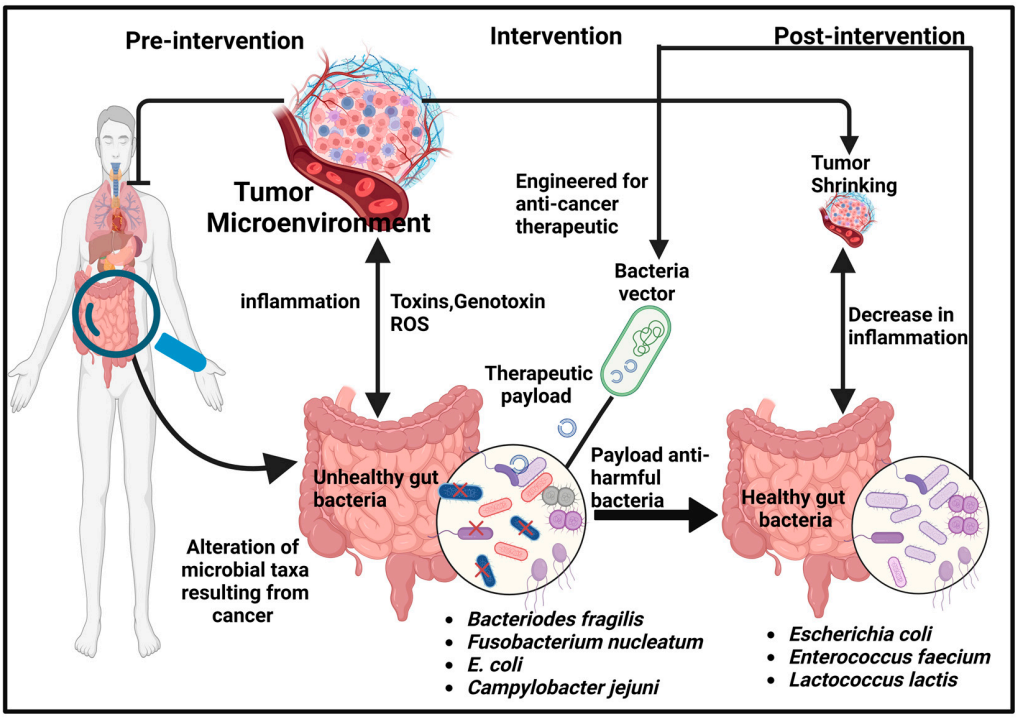

Figure 1. A proposed therapeutic approach targeting the gut microbiome. This approach delivers an efficacious therapeutic payload against bacteria that produce toxins, genotoxins, metabolites, and reactive oxygen species in the gut. This inflammation promotes tumor growth and development. By reducing the populations of these bacteria, healthy bacteria will be promoted, resulting in the shrinking of the tumor. For example, some bacterial species stimulate an inflammatory response supporting carcinogenesis by producing genotoxic metabolites from *Bacteroides fragilis*, *E. coli*, and

Campylobacter jejuni. Bacteria, especially those that are part of the resident flora, can be engineered to produce anti-genotoxic substances that reduce the bacteria responsible for these substances and promote gut-friendly bacteria.

The engineered bacteria vector, which may include *E. coli*, *Salmonella* sp., can be engineered to selectively target and deliver therapeutic plasmid that enables the gut-friendly bacteria to produce bacteriocins that antagonize specific bacteria that have been associated with tumor progression, including *Bacteroides fragilis*, *E. coli*, and *Campylobacter jejuni* (Figure 1). *Bacteroides fragilis* stimulate an inflammatory response that promotes carcinogenesis via toxins, increasing the accumulation of reactive oxygen species subsequently leading to aberrant signaling pathways in human and mouse tumors, which may prevent antitumor immune function [69]. Further, *E. coli* and *Campylobacter jejuni* produce colibactin and cyto-lethal metabolites, which induce carcinogenicity in mice [60,70,71]. By eliminating the harmful bacteria (Figure 1) that produce metabolites that trigger inflammation, we suggest that will enhance the promotion of the growth of gut-friendly bacteria, especially those bacteria that belong to the following taxa that have been associated with good gut health: *Firmicutes*, *Bacteroides*, *Lactobacillus*, and *Enterococcus* [72]. These phyla have been reported to produce short-chain fatty acids (SCFAs), which play a crucial role in health and disease by regulating gut homeostasis. SCFAs are metabolites of specific bacterial taxa of the human gut microbiota, and their production is influenced by foods or food supplements, mainly prebiotics, by the direct fostering of these taxa. The deficiency of these SCFAs contributes to several disorders' pathogenicity, including cancer and cardiometabolic disorders [73]. The reduction in engineered bacteria has the potential to modulate gut microbiota (Figure 1) through the reduction in inflammation by reducing the abundance of the genera that cause host physiology by secreting proteins, such as human interleukin-10, to reduce inflammation.

4. Approach of Invasive Bacteria Cargo Delivery for Application in Cancer Therapy

The conventional therapeutic approach for human malignancy, such as radiotherapies, chemotherapies, and surgery, presents significant health limitations, including, but not limited to, poor tumor-specific targeting, significant adverse effects, insufficient tumor permeability, rapid tumor relapse, and metastasis [74]. However, cancer immunotherapy has emerged as a promising option for promoting the recognition and elimination of tumor cells. It stimulates the immune system by inducing innate and adaptive responses with different treatment strategies [75].

These responses capacitate the immune system to eliminate or protect against several tumors by releasing pro-inflammatory cytokines. Immune therapies such as checkpoint inhibitors, monoclonal anti-cytotoxic T-lymphocyte-associated protein 4 (CTL-4), and programmed death protein 1 (PD1) are now being used clinically, with inspiring results [76–80]. However, immune therapy may decrease immunotolerance, resulting in immune-related adverse events and kidney-related toxicity, which limits its use.

To address these shortcomings, bacteria-mediated anti-tumor therapy provides an alternative option. Bacteria's unique characteristics include the ability to destroy tumor cells from the inside and, subsequently, induce innate and adaptive antitumor immune responses, which help to eliminate tumor cells effectively [81]. Interestingly, compared to most other therapeutics, the efficacy of tumor-targeting bacteria is independent of the tumor's genetic makeup.

Invasive bacteria vectors can be used in different ways in cancer therapy. One approach is to use bacteria to deliver therapeutic genes into cancer cells. These have been used in in vivo studies in phagocytotic and non-phagocytic cells with modified *E. coli* BM2710/pGB2Ωinv-hly carrying pC1ΩTGF-β1. The engineered *E. coli* vector was used to deliver a therapeutic gene (pC1ΩTGF-β1) into the intestinal mucosa through oral administration, significantly reducing the severity of experimental colitis in mice [82]. Further, the re-introduction of a deficient gene sensitizes tumor cells to other chemotherapeutic

agents. Hepatocellular carcinomas (HCCs) are generally highly resistant to chemotherapeutic agents and radiotherapy. It is believed that molecular changes during carcinogenesis, such as the overexpression of the multidrug resistance gene and the loss of tumor suppressor gene p53, may allow tumor cell populations to become resistant to most therapeutic approaches. Using bacteria as a vector, the wild-type p53 gene can be reintroduced into HCCs to sensitize it to a chemotherapeutic agent [83].

Previous research has focused on identifying new pro-drug activating enzymes that produce highly toxic and freely diffusible metabolites. The expression of bacterial enzymes in tumor cells allows to convert non-toxic pro-drugs to highly toxic metabolites with anti-cancer effects. *E. coli* DH5a-lux/bG can hydrolyze glucuronide substrates and emit luminescence. The bG and the luxCDABE gene cluster are used for pro-drug activation, luminance emission, and the specific conversion of the glucuronide pro-drug 9ACG to the topoisomerase I poison 9-amino camptothecin (9AC). The bacteria localize and replicate in human tumor xenografts and produce substantial antitumor activity in combination with systemic 9ACG pro-drug therapy [84]. Cytosine deaminase (CD) converts non-toxic 5-fluorocytosine (5-FU) into the chemotherapeutic agent 5-fluorouracil (5-FU). 5-FU is highly toxic because it is further metabolized into a product that infers with DNA and RNA synthesis. Upon administration of the *S. typhimurium* (VNP20009) strain, cytosine deaminase and 5-FU were expressed in patients, and the conversion of 5-FC to 5-FU indicated the bacterial production of functional CD in the tumor [85].

The use of bacteria in cancer immunotherapy has been demonstrated by several studies, with bacteria preferentially growing within the tumor cores due to the immune-privileged nature and the often hypoxic and necrotic tumor microenvironment with its possibility of locally affecting tumor growth through the recruitment and activation of the host's immune system [86–88]. The cells in this hypoxic region of the necrotic tumor microenvironment are shown to be resistant to chemotherapy and radiotherapy, which provides the opportunity for selective targeting with bacteria [89,90]. In a previous study, an engineered non-pathogenic *Escherichia coli* strain specifically lyses within the tumor microenvironment. It releases an encoded nanobody antagonist of CD47 (CD47nb)12, an anti-phagocytic receptor commonly overexpressed in several human cancer types. It increases the activation of tumor-infiltrating T cells and stimulates rapid tumor regression, preventing metastasis and leading to long-term survival in a syngeneic tumor model in mice. Also, the local injection of CD47nb-expressing bacteria stimulates systemic tumor-antigen-specific immune responses that reduce the growth of untreated tumors [91].

The invasive bacteria vector will offer an exciting alternative to eliminating cancer cells, especially the cells in the core region of the tumor that are resistant to other forms of therapy. The bacteria vector can deliver therapeutic genes, convert non-toxic pro-drugs to highly toxic metabolites, or perform bacteria immunotherapy.

5. Bacteria Immunotherapy and Mechanism of Action

Immunotherapy provides an alternative to traditional cancer treatment. However, it is flawed with limited response rates, acquired resistance, toxicities, and high costs, necessitating the development of new, innovative strategies. Bacteria cancer immunotherapy has attracted much attention due to its unique mechanism and ability to trigger host anti-tumor immunity [92,93]. Bacteria can preferentially colonize the core area of cancer and exert an anti-tumor effect [94]. Using several unique mechanisms, bacterial components may activate innate and adaptive immunity to resist tumor progression. Upon the injection of bacterial cells, the immune system rapidly clears the bacteria that reach normal tissue. In contrast, the bacteria in the tumor can selectively increase due to the uniqueness of the tumor microenvironment, which includes hypoxia, immunosuppression, metabolite deficiency or enrichment, and necrosis [95,96]. For example, the germination of the inert spores of *Clostridium* sp., which is an obligate anaerobe, is restricted to the anoxic region of the necrotic tumor, which helps to confer the tumor selectively (Figure 2A).

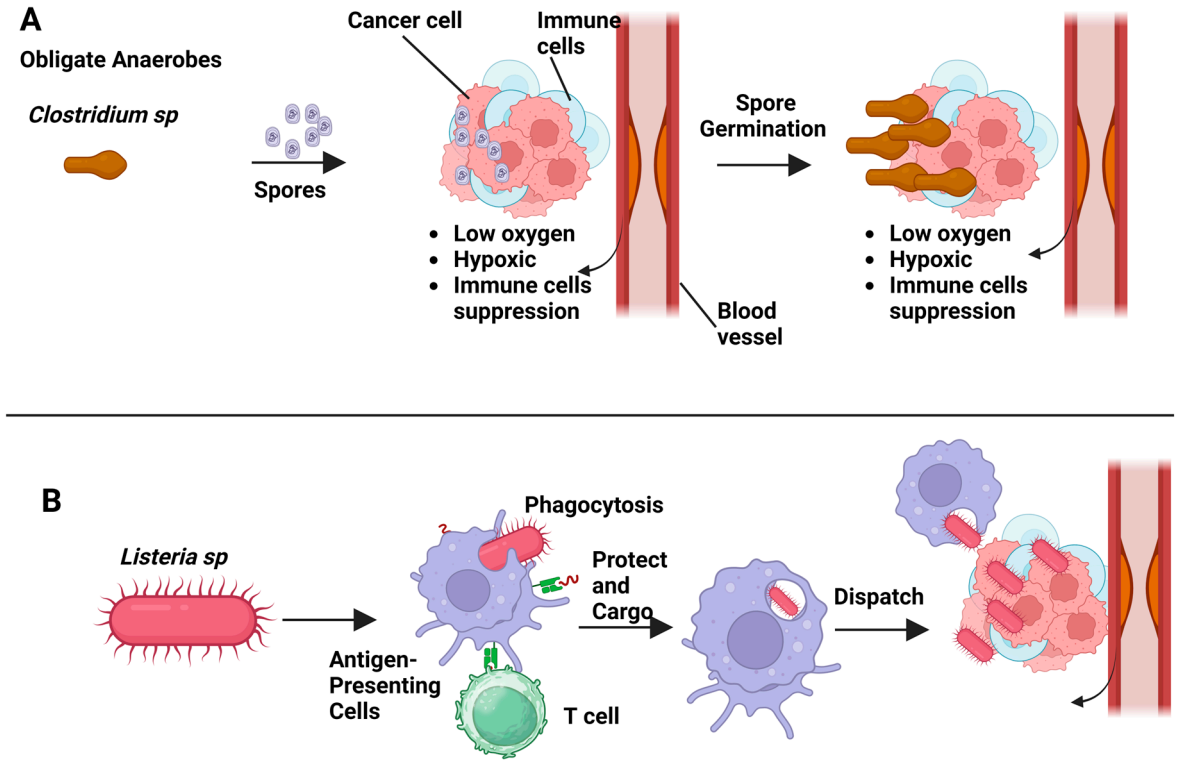

Figure 2. Mechanism of bacterial vectors interacting with cells to achieve tumor selectivity. The bacteria in the tumor can selectively increase due to the uniqueness of the tumor microenvironment, which includes hypoxia, immunosuppression, metabolite deficiency or enrichment, and necrosis. (**A**) The germination of the inert spores of *Clostridium* sp., an obligate anaerobe, is restricted to the anoxic region of the necrotic tumor, helping confer selectivity to cancer. (**B**) *Listeria* sp. uses immune cells, such as antigen-presenting cells and myeloid-derived suppressor cells, which have protective and dispatching roles to reach cancer cells after phagocytosis.

Further, *Salmonella* and *E. coli* are facultative anaerobes that could selectively accumulate in solid tumors' hypoxic and necrotic regions with little penchant for normal cells [97,98]. In the case of *Listeria* sp. (Figure 2B), the immune cells, such as the antigen-presenting cells and the myeloid-derived suppressor cell, can protect and dispatch the bacteria to the immune suppressive tumor microenvironment after its phagocytosis [99]. The tumor microenvironment is characterized by acidic pH, and this was exploited to confer selectively to the *Salmonella* strain STM1787 to deliver Shiga toxin to tumor cells in mice models [100]. The genetic programming of *Salmonella typhimurium* increased its affinity for tumor cells by placing essential genes under promoter elements responsive to hypoxia enhancement, making *Salmonella typhimurium* susceptible to other conditions except anaerobic conditions [101,102]. The innate immune response relies on the detection of conserved motifs from the invading pathogen known as the pathogen-associated molecular pathogen (PAMPs) by a large family of pattern recognition receptors (PRRs) that signal to the host in the presence of infection [103,104]. For example, bacterial PAMPs include cell wall components, such as peptidoglycans and bacterial DNA. However, some invasive bacteria vectors are suicidal and attenuated to prevent a robust immune response. An example is a diaminopimelic acid auxotroph *E. coli*, *Salmonella*, and *Shigella*, respectively, which helps reduce the risk of systemic infection or organ colonization after the delivery of the therapeutic cargo.

Bacteria colonization stimulates immune responses and recruits cytotoxic immune cells to the tumor microenvironment [105]. This process induces several pathways that cause the early host response to infection through the activation of pathways such as nuclear factor-kB (NFkB), mitogen-activated kinase (MAPK), the type 1 interferon (IFN) response,

and inflammasome assembly [106], resulting in the deployment of peripheral blood to the site of infection, including monocytes, neutrophils, basophil eosinophils, and NK cells. These may produce pro-inflammatory antimicrobial reactions, including the synthesis of cytokines and the induction of infected cell death, to prevent the spread of the bacteria.

While delivering therapeutic cargo to the tumor site with the capability to modulate the immune response to induce immunogenicity through the expression of tumor-associated antigens, *E. coli*, *Salmonella*, and *Clostridium* colonization can lead to the increased recruitment of immune cells to the tumor [107–109]. *Salmonella* can enhance antigen presentation by dendritic cells (DCs) through upregulating connexin 43 on tumor cells to form new gap junctions, transferring pre-processed antigenic peptides from the tumor cells to DCs in melanoma cell lines from mice or humans [110,111]. It was suggested that activated $CD8^+$ T cells could be essential in inhibiting tumor growth observed during tumor-targeted therapy by *Salmonella*. Tumor-targeted infection by recombinant *Listeria* can also mount a protective tumor-specific $CD8^+$ T-cell response [112]. This showed that the infiltration of several immune cells is inevitable in the tumor microenvironment following the colonization of bacteria, enhancing both the innate and adaptative immune response and contributing to tumor regression. Innate immune cell infiltration can be triggered by *Clostridium* species (mainly neutrophils and NK cells), resulting in inflammatory cell accumulation at the border between the proliferative and necrotic areas of the tumor when colonized by the most clinically advanced strain, *C. novyi*, the tumor following treatment in several models, including subcutaneous mouse and rabbit allografts.

6. Complementary Use of Bacteria and Other Forms of Cancer Therapy

Complementing bacteria therapy with other types of cancer therapy, such as chemotherapy, radiotherapy, and immunotherapy, has shown remarkable potential for diagnosis and therapeutic application. Conventional chemotherapy and radiotherapy remain cornerstones of cancer treatment. Their significant side effects and the problem associated with drug resistance have called for an urgent search for significantly more effective and less toxic anti-cancer drugs [113,114]. Chemotherapeutic drugs can change gut flora by compromising the gastrointestinal mucosa barrier, causing mucosal inflammation of the digestive tract, known as mucositis [115,116]. Mucositis can drastically affect the quality of life of the patient. However, the underlying mechanisms pinning the association are still unclear. Combining chemotherapy and bacteria therapy would help to reduce the limitation of the monotherapy use of chemotherapeutic drugs (Figure 3). To minimize the induced intestinal damage by 5FU in 5FU treated rats, *streptococcus thermophilus* TH-4 (TH-4), live TH-4, Dead TH-4, and supernatant TH-4 were evaluated for their potential to reduce the severity of 5-FU. However, live TH-4 treatment was the only treatment that exhibited protective effects [117]. The author suggested intestine rejuvenation and repair following live TH-4 treatment.

Radiotherapy is associated with the damage of normal tissue during its application and its ineffectiveness due to the hypoxic nature of the tumor microenvironment that makes cancer cells resistant to radiation [118–120]. Therefore, combining bacteria therapy with another treatment will significantly help alleviate cancer. For example, to improve the limitations of radiotherapy itself, it is essential to combine bacterial treatment and radiotherapy. The unique biological properties of bacteria can improve tumor-related biological characteristics during treatment and increase tumors' sensitivity to radiotherapy. Thus, it enhances the tumor hypoxic microenvironment by regulating cellular processes, including the cell cycle, to enhance the efficacy of radiotherapy [121,122]. *Clostridium novyi-NT* can selectively destroy the hypoxic regions of tumors and enhance the effects of radiation in transplanted tumor mice [123]. The bacteria were reported to improve the efficacy of radiotherapy markedly in several of the mouse models tested, although *C. novyi-NT* spores added little toxicity to the radiotherapeutic regimens, resulting in long-term remissions in a significant fraction of animals.

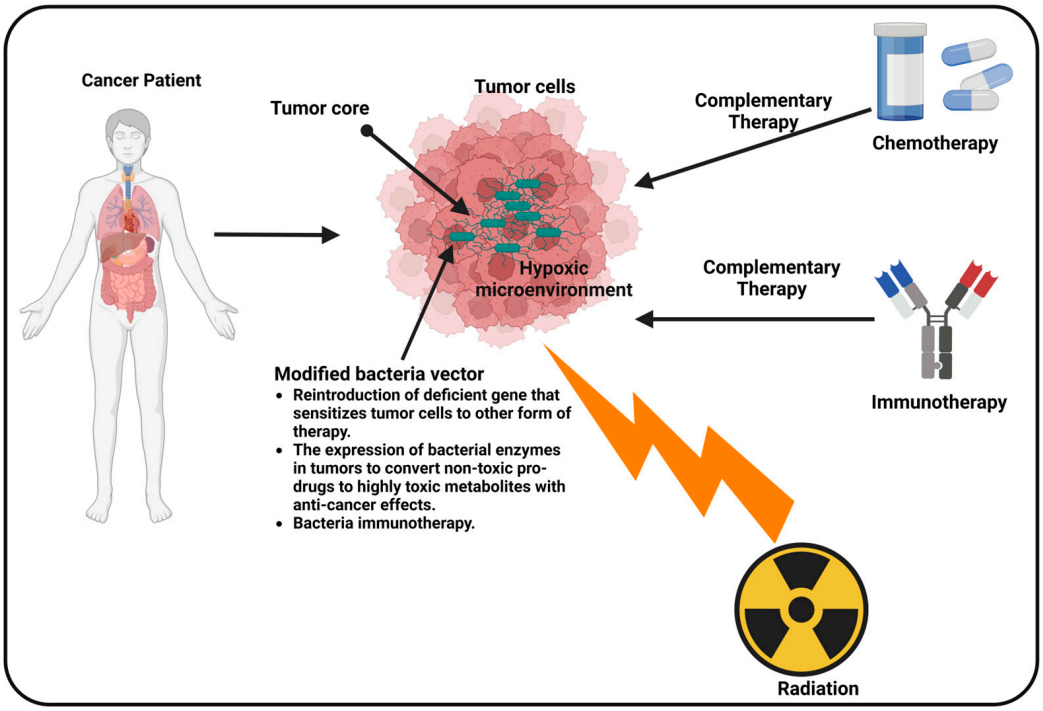

Figure 3. Strategies of tumor treatment, both monotherapy and complementary therapy. Bacterial vectors including *E. coli*, *Salmonella typhimurium*, *Clostridium* sp., and *Bifidobacterium* sp. The tumor microenvironment is a hypoxic region of the necrotic tumor, resistant to chemotherapy and radiotherapy, allowing selective targeting with bacteria. Immunotherapy, on the other hand, results in decreased immunotolerance and other immune-related adverse events, along with kidney-related toxicity, which limits its use.

Furthermore, an engineered *Salmonella typhimurium* (*S.t* ΔppGpp/pBAD-ClyA) that can carry tumor imaging probes (bacterial luciferase, Lux) or therapeutic molecules (Cytolysin A) to kill cancer cells was used in combination with radiotherapy. Radiotherapy helps to sensitize a colon tumor (CT26) model of BALB/c mice to *S. typhimurium* colonization. This showed that combining bacterial therapy and radiotherapy reduced tumor growth compared with only bacterial treatment [124].

7. Perspective, Limitations, and Conclusions

Invasive bacteria used for payload delivery are primarily engineered to acquire improved anti-tumor activities, therapeutic index, and safety [125,126]. To minimize their pathogenicity, significant virulence genes are often deleted. Therefore, understanding the approach of therapeutic cargo delivery, the strategies, and the mechanism would provide us with the necessary insight to improve invasive bacteria vectors for cancer therapeutics.

The deletion of the lipopolysaccharide (LPS) in the outer membrane of the *E. coli* vector makes it a diaminopimelic acid (DAP) autotroph, which helps prevent the stimulation of tumor necrosis factor (TNF) and shock in gram-negative sepsis [27]. Other benefits include an automatic self-targeting ability and the possibility of genetic manipulation to produce newly engineered attenuated strains [127]. Nevertheless, invasive bacteria for anti-cancer treatment have not yet been clinically established and require more research before their use in cancer treatment.

The use of bacteria and bacteria products, including bacteriocins and antimicrobial peptides of microbial origin, has gained significant attention due to their targeted anti-tumor activity [128]. Bacteriocins and some antimicrobial peptides are cationic and amphiphilic, killing tumor cells precisely without harming the surrounding normal cells. Mechanistically, bacteriocins are non-membrane-disrupting with high selectivity affecting the cellular activity of cancer cells through the induction of apoptosis and cell cycle, as well as the

prevention of metastasis. Other review articles provide more comprehensive information about bacteriocin as an anti-cancer agent [128–131]. However, bacteriocins' susceptibility to hydrolysis and hemolysis in vivo limits their clinical application. To overcome these challenges, bacteria vectors can be engineered to deliver this microbial product, namely, bacteriocins and antimicrobial peptides, to the core of the tumor where the radiation therapy cannot reach or sensitize radio-resistance cancer cells to radiation and other forms of treatment.

Interestingly, one of the uniquely safe attributes of bacterial vectors is their sensitivity to clinically available antibiotic treatments, which enables their control post-administration. This is an invaluable property for safe gene therapy. However, despite the potential for bacterial delivery systems, it is crucial to highlight their potential adverse side effects and the need to optimize their delivery into the targeted organ. Recombinant bacteria in humans must be carefully controlled and ascertained to prevent lateral gene transfer to the resident bacteria and limit the vector's environmental spread [132]. The specificity of bacteria vectors, such as *E. coli* [20,23,27], *Salmonella typhimurium* [133,134], and *Shigella flexneri* [135], to bind specifically to integrin receptors for the delivery of therapeutic cargo into cancer cells is one unique characteristic that makes them candidates for cancer gene therapy [136]. The significant expression of integrin on cancer cells provides a focal point for therapeutic cargo delivery into cancer cells. Integrin is a cell adhesion receptor that plays a crucial role in cell proliferation, migration, and survival [137,138]. For example, evidence has shown that β_1 integrin receptors were significantly expressed in melanoma [139], ovarian tumors [140,141], and non-small-cell lung carcinoma [142], which could be explored to deliver plasmid DNA, pro-drug, mRNA, or protein for cancer therapy.

Furthermore, cancer therapy with engineered bacteria provides an alternative option to meet the challenges of late-stage cancers that are initially insensitive to conventional treatments. Manipulating the gut microbiome by combining bacteria with cancer drugs may increase their effective delivery to the cancer sites. Using this strategy, the gut microbiome breaks down and metabolizes oral drugs, helping to dispense and distribute them into lymphatic and blood circulation and the gut–brain axis [143].

Bacteria used as drug carriers face challenges, such as biocompatibility, motility deterioration after drug loading, and a lack of in vivo verification [144]. Industrially, bacterial vectors are cheap to manufacture and practical compared to viral vectors, which are particularly cumbersome, time-consuming, and more expensive for gene therapy [145]. The necessary infrastructure and expertise already exist for low-cost bacterial vector manufacturing on an industrial scale, as bacterial culture systems have long been in operation in the biotechnology industries [146].

Bacterial therapy has demonstrated promising effects both preclinically and clinically. Many things could still be improved in using bacteria as anti-cancer therapeutics in clinical practice, including problems such as toxicity, limited targeting, safety, and effective use with other conventional therapies [147,148]. Although traditional therapies are still the mainstream treatment, the distinctive physiopathology of solid tumors has made these anti-cancer therapies inefficacious. Therefore, the sophistication of the metagenomics and transcriptomics sequencing of diverse cell populations to quantify the microbial community in cancer patients could be used to identify the gut microbiome composition, function, and dynamics, which will be critical to developing personalized cancer treatment using bacteria resident flora in the gut (Figure 4).

Overall, developing bacterial vectors with the potential to deliver therapeutic agents is an exciting area of research, but extensive work is still needed. Cargo delivery or the use of invasive bacteria in anti-cancer therapy is gaining acceptance because of its potential to provide positive clinical outcomes. However, more work needs to be done to improve some systems' safety and efficacy so that this approach can yield dividends in the coming years.

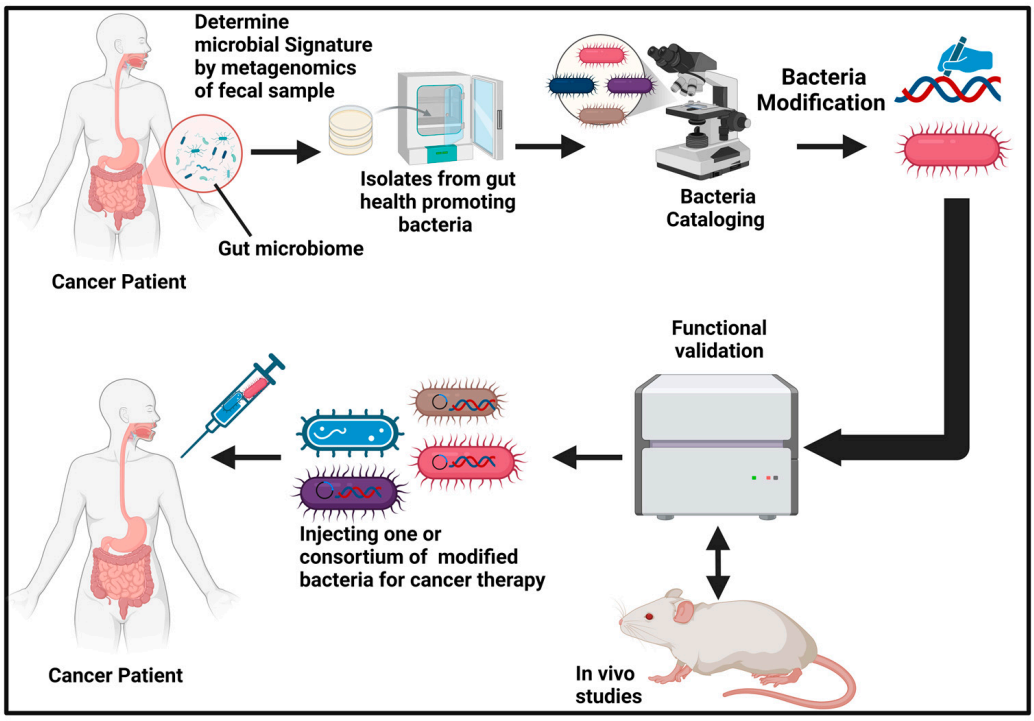

Figure 4. Personalized bacteria isolated from patients are used for cancer therapy. Fecal samples are collected from the patients, and metagenomics is used to determine the microbial signature. The known gut-health-promoting bacteria are isolated and cataloged before being modified by introducing therapeutic genes. Molecular techniques and in vivo testing validate the modified bacteria. The bacteria can be used singly or in a consortium as cancer therapy.

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