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Clostridium bacteria: The team of microscopic oncologists

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Abstract

As we approach the year 2023, the global rise in cancer mortality remains a pressing concern. Recent studies have demonstrated the remarkable potential of bacteria in combating cancer by stimulating the immune system. Exciting evidence suggests that bacterial therapy can revolutionize both the treatment and diagnosis of tumors. To effectively classify and treat tumors, the introduction of obligate or optional anaerobic bacteria into solid tumors may be necessary. Notably, certain strains of Clostridium have proven to be particularly effective in cancer treatment. A fascinating natural phenomenon lies in the ability of Clostridium spores to infiltrate tumors and selectively germinate in hypoxic regions within dense tumors upon injection into a vein. This bacterial invasion directly eliminates tumor cells by enhancing the presence of tumor-specific antigens, enabling the immune system to recognize and attack cancerous cells. Although these bacteria do not directly destroy tumor cells, their activation of the immune system holds great promise for eradicating them. Currently, an extensive range of bacteria is employed for cancer treatment, designing bacteria-carrying pharmaceutical compounds, and facilitating radiotherapy or radiation therapy. Additionally, genetic manipulation techniques can enable bacteria to specifically target tumor tissue and inhibit angiogenesis. In this comprehensive review, we delve into the potential advantages of utilizing Clostridium bacteria in cancer medications. Specifically, we explore the abilities of Clostridium perfringens and Clostridium novi to induce angiogenesis, provoke immune responses, and operate within oxygen-deprived environments.

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1. Introduction

According to the latest report from the World Health Organization (WHO), breast, lung, colon, rectum, and prostate cancer are projected to be the most prevalent types of cancer in 2020. Cancer, being a complex disease, necessitates a diverse range of treatment options. Surgery, radiotherapy, and chemotherapy, individually or in combination, are the primary treatment modes. Apart from cancer's intricate mechanisms, microorganisms are associated with cancer. Furthermore, bacterial toxins could be employed in cancer treatment (1-4). This review focuses exclusively on Clostridium perfringens, diphtheria, and novi. Additionally, extensive research has been conducted to comprehend the characteristics of Clostridium bacteria and the strategies employed for cancer and tumor treatment using this bacterium. **Table 1** presents a chronological overview of physicians' reports regarding Clostridium's positive effects on cancer treatment over the years. This indicates a long history of cancer therapy application. Historical evidence suggests that both acute and chronic infections are associated with spontaneous tumor regression. The immune system plays a pivotal role in this process. Anaerobic bacteria are anti-cancer agents that dates back over a century. Although bacteria can pose risks to the body, attenuated strains can benefit the immune system.

2. Immunological Factors and Angiogenesis in Cancer: Impact on Tumor Growth, Metastasis, and Treatment Strategies

Tumor cells recruit lymphocytes and antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs). The innate immune system detects and responds to bacterial surface molecules called Pathogen-associated molecular patterns (PAMPs) through specific receptors like toll-like receptors (TLRs). PAMPs stimulate the immune system and generate molecules such as B7 and IL-12, which further activate defense responses (5). Cellular immunity relies on T lymphocytes, while B lymphocytes play a crucial role in humoral immunity. The presence of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells is essential for tumor lysis. Additionally, NK and CTL cells can detect tumor cell antigens through the major histocompatibility complex (MHC), playing a vital role in preventing metastasis (20). NK cells have a critical role in innate immunity against tumors by inhibiting tumor growth, activating macrophages, and increasing T helper-1 (TH-1) expression through the release of cytokines such as interferon- gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which actively participate in anti-tumor responses (18). Furthermore, DCs can be stimulated not only by lipopolysaccharides (LPSs) but also by other bacterial and viral products. Therefore, any factor capable of stimulating the immune system to eliminate cancer cells can be effective in their treatment. The immune response to tumors is influenced and interacts with various factors, including NK cells (21), TCD8+ lymphocytes (22), macrophages, DCs, Tγδ cells, and FOXP3+ regulatory T cells (T-reg). Several of these anti-tumor cells inhibit tumor cell growth and shape the tumor microenvironment (23). Tumor cells produce specific antigens, including modified surface proteins known as tumor-specific antigens (TSA), which reduce immunological tolerance to the tumor. Detection of tumor antigens triggers appropriate immune responses, stimulating the immune system (18).

Angiogenesis and hypoxia are widely recognized as prominent pathological indicators in solid tumors (27). Arterial hypoxemia during these processes leads to macrophage and immune cell accumulation. Numerous studies have demonstrated inflammation's direct involvement in cancer initiation and progression (28, 29). The rapid proliferation of solid tumors outpaces blood vessels' ability to adequately supply oxygen and nutrients to the cells. Cancer cells release catabolites, accumulating on the tumor surface. This accumulation, combined with oxygen deprivation, induces hypoxia, leading to a decrease in pH levels and glucose levels (31). Hypoxic regions present a significant challenge in cancer treatment as they adversely affect therapeutic agent distribution. Inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) play critical roles in angiogenesis. IL-1 is primarily secreted by activated lymphocytes and macrophages and exhibits a potent effect on tumor cells upon secretion. It stimulates endothelial cell proliferation, enhances adhesion molecules expression, and produces cytokines that regulate angiogenesis. IL-1α is also known to stimulate angiogenesis and increase vascular endothelial growth factor (VEGF) expression (38). Tumor cells themselves produce VEGF as one of their growth factors, with neutrophils serving as a significant source of VEGF in angiogenesis. Macrophages are well-established

Table 1. **A history of medical reports about bacteria species in cancer treatment**

Year	An Overview of Reported Cases	Ref
A.D. 2600	In ancient Egypt, Imhotep, a physician, and chancellor of Pharaoh Djoser, employed a novel approach to treating cancer. He would make an incision on the tumor and apply an ointment internally, deliberately inducing an infection to treat the cancer.	(2)
Over Two Centuries Ago	There have been documented cases of cancer exhibiting spontaneous regression or successful treatment.	(3)
Over a Century Ago	After surgery performed by William B. Coley, a 19-year-old female patient afflicted with sarcoma passed away. However, Coley's subsequent examination of 100 cases of successfully treated sarcoma patients revealed a noteworthy correlation: those patients who had contracted Saint Anthony's fire infection had experienced a complete cure and subsequent recovery.	(3)
1813	Vautier observed the remarkable recovery of a cancer patient who had developed gangrene. Upon investigation, it was determined that the neoplastic tissue in this particular case was infected with *Clostridium perfringens*.	(2)
1829	In a study conducted by Dupuytren, a significant association between infectious diseases and cancer spontaneous regression was demonstrated. A case was reported involving a woman with breast cancer who declined surgery. After approximately 18 months, her condition worsened, leading to hospitalization. Progressive deterioration, accompanied by symptoms such as nausea and fever, indicated a decline in her health. Subsequent examination revealed that the tumor had become infected with gangrene. To alleviate viscous fluid accumulation, multiple incisions were made in the tumor. Over eight days, the tumor regressed, reducing its size to approximately one-third of its original dimensions. Notably, after four weeks, no clinical indications of a tumor were evident. Similarly, another French physician reported an intriguing case where bandages soaked in gangrene discharges were applied to cancerous lesions on a woman's breast. Astonishingly, within 19 days, the lesions completely healed, and the woman recovered from her cancer.	(2, 3)
1866	Fehleisen and Busch reported an intriguing finding that sarcoma can be cured with intermittent relapses of Saint Anthony's fire infection. Notably, they observed tumor regression in patients infected with *Streptococcus pyogenes*.	(4)
1887	Bruns documented a significant observation whereby malignant metastatic melanoma patients experienced a cure when afflicted with Saint Anthony's fire infection.	(3)
1918	In a comprehensive study examining 166 instances of spontaneous tumor regression in humans, Rhodenburg discovered that 72 out of the 166 cases were accompanied by fevers attributed to infections.	(3)

contributors to angiogenesis (37), and under hypoxic conditions associated with tumors, the expression of multiple growth factors, including VEGF, Placental growth factor (PIGF), fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), Hepatocyte growth factor (HGF), and angiopoietin, is increased (28). Cancer therapy targets neutrophils and macrophages to inhibit angiogenesis (34). TNF-α, an angiogenic agent, induces angiogenesis by upregulating VEGF-A and FGF-2 (39). High doses of TNF-α inhibit angiogenesis, while low doses promote vascularization in the affected area. IL-6, released by tumor cells, is another angiogenic agent. IL-6 levels are associated with inflammation and cancer. Evidence suggests that IL-8 expression by tumor cells and endothelial cells contributes to tumor growth, angiogenesis, and metastasis. Metastasis, a critical factor in cancer survival, accounts for approximately 90% of cancer-related deaths. Oxygen deficiency in the affected tissues contributes to metastatic progression due to aberrant angiogenesis. Lipopolysaccharides directly induce angiogenesis, a process closely linked to tumor growth. Additionally, macrophage- and neutrophil-dependent angiogenesis influences tumor expansion. These findings underscore the potential impact of controlling oxygen supply and regulating the inflammatory

response on tumor metastasis. Therefore, gaining a deeper understanding of the mechanisms underlying tumor metastasis holds promise for advancing cancer treatments (34).

3. Exploring the Potential of Anaerobic Bacteria in Cancer Treatment

In 1960, a hypothesis emerged proposing the use of anaerobic bacteria as a potential treatment for cancer. This groundbreaking idea suggests that in the context of severe cancers, anaerobic bacteria thrive in oxygen-deprived environments. Additionally, the process of angiogenesis triggers the production of a transcription factor called hypoxia-inducible factor alpha (HIF-1α), which promotes angiogenesis, specifically involving VEGF and angiopoietin-2. The presence of a hypoxic environment within cancerous tissues is believed to contribute to the preferential growth of anaerobic bacteria. This article delves into the potential of utilizing anaerobic bacteria in cancer treatment, exploring their advantages, challenges, and promising developments (35). In contrast to their potential benefits, bacteria can have adverse effects on the host. Bacterial infections not only directly damage the host's DNA but also indirectly activate oxygen and nitrogen, posing harm to the host. These infections trigger phagocytosis and oxidative stress, disrupting the balance between free radicals and antioxidants, and ultimately favoring free radicals (36,37). Free radicals are atoms with unpaired electrons that can readily react with other atoms, including oxygen molecules. Bacterial infections stimulate macrophages and DCs associated with cancer cells, leading to elevated levels of reactive oxygen species (ROS). These ROS contribute to drug resistance and promote phagocytosis, prolonging the survival of tumor cells. As a result, the challenges in treatment intensify, increasing the risks of recurrence and metastasis. Toxicity and invasiveness are crucial characteristics exhibited by bacteria. Toxic bacteria produce harmful toxins that target specific tissues and disrupt the body's metabolic machinery. Invasive bacteria rapidly multiply at the site of infection and spread quickly (38). When a host cell undergoes cell death or experiences DNA damage that leads to cancer, the extent of DNA damage becomes too extensive for the cell to repair. Bacteria play a significant role in both the development (carcinogenesis) and prevention of cancer (39).

In the pursuit of more effective cancer treatments, scientists have been exploring the potential of harnessing bacteria with genetically modified properties. By manipulating bacteria, researchers aim to induce specific cytokines that possess anti-tumor properties. This innovative approach offers several advantages, including the ability to enhance the effectiveness of bacterial anti-cancer drugs through genetic modification. In this article, we will delve into the strategies and mechanisms employed in designing bacteria for targeted cancer treatment (40).

4. Unleashing the Power of Bacterial Anti-Cancer Drugs

Bacterial anti-cancer drugs employ two primary mechanisms to combat tumors. The first mechanism involves the direct expression of proteins with physiological activities against tumors (40-43). The second mechanism revolves around carriers that express eukaryotic genes within infected cancer cells. A large amount of research focuses on three classes of anti-cancer drugs. These include cytotoxic agents designed to destroy cancer cells (44). These potent substances act by targeting and eliminating malignant cells, effectively inhibiting tumor growth. Next, cytokines play a vital role in stimulating immune cells to eliminate cancer cells. By harnessing cytokines, bacterial anti-cancer drugs can activate the immune system and enhance its ability to combat tumors effectively (45). Moreover, tumor antigens sensitize the immune system to recognize and target cancer cells specifically. This targeted approach facilitates a robust anti-cancer response against cancer, bolstering the body's defenses in the fight against the disease (46-48). The secretion of cytokines is triggered through various mechanisms, such as the activation, proliferation, and migration of immune cells (49). Upon injection, bacteria activate the function of NK cells, leading to the release of lymphokines and the proliferation of lymphocytes. This orchestrated response enhances the immune system's ability to combat cancer cells effectively. Among the plethora of bacterial cytokines studied, IL-2 stands out as one of the most abundant. IL-2 stimulates NK, T, and dendritic cells, further bolstering the immune response against tumors (50-53). Additionally, IL-12 stimulates neutrophils and macrophages to attack tumor cells, reducing tumor growth and minimizing systemic toxicity. Furthermore, the utilization of bacteria exposed to LPS triggers cytokine production. Notably, bacterial LPS exhibit varying toxicities, prompting different immune cells to secrete specific cytokines (52-55). One of the most potent cytokines is TNF-α, which induces apoptosis within cancer cells. This apoptotic pathway is activated through FS-7-associated surface antigen (Fas) Ligand (FASL) production and the subsequent binding to its

FASL ligand. TNF-related apoptosis-inducing ligand (TRAIL) and TNF-α have shown remarkable efficacy in inducing apoptosis in various cancer cell types (56,57). It is noteworthy that cancer cells exhibit greater temperature sensitivity than normal cells. Therefore, febrile diseases play a vital role in cancer treatment. Fever stimulates the release of inflammatory cytokines like IL-1, IL-6, and TNF-α from immune cells. These cytokines, in turn, activate other immune cells, such as dendritic cells and cytotoxic T lymphocytes (CTLs). Furthermore, fever can induce tumor vessel collapse, leading to hemorrhagic necrosis (58,59). So, fever's role in stimulating immune cells and promoting tumor destruction is significant. When considering cell-based therapies for cancer treatment, it is crucial to ensure that the cells employed are not toxic to the host. They should selectively replicate within the tumor site and navigate and eradicate tumor cells in necrotic and hypoxic areas. Moreover, it is desirable to eliminate microorganisms from the living organism gradually and entirely (60-61). CD47, a signaling molecule found in abundance in most solid human tumors, poses a significant challenge to traditional cancer treatments. It acts as a shield, protecting cancer cells from destruction by innate immune cells like macrophages and dendritic cells. However, by engineering bacteria to specifically target CD47 within tumors, researchers aim to overcome this obstacle and minimize systemic side effects. Treatment with these engineered bacteria leads to the development of tumor-specific T cells within the tumor, presenting a potential breakthrough in treating both primary and distant tumors (62-64).

Bacterial-mediated tumor therapy has shown promise as an effective approach in combination with other cancer treatments. This article explores the potential of bacterial therapy in cancer treatment, focusing on the use of Clostridium bacteria and their unique mechanisms of action.

5. Clostridium Bacteria in Cancer Treatment: Targeting Tumors with Anaerobic Properties and Therapeutic Mechanisms

Clostridium is a group of motile gram-positive bacilli that possess a peritrichous flagella, except for Clostridium perfringens, which lack a flagellum (65). These bacteria are characterized by their ability to form spores and thrive under anaerobic or microaerophilic conditions. Clostridium is a diverse group of rod-shaped, anaerobic, gram-positive bacteria found abundantly in soil and freshwater environments. Some Clostridium subspecies, such as C. acetobutylicum, C. Butyricum, C. Tetani, C. Beijerinckii, and C. Histolyticum, have been studied for their potential anti-cancer properties (66). There are four major groups of Clostridium bacteria that are of significant medical importance. C. histolyticum causes tissue infections following wounds or trauma. Invasive Clostridium species cause food poisoning and gastrointestinal diseases. C. tetani causes tetanus, while C. botulinum causes botulism (65).

These spore-forming bacteria can cause serious conditions such as botulism (C. botulinum), tetanus (C. tetani), gangrene, and intestinal infections like pseudomembranous colitis (C. perfringens or C. welch) (47). Clostridium is primarily transmitted through the oral-fecal route, as it is part of the normal gastrointestinal flora in humans and animals. Clostridium infections can cause mild diarrhea to life-threatening colitis (67).

The year 1947 marked a significant milestone as Clostridium Histolyticum spores were introduced into clinical trials (46). While these bacteria selectively accumulated in cancerous tissues, the exotoxins they released posed a grave systemic toxicity concern. Bacterial-mediated tumor therapy (BMT) emerged as a novel approach in 2002 (3). To effectively combat cancer, various treatment methods have been proposed, including a combination of orthomolecular drugs, a balanced diet rich in vitamin C (at least 12 grams per day), vaccination, and a cautious approach to antibiotic usage (which has proven highly effective in cancer treatment). These treatments have demonstrated their effectiveness when combined with other therapies like surgery-radiotherapy and chemotherapy (47). During the latter half of the twentieth century, researchers examined spores of obligate anaerobic bacteria, such as Clostridia. They treated cancer by strategically placing bacterial spores within solid tumor sites. These spores would then germinate in an oxygen-deprived environment, assuming a vegetative form and initiating tumor destruction. However, the growth process involved the secretion of an alpha-toxin, which led to high toxicity and mortality in humans. However, researchers successfully reduced the toxicity of this treatment by eliminating alpha-toxin (48, 49). It is worth noting that approximately 90% of cancers manifest as solid tumors. Studies have revealed that many types of solid tumors contain hypoxic areas within the tumor mass, making them ideal

environments for anaerobic bacteria like Clostridium (23). The concept of bacteria as an adjuvant in cancer treatment is gaining traction (50). Ultimately, bacteria, alone or as an adjunct or component (with the toxic part removed), promises tumor growth.

Bacterial toxins play a crucial role in killing cells and altering cellular processes that regulate cancer progression, such as proliferation, apoptosis, and differentiation. These processes are intimately linked to cancer development mechanisms. Bacteria can be harnessed as carriers for delivering therapeutic compounds to target tumors or as vehicles for the efficient production of enzymes. Cancer treatment uses bacterial toxins conjugated with tumor-specific surface antigens or applied alongside bacterial spores. Generally, live or genetically modified bacteria are employed for therapeutic purposes. Bacteria can also serve as immunotherapy agents in cancer treatment (51). Microorganisms' inhibitory effect on tumors has been the subject of numerous studies (5). Various bacterial species, including Clostridium, have been tested as carriers in animal models to deliver tumor-inhibitory genes, anti-angiogenic genes, cell suicide genes, or tumor-specific antigens. These bacteria can be employed as immunotherapeutic agents in cancer treatment. Three notable applications in this regard include infection with bacteria such as Clostridium novyi can induce heat shock protein 70 (Hsp70) (52). Additionally, infection with Clostridium novyi triggers the release of bacterial-released PAMPS that bind TLRs in the body, activating them and stimulating the upstream regulation of inflammatory cytokines, such as IL-12 (53). Next, bacterial spores can be utilized as an anti-tumor agent. Moreover, bacterial toxins or enzymes, including cytolysin A (CLY A), can impede tumor growth through the formation of pores in the eukaryotic cell membrane. Moreover, they can induce cell necrosis via intracellular caspases or cytokines belonging to the TNF-α family through apoptosis (54). One of the notable advantages of using bacteria for delivering therapeutic compounds lies in their ability to precisely target cancer cells. There are two primary methods of inhibiting tumors using bacteria (3). The first method involves utilizing enzymes produced by bacteria, while the second method relies on the release of compounds secreted by bacteria during colonization (3). In the context of enzymatic therapy, a low level of enzyme expression in the cancerous environment may indicate a lack of therapeutic efficacy (55, 56, 57). On the other hand, the second method involves the use of bacterial toxins with properties such as hemolysin (specifically the alpha-hemolysin type) and Azurine (58, 59). Additionally, effective recombinant proteins like mTNF-α and recombinant Interleukin-2 (RIL-2) (8) or shRNA (60, 61) (62) can be employed. However, a challenge arises when these molecules need to be transported across the bacterial membrane into the external environment. They are further distributed within the body (3). When proteins such as toxins, cytokines, enzymes, and immunogens are used as anti-cancer agents, the issue of effectively delivering them to the tumor mass becomes apparent (63).

Currently, two active transfer strategies are employed for gram-negative bacteria. The first approach involves a delayed-stage lysis system where compounds are released passively through the lysis process. This system delivers high-concentration compounds in a short period of time. However, it also has limitations, including continuity, discontinuity, low immune system activation, or rapid compound degradation. This method has shown success in vaccination and cancer treatment (3). The second strategy is based on the incorporation of therapeutic compounds into signaling molecules, a mechanism present in almost all bacteria. In this system, compounds must be continuously secreted. However, the binding of compounds to peptides can pose a limiting factor, potentially resulting in therapeutic activity loss due to conformational changes or improper folding (64). Bacteria harbor valuable biologically active compounds, including proteins and anti-cancer peptides (70). Anaerobic bacteria like Clostridium in cancer treatment offer significant benefits. It allows for targeted therapy by selectively destroying a small portion of the tumor and provides opportunities for combination therapies (68). Clostridium-based cancer treatment shows promise as a viable approach to combating solid tumors (68).

Research on the application of Clostridium bacteria in cancer treatment has shown promising results due to their anaerobic nature and ability to target tumors with necrotic areas. One study revealed that by removing the alpha toxin gene from Clostridium novyi, researchers were able to achieve selective colonization of tumor tissues (71). In in-vitro models of various cancers such as colorectal cancer, renal cell carcinoma, glioma, and sarcoma, C. novyi exhibited the ability to selectively colonize and release cytokines and immune cells, leading to necrosis of tumor tissue (72, 73, 74, 75). Hypoxic areas within tumors, characterized by

inadequate blood flow, acidity, and nutrient deprivation, are closely associated with tumor malignancy, angiogenesis, and increased metastasis. The unique property of anaerobic bacteria like Clostridium is their ability to multiply specifically in these hypoxic and necrotic regions of the tumor. This process activates the immune system, promoting appropriate immune responses that aid in the elimination of the tumor (76). Bacterial toxins can exert their effects locally or systemically. One example is toxin-A of C. botulinum, which has shown promise in the treatment of prostate cancer (PC-3 and LNCaP cell lines), breast cancer (T47D cell line), and neuroblastoma (SH-SY5Y cell line). This toxin has apoptotic functions and has been utilized specifically for prostate cancer by initiating caspase-3 and caspase-7-dependent processes, while in breast cancer, it induces apoptosis through caspase activation (70).

Clostridium perfringens enterotoxin (CPE), a potent cytolytic toxin released during the sporulation process, targets cells expressing claudin proteins involved in tight junction formation and cell diffusion (77). Claudin proteins (CLDNs) are membrane proteins with intracellular and extracellular domains, including extracellular loop domains. Specifically, claudin-3 and claudin-4 possess extracellular loop domains that serve as receptor sites for the CPE ligand (78). CPE naturally binds to CLDN-4 through its C-terminal amino acids (4). This binding results in the formation of permeable channels for calcium ions, ultimately leading to the opening of epithelial cell-extracellular gates. CLDNs not only play a role in cancer treatment but also have diagnostic value, as specific claudins have been associated with prognostic indicators in various tumors (4). Some of these cancers are presented in **Table 2.** CLDN-4, highly expressed in breast, colon, prostate, pancreas, and ovarian cancers, holds potential as a therapeutic target (68, 79). In ovarian cancers, claudin-3 and claudin-4 are frequently expressed, and studies have demonstrated the potential suppression of primary and chemotherapy-resistant metastatic ovarian cancers through CPE in vivo (80, 65). CPE, being a bacterial protein, can elicit potent immune responses from T lymphocytes. CPE-mediated cell lysis has emerged as a promising antitumor therapy for cancers with high CLDN expression that are sensitive to CPE. Additionally, the recombinant C-terminal components of CPE (C-CPE) have been utilized as adjuvants in cancer treatment to enhance the permeability of antitumor drugs within tumor tissues and increase drug sensitivity (101). In a study focusing on targeted gene therapy of colon cancer cell lines expressing claudin-3 or claudin-4, researchers used an optimized CPE (optCPE)-expressing vector and recombinant CPE. The study demonstrated a positive correlation between Clostridium perfringens enterotoxin cytotoxicity and the level of colorectal cancer, breast cancer, and ovarian cancer (4). Furthermore, studies have shown that the overexpression of claudin-3 and claudin-4 is associated with aggressive tumor behavior and poor prognosis in various cancer types (68).

Clostridium bacteria equipped with prodrug converting enzymes (PCEs) have shown promise in converting non-toxic prodrugs into active chemotherapeutic agents within the tumor microenvironment. The PCE is selectively expressed within the tumor, taking advantage of the anaerobic conditions in which Clostridium can thrive. This localized activation of prodrugs minimizes the systemic side effects typically associated with conventional chemotherapy, as the conversion occurs specifically within the tumor. This approach holds great potential for improving the efficacy and safety of cancer treatments. Figure 1 illustrates the concept of using Clostridium bacteria and prodrug-converting enzymes for targeted cancer therapy (102).

Clostridium difficile is a bacterial pathogen known to cause diarrhea, and its impact is of significant global concern due to the associated mortality rate and financial burden on healthcare systems. Individuals with cancer are particularly susceptible to Clostridium difficile infection (CDI) (103). Interestingly, if CDI occurs at the site of a tumor, the tumor tends to regress. C. difficile produces two toxins called Clostridium difficile toxin A (TcdA) and Clostridium difficile toxin B (TcdB), which have inflammatory properties and can induce the secretion of cytokines and chemokines (104). Through the activation of acquired and innate immunity, C. difficile triggers inflammatory responses mediated by the nuclear factor kappa B (NF-κB) pathway. NF-κB acts as a link between inflammation and tumor regression. The NF-κB-dependent pathways lead to the secretion of pro-inflammatory cytokines that play critical roles in initiating and propagating inflammatory responses (101). Specifically, NF-κB activation induces the expression of genes such as cyclooxygenase-2 (COX-2), which is involved in

Table 2. **Decoding the Prognostic Significance of Isoforms in Cancer**

Claudins	Genes	Cancer Type	Ref
Claudin 1	*CLDN1*	• Colorectal Cancer • Liver Cancer (Hepatocellular Carcinoma)	(81-87)
Claudin 2	*CLDN2*	• Colorectal Cancer • Liver Cancer (Hepatocellular Carcinoma)	(79)
Claudin 3	*CLDN3*	• Ovarian Cancer • Non-Small Cell Lung Cancer (NSCLC) • Breast Cancer	(81-83), (87-92)
Claudin 4	*CLDN4*	• Ovarian Cancer • Lung Cancer	(78)
Claudin 7	*CLDN7*	• Ovaries-endometrium • Colorectal Cancer • Breast Cancer	(82), (87), (90), (93), (94-96)
Claudin 10	*CLDN10*	• Hepatocytes-ovaries	(77)
Claudin 18	*CLDN18*	• Stomach Cancer	(89), (96-97)

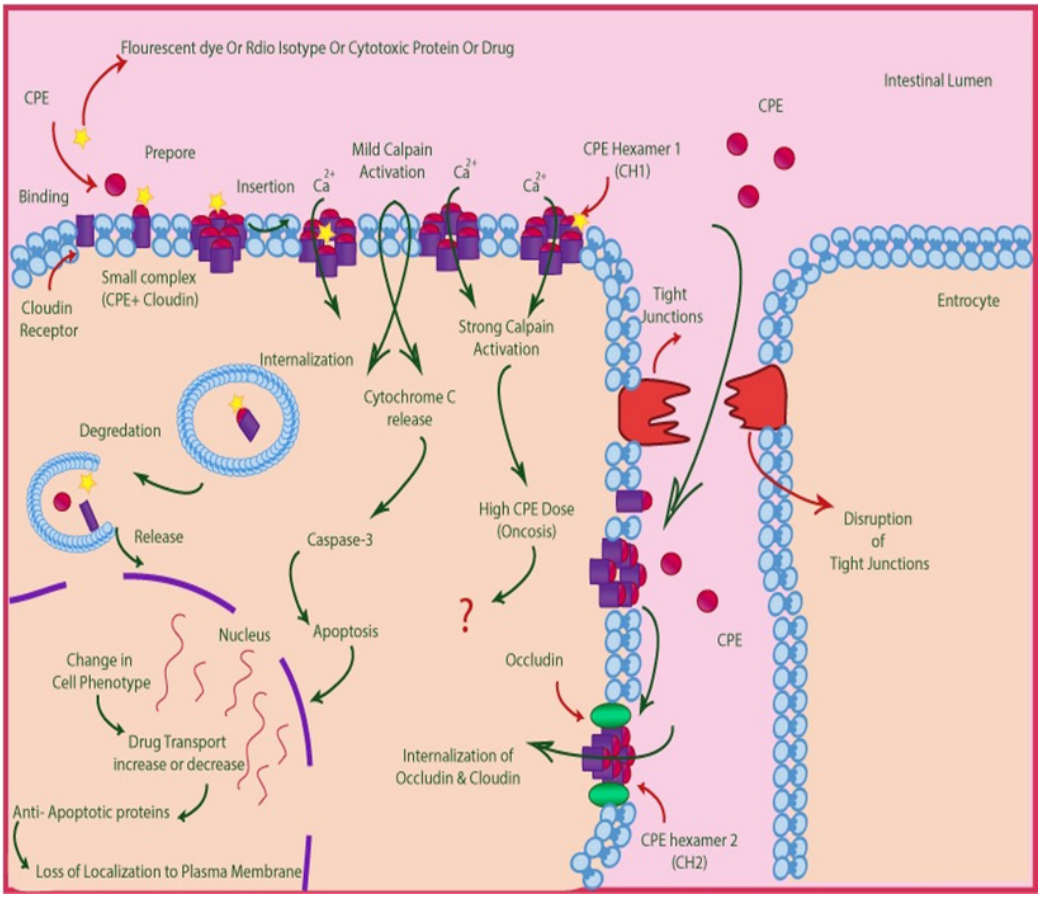

Figure 1. **Shining a Light on Cancer by CPE Cytolysis as a Fluorescent Diagnostic.** Tool CPE cytolysis is a process where Clostridium perfringens enterotoxin (CPE) binds to claudins (CLDNs), forming a hexamer complex in the cell membrane. This complex acts as a pore, allowing calcium ions to enter the cell. The rapid influx of calcium activates calcium-dependent proteases, leading to cellular necrosis. C-CPE, a recombinant C-terminal component of CPE, is used as an anticancer drug and a fluorescent dye for cancer diagnosis and treatment. It is binding to CLDNs helps target cancer cells specifically, aiding in the delivery of therapeutic agents or visualization of tumor tissues during diagnostic procedures.

inflammation, as well as cytokine genes essential for tumor regression. Additionally, genes associated with tumor metastasis and angiogenesis, such as matrix metalloproteinase (MMP6), chemokine receptors, and VEGF, are also activated (105). TcdA and TcdB stimulate neutrophils and immune cells in the colon, thereby activating the immune system (101). The immune system has a dual role, as it can either suppress or promote tumor development. Bacterial infections promote T-cell receptor signaling in CD8+ T cells, which are crucial players in the host's anti-tumor response, enabling them to detect small amounts of tumor antigens (106). TcdA induces apoptosis in the CT26 cell line (a murine colorectal carcinoma cell line is from a BALB/c mouse), leading to the expression of calreticulin on the cell surface and the initiation and reinforcement of anti-tumor immunity (104, 107). Both TcdA and TcdB are cytotoxic to cells, but TcdB is more potent in this regard. TcdB-infected tumor cells exhibit high immunogenicity and can activate dendritic cells, thereby eliciting a long-lasting and robust anti-tumor response in mice. The toxins of C. difficile can induce apoptosis or dose-dependent necrosis. Studies using TcdB mutant bacteria with impaired glycosyltransferase activity have shown that proper glycosyltransferase activity of C. difficile toxin is essential for the secretion of TNF-α from macrophages (108, 109). Similarly, in mouse melanoma, TcdB-affected CT26 cells efficiently activate bone marrow-derived dendritic cells (BMDCs) and subsequent T-cell functionality. Moreover, TcdB can be employed to broaden the anti-tumor response against various types of cancer. For instance, TcdB can induce colorectal cancer in mouse models, and the infected cells can stimulate both T-cell and BMDC activity (104). The apoptosis process of the CT26 cell line induced by TcdB involves the generation of several types of reactive oxygen species (110).

In addition to its direct effects on tumor regression and immune response, C. difficile infection has been found to have implications for cancer therapy and immunotherapy. The presence of Clostridium difficile in the tumor microenvironment can enhance the efficacy of certain cancer treatments. Studies have shown that the presence of C. difficile and its toxins, TcdA and TcdB, can modulate the tumor microenvironment and improve the response to chemotherapy. The toxins can induce tumor cell death and release tumor antigens, which can then be recognized by the immune system, leading to an anti-tumor immune response (111). This phenomenon has been observed in colorectal cancer, where the presence of C. difficile has been associated with improved response to chemotherapy drugs such as oxaliplatin (112). Furthermore, C. difficile has been investigated as a potential vehicle for targeted delivery of therapeutic agents to the tumor site. The ability of C. difficile to selectively colonize hypoxic and necrotic areas of tumors makes it an attractive candidate for delivering drugs specifically to the tumor microenvironment (113). Researchers have explored the use of engineered strains of C. difficile to deliver therapeutic agents, such as cytotoxic drugs or immunomodulatory molecules, directly to the tumor site (114). This approach holds promise for enhancing the efficacy of cancer treatments while minimizing off-target effects on healthy tissues. Moreover, the unique ability of C. difficile to colonize the tumor microenvironment and stimulate immune responses has sparked interest in using this bacterium as a platform for cancer immunotherapy. Researchers are exploring the potential of Clostridium difficile-based vaccines or immunotherapeutic strategies to enhance anti-tumor immune responses and improve patient outcomes. By harnessing the immune-stimulating properties of C. difficile, it may be possible to develop novel approaches for cancer treatment that exploit the bacteria's natural capabilities (115).

Clostridium novyi-NT is a bacterium known to cause gangrene and sepsis. Interestingly, its growth and germination are hampered by insufficient oxygen pressure in its tissues. This characteristic led researchers to explore its potential in cancer treatment. The hypothesis was that by injecting C. novyi-NT directly into tumors, it would attack cancer cells and trigger immune responses against them (47). Similar to oncolytic viruses, C. novyi-NT causes an infection that results in tumor lysis and the release of tumor-dependent antigens, leading to an anti-tumor immune response. In addition to this mechanism, two important bacteria-dependent signaling pathways, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), are activated. These pathways induce the maturation of antigen-presenting cells (APCs) through various cytokines and promote the activation of CD4+ and CD8+ T-cells against cancer cells (111). The attenuated

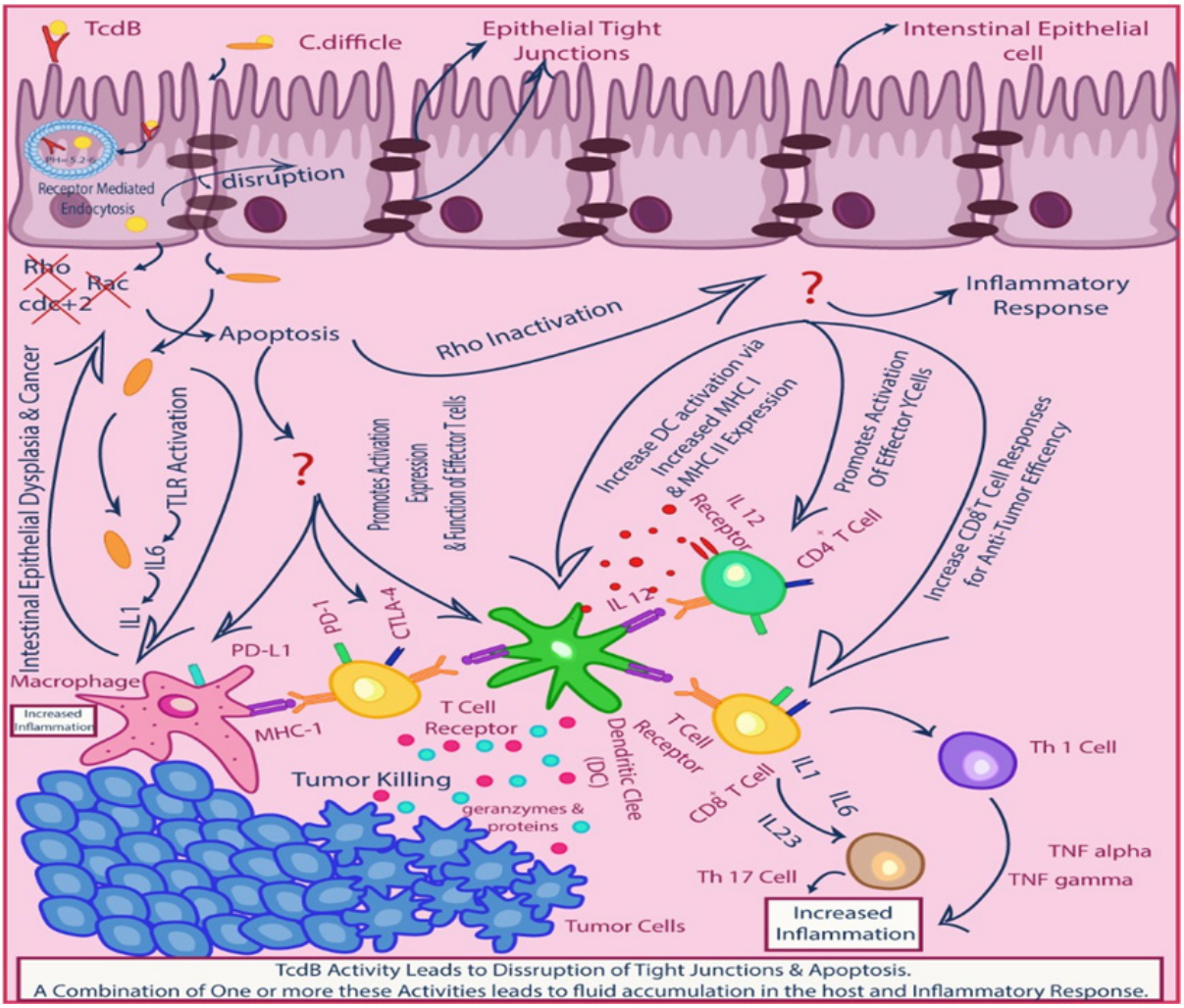

Figure 2. **TcdB's Role in Unleashing the Immune Attack on Cancer.** TcdB disrupts the intestinal epithelial cell barrier, which normally protects against harmful substances. Once the barrier is compromised, C. difficile or its components trigger Toll-like receptors (TLRs) on immune cells, leading to immune responses. These responses involve interleukins, which induce antitumor responses. Macrophages and dendritic cells are then activated to target and eliminate cancer cells, removing them from affected tissues. Next, the adaptive immune response activated by TLRs increases the number of cytotoxic T cells and T CD4+ cells. These cells are essential for cancer cell destruction. These immune cells also produce cytokines and chemokines to further boost the immune system's ability to fight cancer.

form of C. novyi-NT has shown efficacy in eliminating treatment-resistant hypoxic tumors in mouse models. Spores of the bacteria can be injected systematically or directly into the tumor. By thriving in low-oxygen conditions, the spores target and eliminate the tumor (66). It is worth noting that C. novyi-NT spores can cross the blood-brain barrier, allowing them to target tumors in the brain without causing significant damage to the host or healthy tissues. However, it should be noted that tumors without necrotic areas or those that have metastasized may not be suitable for colonization by these bacteria and can be more effectively targeted using other therapeutic approaches (3). C. novyi-NT relies on selective anaerobic spore growth. After injecting C. novyi-NT spores into the tumor and allowing the bacterium to colonize the site, it leads to hemorrhagic necrosis, tumor lysis, and regression. In clinical experiments, non-pathogenic Clostridium species like C. butyricum M-55 or C. novyi, combined with liposomal doxorubicin, have shown success in removing well-developed tumors (7). Given the relative rarity of treatment with C. novyi-NT, it can be combined with chemotherapy drugs or radiotherapy protocols in a treatment approach known as combined bacteriolytic therapy (COBALT) (71). In the COBALT method, C. novyi-NT can target necrotic and hypoxic regions of tumors, while normoxic sites trigger a typical cytotoxic immune response (66, 112).

One of the advantages of using C. novyi-NT in cancer treatment is the germination of spores in hypoxic regions of solid tumors. Systemic injection of these spores allows them to infiltrate the tissue and germinate into bacteria (71, 112). Once germination occurs, Clostridium undergoes vegetative growth and secretes extracellular enzymes, including proteases, lipases, and phospholipases. The protease activity leads to cellular digestion and tumor lysis, resulting in a long-term anti-tumor response and tumor regression. Lipase activity plays a crucial role in membrane lysis, activation of NK cells and CD8+ T cells, and subsequent tumor regression (112). By inducing long-term immunity in the host, C. novyi-NT promotes a sustained anti-tumor immune response (112). In experimental models, the next gene from C. novyi-NT spores was extracted and prepared and then injected into model mice. After spore infiltration, a prodrug was administered. Through the germination process, the noxC gene was expressed, leading to the conversion of the prodrug into cytotoxic derivatives (63, 102). Increased therapeutic effects have been observed when C. novyi-NT spores are combined with radiotherapy (113). In 2006, it was demonstrated that C. novyi-NT enhances liposomal drug release within tumors (114). It is speculated that spore germination aids in the release of encapsulated drugs from liposomes (114). For detection purposes, genes encoding light-emitting fluorescence signals, such as luciferase, have been used to track the presence of bacteria in model organisms (115).

C. novyi-NT also inhibits tumor-inhibiting immune responses (116). TNF-α initiates apoptosis pathways, while NF-κβ inhibits tumor growth and metastasis, making them potential targets for future cancer treatments (68). C. novyi-NT secretes extracellular enzymes, including proteases that digest proteins on the periphery of cancer cells, impairing their adhesion properties. The lipases produced by C. novyi-NT dissolve the plasma membrane of tumor cells, and phospholipase C induces immune responses and further membrane dissolution. These activities, combined with chemotherapy and radiotherapy, contribute to tumor regression (63). NK cells and CD8+ T-cells are activated as a result of tumor regression, which further promotes cancer regression (63). Genomic and transcriptomic analysis of C. novyi-NT has revealed highly expressed genes that encode extracellular proteins with lipase activity. These proteins can alter the structure and permeability of the lipid bilayer, leading to cytotoxicity. Phospholipases are responsible for inducing immune responses and anti-tumor immunity. C. novyi-NT induces inflammatory reactions through the release of granulocyte colony-stimulating factor (G-CSF), Tissue inhibitor of metalloprotease-1 (TIMP-1), macrophage inflammatory protein (MIP)-2, and IL-6, attracting immune cells to the infection site and contributing to long-term anti-tumor responses (112). Promising initial results have been reported regarding the application of genetically modified C. novyi-NT spores in cancer treatment, with ongoing research in this area (102). Several challenges need to be addressed for the clinical use of C. novyi-NT spores. These include managing tumor lysis syndrome, considering tumor size and suppression rate, optimizing the growth rate of C. novyi-NT, determining the appropriate utilization of the bacterium, and effectively targeting hypoxic tumor niches (63, 112). Achieving the right balance between bacterial lysis, angiogenesis, immune response, and tumor size is crucial for treatment efficiency and minimizing adverse effects such as tumor lysis syndrome. Further research and clinical trials are necessary to fully understand the potential of C. novyi-NT as a therapeutic agent in cancer treatment. The unique ability of C. novyi-NT to target hypoxic tumor regions and induce tumor regression, along with its capacity to activate immune responses, makes it a promising candidate for combination therapies and future cancer treatment approaches (117). Extensive preclinical studies have demonstrated Clostridium bacteria's efficacy in inhibiting tumor growth and prolonging survival in animal models. These studies have also revealed the potential synergistic effects of combining Clostridium-based therapies with conventional treatments, such as chemotherapy and immunotherapy. By augmenting the tumor microenvironment and promoting immune response, Clostridium bacteria can enhance cancer treatment effectiveness (**Table 3**). While Closterium bacteria and cancer treatment research is still in its early stages, there have been encouraging findings from preclinical studies and animal models. Clinical trials are now being conducted to further investigate the potential of these bacteria in human cancer patients. These trials aim to evaluate Closterium bacteria safety, efficacy, and optimal treatment strategies. The results of these trials will provide valuable insights and pave the way for potential future therapies (**Table 4**)

Table 3. **Crucial Functions of Clostridium Bacteria in Cancer Treatment.**

Method of action	Mechanism	Clostridium spp.	Anti-tumor Effects	Ref
Bactofection	• Bacteria carry cytotoxic genes within plasmids and transfer them to the cancerous cells.	Various species	Cancer apoptosis	(2)
Combined Bacteriolytic Therapy (COBALT)	• Cytotoxic genes-free anaerobic bacteria combined with routine treatments like chemotherapy and radiotherapy are used. • When combined with other cancer therapeutics, Clostridium spp. has a direct anti-tumor effect against tumor antigens.	• *C. novyi,* • *C. oncolyticum,* • *C. acetobutylicum*	• Long-term enhancement of tumor regression • Increased hypoxia	(2, 68)
Clostridium-Directed Enzyme Prodrug Therapy (CDEPT)	• Prodrug activation via bacterium enzymes. • Genetically engineered Clostridium spp. Express enzymes that convert a prodrug to an active drug. Clostridium spp. are used as vectors.	Various species	• Cytosine and 5-fluorocytosine (5FC) deaminase are two enzymes cloned in the clostridium species, converting 5-fluorouracil to 5-fluorocytosine, hindering tumor growth.	(2, 68)
Clostridium-Directed Antibody Therapy (CDAT)	• Modified Clostridium spp. Induce antibodies secretion against tumor antigens in large quantities.	Various species	• There are various types of cells that can be stimulated by HIF-1, including tumor cells as well as immortal cells. HIF-1 activates under both normal and hypoxic conditions. HIF-1 inhibits antibodies' functions at the VHH site.	(68, 69)
Tumor recognition and immune system reinforcement	• Using genetic engineering in bacteria, cytokines such as IL-2 and TNF-⊠ are synthesized in order to promote tumor regression.	Various species	• A variety of *Clostridium* species have been successfully used to deliver cytokines or to enhance the immune response to tumor antigens.	(68)
Diffusion of liposome drugs in solid tumors	• This method uses *Clostridium* spp. Secreting lipase to deliver liposome-encapsulated drugs directly to tumors.	*C. novyi*-NT	• Because of its ability to express multi-substrate lipases, the bacteria exhibit some oncologic characteristics as it colonizes within a tumor.	(68)

Table 4. **Clostridium Bacteria in Cancer Treatment and Historical Evidence of Tumor Regression**

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2001	Nontoxigenic *Clostridium novyi*	• Lung • Breast • Prostate	Mice	• The bacterium is capable of selectively targeting and infecting the tumor cells within solid tumors. Once inside the tumor, the bacteria multiply and cause the tumor cells to burst, leading to tumor destruction. This process also induces an immune response against the tumor, aiding in its regression.	(132)
2002	• *C. butyricum* • Recombinant *C. acetobutylicum*	• Colorectal • Gastric • Hepatocellular • Breast Glioma.	Mice	• The study explores the use of recombinant clostridia for targeted drug delivery in tumors, specifically anti-tumor agents and suicide genes. The production of therapeutic molecules within the tumor shows the potential for achieving a complete cure. Different enzymes and prodrug/drug combinations are proposed for directed-enzyme prodrug therapy (DEPT). Clostridia-based delivery systems offer high tumor specificity by targeting hypoxic/necrotic regions. The paper also discusses the use of clostridia in delivering therapeutic agents, such as tumor necrosis factor (TNF)-a, which has demonstrated anti-tumor capabilities. The production of biologically active TNF-a by modified clostridia is demonstrated, highlighting its potential for localized tumor production.	(133)
2004	*C.* perfringens	Breast	Immunodeficient Mice	• *In vitro* experiments demonstrate that breast cancer cell lines expressing CLDN 3 and 4 undergo rapid and dose-dependent cytolysis upon treatment with CPE. *In vivo,* experiments using xenografts of breast cancer cells show a significant reduction in tumor volume and necrosis following intertumoral administration of CPE. Additionally, primary breast carcinoma samples treated with CPE exhibit necrotic reactions, and isolated carcinoma cells undergo complete cytolysis within a short duration. These findings highlight the sensitivity of primary breast carcinomas expressing CLDN 3 and 4 to CPE-mediated cytolysis and suggest the potential of CPE in breast cancer therapy.	(134)
2005	*C.* perfringens	Ovarian	Mice	• In this study, chemotherapy-resistant/recurrent ovarian tumors showed significantly higher expression levels of claudin-3 and claudin-4 genes compared to chemotherapy-naive ovarian cancers. *In vitro,* experiments demonstrated that primary ovarian tumors, regardless of their resistance to chemotherapy, were highly sensitive to CPE-induced cell death within 24 hours of exposure to 3.3 microg/mL CPE. Additionally, researchers evaluated the therapeutic efficacy of intraperitoneal (i.p.) CPE administration in a clinically relevant mouse xenograft model of chemotherapy-resistant ovarian cancer. Repeated i.p. doses of sublethal CPE significantly inhibited tumor growth and prolonged survival in mice harboring both early-stage and advanced-stage ovarian tumors. These findings suggest that CPE has the potential to be a novel treatment for chemotherapy-resistant/recurrent ovarian cancer.	(135)

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2006	*C. sporogenes* NCIMB 10696	Human colorectal carcinoma	Mice	• In this study, the researchers aimed to enhance the potential of clostridia as gene delivery vectors for targeted tumor therapy. They focused on improving the efficacy of recombinant Clostridium species by evaluating different nitroreductase (NTR) enzymes for their ability to convert the CB1954 prodrug into its toxic form. Among the tested enzymes, one showed superior characteristics in activating the prodrug. Additionally, they developed an efficient gene transfer procedure using conjugation, allowing for successful genetic engineering of Clostridium strains with enhanced tumor colonization properties. Using this procedure, they created a recombinant *C. sporogenes* strain overexpressing the selected NTR enzyme. To mimic a clinical setting, the researchers conducted multiple consecutive treatment cycles with antibiotic clearance between cycles. Importantly, the intravenous administration of spores from the NTR-recombinant *C. sporogenes* strain, combined with prodrug administration, demonstrated significant antitumor efficacy. These • findings highlight the potential of this approach for targeted tumor therapy using recombinant clostridia.	(136)
2007	*C. perfringens*	Uterine Serous Papillary Carcinoma	SCID Mice	• Findings showed that all primary flash-frozen USPC samples tested (20 out of 20) exhibited overexpression of one or both CPE receptors, as confirmed by RT-PCR. Immunohistochemistry analysis revealed membranous expression of claudin-4 protein in the majority of USPC specimens, while normal endometrial control tissue samples displayed minimal membranous staining. *In vitro,* experiments using primary and metastatic USPC cell lines demonstrated a dose-dependent cytotoxic effect when treated with various concentrations of CPE. *In vivo,* studies involving intratumoral injections of well-tolerated doses of CPE in large subcutaneous USPC xenografts resulted in extensive tumor cell necrosis and complete tumor regression in all treated animals. Similarly, sublethal intraperitoneal injections of CPE significantly inhibited tumor progression and prolonged the survival of animals with chemotherapy-resistant intra-abdominal USPC carcinomatosis. Based on these findings, claudin-3 and claudin-4 receptors hold promise as potential targets for CPE-based therapy in the treatment of this highly aggressive and chemotherapy-resistant variant of endometrial cancer, USPC.	(137)
2008	*C. sporogenes*	Human Cervical Carcinoma	Immunodeficient Nude Mice	• In a previous study, researchers showed that genetically modified spores of the non-pathogenic Clostridial strain *C. sporogenes*, expressing the E. coli-derived cytosine deaminase (CD) gene, could effectively convert systemically injected nontoxic 5-fluorocytosine (5-FC) into the toxic anticancer drug 5-fluorouracil (5-FU), leading to tumor-specific antitumor activity. In this new study, their aim was to improve the expression of E. coli-derived genes within this system.	

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2009	*C. perfringens*	Glioma	Inbred Male Fischer Rats	• Researchers employed non-tumor-bearing inbred Fisher rats and administered a photosensitizer followed by a non-toxic intraperitoneal dose of *Clostridium perfringens* epsilon prototoxin (ETXp) and subsequent light exposure. We monitored the degree of blood-brain barrier (BBB) disruption using post-contrast T1 MRI scans. Additionally, we implanted F98 tumor cells into the brains of other animals, which were treated 24 hours later with ETXp-PCI BBB opening, followed by the intraperitoneal administration of bleomycin (BLM). Findings indicate that photochemical internalization (PCI)-mediated BBB opening using ETXp was effective in a localized region of the brain. Furthermore, this BBB disruption significantly increased the efficacy of BLM therapy. By selectively opening the BBB in the targeted area, PCI holds promise for overcoming the limitations imposed by the BBB and improving the delivery and effectiveness of anti-tumor therapies for malignant brain tumors.	(138)
2010	*C. perfringens*	Ovarian	SCID mice	• The results of the study showed specific binding of the FITC-conjugated CPE peptide to multiple primary chemotherapy-resistant ovarian cancer cell lines both *in vitro* and *in vivo*. Biodistribution studies in mice with clinically relevant animal models of chemotherapy-resistant ovarian carcinoma revealed higher uptake of the peptide in tumor cells compared to normal organs. Confocal microscopy confirmed the presence of the peptide in discrete accumulations, such as tumor spheroids, and even in single chemotherapy-resistant ovarian cancer cells floating in the ascites of xenografted animals. Researchers also observed a time-dependent internalization of the FITC-conjugated CPE peptide in chemotherapy-resistant ovarian tumor cells. Based on the high expression levels of claudin-3 and claudin-4 in chemotherapy-resistant ovarian cancer, as well as in other aggressive epithelial tumors like breast, prostate, and pancreatic cancers, the CPE peptide shows promise as a potential lead peptide for the development of new diagnostic tracers or alternative anticancer agents.	(139)
2011	*C. Perfringens*	Ovarian	SCID Mice	• The results revealed significantly higher levels of the claudin-4 gene in $CD44^{+}$ ovarian cancer stem cells compared to autologous $CD44^{-}$ ovarian cancer cells. Despite the inherent resistance of these stem cells to chemotherapy, they exhibited sensitivity to CPE treatment, with cell death occurring within one hour after exposure to 1.0 μg/mL of CPE *in vitro*. To further validate the role of claudin-3/-4 in mediating the cytotoxic effects of CPE, they performed small interfering RNA (siRNA) knockdown experiments. The knockdown of claudin-3/-4 expression in $CD44^{+}$ cancer stem cells significantly protected them from CPE-induced cytotoxicity, suggesting that these tight junction proteins play a crucial role in CPE-mediated cell death. Importantly, *in vivo,* experiments using C.B-17/SCID mice harboring xenografts of chemotherapy-resistant $CD44^{+}$ ovarian cancer stem cells demonstrated the therapeutic activity of CPE.	(140)

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2012	*C. Perfringens*	Prostate	Mice	• The results of the study showed that both Cldn4 and Cldn3 were expressed in primary human prostate cancer tissues, as well as in the cell lines 22Rv1, DU145, and PC3. Cldn4 protein was also present in PrECs, but its distribution differed from that in cancer cells. While Cldn4 was distributed throughout the cell membrane of the cancer cell lines, it specifically localized to tight junctions in PrECs. CPE treatment induced significant cytotoxicity in PC3 cells, but minimal effects were observed in PrECs. Knocking down the expression of Cldn4 led to a substantial decrease in cytotoxicity in both PC3 and 22Rv1 cells, whereas the knockdown of Cldn3 did not have a significant impact. Furthermore, when CPE was injected around PC3 xenografts in mice, it significantly suppressed tumor growth.	(141)
2013	*C. butyricum* MIYAIRI 588	Bladder	C3H/HeN Mice	• Researchers stimulated PMNs or PBMCs with BCG or *Clostridium butyricum* MIYAIRI 588 (CBM588) and analyzed the culture supernatants or cell lysates for the presence of TRAIL. Their findings indicate that CBM588 can induce the release of endogenous TRAIL from PMNs, similar to BCG. They have also identified matrix metalloproteinase 8 (MMP-8) as one of the key factors responsible for this release. Interestingly, the Toll-like receptor 2/4 (TLR2/4) signaling pathway has been implicated in the release of TRAIL-mediated by MMP-8. Furthermore, their research demonstrates that CBM588 is as effective as BCG in inducing apoptosis in cancer cells both *in vitro* and in *vivo*.	(142)
2014	*C. Perfringens*	Prostate	Human Prostate Cancer Cell Lines: LNCaP, PC-3, and DU-145	• Prostate cancer cells produce prostate-specific antigen (PSA), which is an enzyme with proteolytic activity. However, when PSA is released into the bloodstream, it becomes inactivated due to its binding to protease inhibitors. To address this issue and minimize the potential systemic toxicity of CPE, scientists have developed a modified form of the toxin called a protoxin. The engineered protoxin is created by attaching a specific ligand to the C-terminus of the toxin. This ligand is connected to the toxin via a flexible linker that contains a cleavage site for PSA-specific proteases. As a result, the modified protoxin can selectively target and destroy prostate cancer cells that produce PSA, while cells expressing claudin 3 and claudin 4 but lacking PSA remain resistant to cytolysis.	(143)
2015	*C. sporogenes*	Colorectal	• CT26 Murine Colorectal Cancer Cells: ATCC CRL-2638 HCT116 • Human Colorectal Cancer Cells: ATCC CCL247	• The study examines the effects of the heat-inactivated form of the bacteria (referred to as IB) and the proteins secreted by the bacteria in culture media (known as conditioned media or CM) on CT26 and HCT116 colorectal cancer cells using both a 2-dimensional (2D) monolayer culture and a 3-dimensional (3D) spheroid culture. The results show that IB significantly inhibits the proliferation of CT26 cells, reducing it to only 6.3% of the control after 72 hours in the 2D culture. In the 3D spheroid culture, the proliferation of HCT116 spheroids is notably reduced to 26.2%. Similarly, CM also demonstrates a significant reduction in cell proliferation, with CT26 cells showing a decrease to 2.4% and 20% in the 2D and 3D models, respectively. This study establishes the inhibitory effects of inactivated *C. sporogenes* and its conditioned media on colorectal cancer cells. By utilizing these non-viable bacterial derivatives, researchers aim to overcome tumor hypoxia and provide a potentially safer more and effective approach for cancer therapy.	(144)

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2016	*C. Perfringens*	Bladder	Human BC RT4, 5637 and T24 cells	• The cytotoxicity of CPE was examined against bladder cancer (BC) cell lines and 3D cultures of cells derived from surgical samples. To gain a better understanding of the cellular mechanisms activated by CPE and to explore the use of a non-toxic fragment of CPE (C-CPE) in combination with other drugs, C-CPE was synthesized. Its cytotoxic activity was compared with that of CPE, and the expression and intracellular localization of claudin 4 was examined after C-CPE treatment. Results showed that CPE induced cell death within 1 hour in low-aggressive RT4 cells, moderately aggressive 5637 cells, and primary 3D cultures of BC cells derived from NMIBC. However, non-transformed urothelial cells and cells derived from highly aggressive tumors (T24) survived this treatment. The resistance of these cells to CPE may be due to lower expression of claudins or their inaccessibility to CPE. Treatment with C-CPE for 48 hours did not affect cell viability but reduced CLDN4 expression in RT4 cells. C-CPE also increased the sensitivity of RT4 cells to Mitomycin C and Dasatinib.	(145)
2018	*C. difficile*	Breast	Mice	• In this study, the results showed that *Clostridium difficile* toxin B (rcdtB) significantly induced cell death in MDA-MB-231 cells, inhibited cell growth, and decreased the proportion of cells in the S-phase compared to the normal control group. rcdtB also significantly induced both early and late apoptosis and reduced Bcl-2 levels compared to the normal control group. Additionally, rcdtB significantly inhibited cell migration and tumor growth and activated inflammation in the breast cancer mouse model compared to the normal control group. Furthermore, rcdtB significantly reduced the expression of C-erbB-2 and Cox-2 in tumor tissues compared to the normal control group.	(146)
2019	*C. sporogenes*	Lung	Mice	• The study highlights the anticancer effectiveness of methionine □-lyase (MGL) derived from *Clostridium sporogenes*. MGL exhibited activity against cancer cells both *in vitro* and *in vivo*. The combination of MGL and doxorubicin (DOX) demonstrated enhanced efficacy in inhibiting the growth of A549 human lung cancer cells compared to either agent alone, both *in vitro* and *in vivo*.	(147)
2020	*C. butyricum* MIYAIRI 588	Non-Small Cell Lung Cancer	-	• In this study, the researchers hypothesized that probiotic *Clostridium butyricum* therapy (CBT) may influence the effectiveness of immune checkpoint blockade therapies. They conducted a retrospective analysis of 118 patients with advanced non-small cell lung cancer. Survival analysis was performed, comparing patients who received CBT before and/or after immune checkpoint blockade (ICB) treatment. The results of the propensity score analysis confirmed that probiotic CBT significantly improved both progression-free survival (PFS) and overall survival (OS) rates. Notably, the positive impact of probiotic CBT on PFS and OS was observed even in patients who had received antibiotic therapy. These findings suggest that probiotic CBT may enhance the therapeutic efficacy of immune checkpoint blockade in cancer patients.	(148)

Year	Clostridium spp.	Cancers	Animal Model or Cell Line	Therapy Mechanisms	Ref
2021	*C. novyi*	Breast	Mice	• To begin, the lethal toxin gene in *C. novyi* type B was eliminated. Colonies were isolated and confirmed using PCR testing. Plasmid extraction and *in vivo* assays were conducted to ensure the removal of the alpha-toxin. Subsequently, a single dose of non-toxic *C. novyi* spores was administered to treat breast cancer models of varying tumor sizes. The results indicated that the non-toxic *C. novyi* strain had lost its lethal toxin and appeared to be safe for use. In the case of tumors smaller than 1000 mm3, a single dose of non-toxic *C. novyi* spores resulted in a complete cure of breast tumors in 100% of the mice. Furthermore, the mice remained tumor-free without relapse. However, tumors larger than 1000 mm3 were not effectively cured by a single dose of non-toxic *C. novyi* treatment. The study concluded that non-toxic *C. novyi* could be a suitable and safe candidate for a novel therapeutic approach to target hypoxic regions found within the central areas of tumors.	(149)
2022	*C. butyricum*	• Clone • Lung • Liver	Cell Lines: • HT-29 & HCT 116 • A549 • HepG2	• In this study, the researchers investigated the *in vitro* effects of two probiotic strains, *Lactobacillus sporogenes* and *Clostridium butyricum* (referred to as PBT), individually or in combination with the chemotherapy drug 5-fluorouracil (5FU), against colon (HT-29 and HCT 116), lung (A549), and liver (HepG2) cancer cell lines. The objective was to assess the potential anticancer activity of these probiotic strains. The study also examined the underlying mechanism of action of PBT and PBT-5FU combination treatment against the HT-29 colon cancer cell line. Using Hoechst 33342 staining, the researchers observed characteristic apoptotic changes in the cells, including chromatin condensation, nuclear fragmentation, and membrane blebbing. Additionally, the expression levels of various pro-apoptotic (Bax, Bid, Bad, and Bak) and anti-apoptotic (Bcl-2 and Bcl-XL) proteins were analyzed. The results showed an increase in the expression of pro-apoptotic proteins and a decrease in the levels of anti-apoptotic proteins.	(150)
2023	*C. butyricum*	Colorectal	Mice	• Researchers initially discovered a positive correlation between methyltransferase-like 3 (METTL3) and various CRC-related processes, including proliferation, epithelial-mesenchymal transition (EMT), DNA repair, metastasis, and invasion. They conducted experiments wherein we overexpressed METTL3 in CRC cells. This overexpression resulted in enhanced proliferation, migration, invasion, and the formation of vasculogenic mimicry (VM) structures. However, treatment with *C. butyricum* led to the downregulation of METTL3 expression in CRC cells, along with reduced expression of vimentin and vascular endothelial growth factor receptor 2, ultimately suppressing EMT and VM formation. Additionally, *C. butyricum* counteracted the pro-oncogenic effects induced by METTL3 overexpression in CRC cells. To explore the specific mechanism involved, they investigated the role of G-protein coupled receptor 43 (GPCR43), which is a downstream effector of butyrate signaling. They found that knocking down GPCR43 attenuated the anti-EMT effect mediated by METTL3 reduction in response to *C. butyricum* treatment. Furthermore, in a mouse model, *C. butyricum* prevented EMT and VM formation and inhibited tumor metastasis.	(151)

6. Current advances, Challenges and Future Directions in Bacterial Therapy

The Bacteria Ghost (BG) is a novel technology used to deliver drugs, vaccines, and DNA via delivery vectors. BGs are non-living, empty bacteria surrounded by an envelope of gram-negative bacteria, achieved through controlled expression of the cloned lysis gene. These E.BGs lack cytoplasmic content, including chromosomal and plasmid DNA, but retain their cellular surface structure, including the outer membrane, inner membrane, and peptidoglycan.

The gene E's function in the lysis of Escherichia coli was extensively studied and understood in 1966. It was the first lethal gene in bacteria that could be silenced within the plasmid. Gene E encodes a membrane protein that forms a transmembrane tunnel structure through oligomerization. The N-terminal end of protein E contains a hydrophobic region that integrates co-translationally into the cytoplasmic membrane of E. coli. The analysis of protein E's hydropathicity regions revealed an E-specific lysis tunnel spanning the inner membrane (IM) and outer membrane (OM) adhesion sites within the host cell. The process of E-mediated tunnel formation follows a three-phase model as described by Schön et al. Firstly, protein E is integrated into the IM with the C-terminal region. Secondly, conformational changes in protein E lead to the translocation of the C-terminal domain to the periplasmic space, accompanied by oligomerization. Lastly, the fusion of IM and OM at the membrane adhesion sites is initiated by the exposure of the C-terminus of protein E to the cell surface. This lysis process causes the release of the entire cytoplasmic content and the drug into the host cell, while the periplasmic content remains in the empty cell envelope (118,119).

Severe toxicity remains a significant challenge in cancer chemotherapy, despite advancements in the pharmaceutical industry. The need for high-dose administration of chemotherapeutic agents arises from their low specificity towards cancer cells, rapid clearance, and non-specific drug distribution, leading to adverse side effects. To address this, targeted drug delivery systems (DDSs) have been developed, involving the use of liposomes, polymers, and nanoparticles to encapsulate chemotherapeutic agents and target tumor cell receptors. This approach enhances specificity to cancer cells while reducing the side effects of chemotherapy (120). Researchers have explored the potential of using bacteria as carriers for chemotherapeutic agents in cancer therapy, with a focus on targeting primary and metastatic tumors. A new technology involves packaging cytotoxic agents into nanosized particles derived from bacteria, known as minicells. These minicells, ranging from 100 to 400 nm in diameter, offer a promising approach for intracellular and selective targeted delivery to cancer cells (121). Minicells were first described in 1967 by Howard Adler and colleagues. They are non-living, nanosized cells that lack a nucleus and are produced through mutations in genes that regulate bacterial cell divisions, preventing cell fission. Initially used for studying various cellular processes, such as isolating bacterial cell plasmid DNA and protein synthesis, minicells have now gained attention for their potential in drug delivery and immune-activating vaccines. Importantly, minicells retain the virulence properties of their parent cells but cannot proliferate (122).

Despite facing various challenges like complex production, limited stability, side effects, and mutations, several bacterial drug delivery systems for cancer treatment are currently undergoing clinical trials. Among various bacterial species, Listeria vaccine strains have shown promising results either alone or in combination in human trials. For example, the S. typhimurium VNP20009 strain, designed by Vion Pharmaceutics, was tested on 24 patients with metastatic melanoma in a phase I trial. Although the maximum tolerated dose was identified at 3.0×108 CFU/m2 after intravenous administration, there was no objective tumor regression. However, there was an increase in proinflammatory cytokines (123,124). In another trial involving four patients with metastatic melanoma treated with S. typhimurium VNP20009, no objective tumor response was observed. Consequently, the VNP20009 strain was modified to express E. coli CD to enhance therapeutic efficacy. This modification allowed the expressed E. coli CD to convert 5-FC to toxic 5-FU. Patients suffering from oesophageal adenocarcinoma and neck squamous carcinoma were treated with these modified bacteria via intratumoral injection and oral route for multiple cycles, three times a day. The results indicated that bacterial colonization at the tumor was three times higher compared to non-colonized patients, and no significant adverse effects were reported after six cycles of treatment. However, there were discrepancies observed between clinical (human model) and preclinical (animal model) outcomes. These discrepancies could be attributed

to the lack of lipid A function, as discussed earlier. Despite the challenges and setbacks, ongoing clinical trials of bacterial drug delivery systems hold promise for the future of cancer treatment. Efforts to optimize bacterial strains and delivery methods continue to be explored in the hope of achieving more effective and targeted cancer therapies (125,126).

On the other hand, the application of Clostridium novyi-NT spores has progressed to Phase I clinical trials, showing promising therapeutic effects when administered via intratumoral injection. The treatment led to extensive tumor destruction through the formation of gas pockets by C. novyi-NT spores. However, it was observed that Clostridium cells were unable to completely eradicate all cancerous cells, resulting in tumor relapse. To address this issue, a clinical trial was initiated, combining the C. novyi-NT strain with pembrolizumab, to treat patients with refractory advanced solid tumors. By combining these treatments, researchers aim to enhance the efficacy of the therapy and improve the outcomes for patients Similarly, bacterial minicells designed to deliver paclitaxel into cancer cells were evaluated for safety in a human phase I clinical trial on patients with advanced solid tumors. The patients received five weekly infusions of the minicells, and the study followed a dose escalation design with seven dose levels to determine safety, tolerability, and antitumor effects. Common treatment-related adverse events reported were rigors and pyrexia, but fortunately, no deaths occurred during the treatment. The results indicated that bacterial minicells are safe to use and demonstrated modest clinical efficacy. These clinical trials showcase the potential of bacterial-based therapies for cancer treatment, and ongoing research and refinement of these approaches hold promise for improving the outcomes of patients with advanced solid tumors (127,128).

Using bacteria as therapeutic or delivery agents in cancer treatment presents various challenges, particularly related to adverse effects and toxicity. Introducing live, attenuated, or genetically engineered bacteria into the human system may lead to systemic infections, especially in immunocompromised patients. This can cause undesired biodistribution of bacteria, leading to off-target effects and the release of anticancer drugs in normal cells instead of tumor cells The success of cancer therapy with bacteria depends on the extent of toxin uptake and its selectivity for tumor cells. Biotechnological advances have shown that non-pathogenic bacteria and their products can induce tumor cell necrosis. Some strains of viruses carrying altered genes and certain bacterial species expressing therapeutic proteins have been found to selectively replicate in malignant cells. However, their effectiveness is hindered by the human body's ability to fight and eliminate these microorganisms and their anti-tumor effects by producing neutralizing antibodies (129).

Another significant issue with bacterial drug delivery is the lack of a comprehensive understanding of the exact mechanism of preferential tumor colonization by bacterial cells with innate anti-tumor effects. Clinical studies have shown insufficient bacterial colonization, possibly due to robust immune reactions countering bacterial accumulation. Rapid clearance of bacteria from the blood before reaching the tumor site and contrasting results between clinical and preclinical data on bacterial maintenance in the bloodstream for a longer duration have also been observed. Additionally, bacteria have limited cytotoxic properties, necessitating their conjugation with chemotherapeutic agents. Genetic modification poses a risk of mutation, which needs to be addressed to utilize bacteria as potential therapy in cancer patients (130). On the other hand, Bacteria Ghosts (BGs) also have limitations as a delivery system. Drugs weakly bound to bacterial membranes, membrane proteins, and cell walls can cause significant drug loss due to the large pores on the BG surface. BGs may induce potent proinflammatory cytokines among immune cells, making them unsuitable for use in immunocompromised patients. Bacterial clearance, systemic infection, mutation, and toxicity can also compromise their therapeutic effects. The physiochemical and structural complexity of BG formulations is another factor contributing to the slow pace of their clinical transition as a bacterial drug delivery system, with complex fabrication procedures and scale-up limitations in large-scale manufacturing (131).

7. Conclusion

In conclusion, Clostridium bacteria hold great promise in the field of cancer treatment. Their immunomodulatory effects, anti-inflammatory properties, and potential to enhance drug efficacy make them an exciting area of research. As we continue to unravel the intricate

relationship between bacteria and cancer, it is clear that Closterium bacteria have the potential to revolutionize cancer treatment and improve patient outcomes. Further research and clinical trials are needed to fully understand the mechanisms and therapeutic applications of these remarkable bacteria. As the research continues, the potential of Clostridium bacteria as a natural and effective treatment for cancer is becoming increasingly clear. The implications of this research are immense and could revolutionize cancer treatment as we know it.

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Ethical approval statement

This study does not need any ethical approvals.

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Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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