Review

Bacterial-Based Cancer Therapy (BBCT): Recent Advances, Current Challenges, and Future Prospects for Cancer Immunotherapy

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Abstract: Currently approximately 10 million people die each year due to cancer, and cancer is the cause of every sixth death worldwide. Tremendous efforts and progress have been made towards finding a cure for cancer. However, numerous challenges have been faced due to adverse effects of chemotherapy, radiotherapy, and alternative cancer therapies, including toxicity to non-cancerous cells, the inability of drugs to reach deep tumor tissue, and the persistent problem of increasing drug resistance in tumor cells. These challenges have increased the demand for the development of alternative approaches with greater selectivity and effectiveness against tumor cells. Cancer immunotherapy has made significant advancements towards eliminating cancer. Our understanding of cancer-directed immune responses and the mechanisms through which immune cells invade tumors have extensively helped us in the development of new therapies. Among immunotherapies, the application of bacteria and bacterial-based products has promising potential to be used as treatments that combat cancer. Bacterial targeting of tumors has been developed as a unique therapeutic option that meets the ongoing challenges of cancer treatment. In comparison with other cancer therapeutics, bacterial-based therapies have capabilities for suppressing cancer. Bacteria are known to accumulate and proliferate in the tumor microenvironment and initiate antitumor immune responses. We are currently well-informed regarding various methods by which bacteria can be manipulated by simple genetic engineering or synthetic bioengineering to induce the production of anti-cancer drugs. Further, bacterial-based cancer therapy (BBCT) can be either used as a monotherapy or in combination with other anticancer therapies for better clinical outcomes. Here, we review recent advances, current challenges, and prospects of bacteria and bacterial products in the development of BBCTs.

Keywords: bacterial therapy; bacteriotherapy; tumor targeting bacteria; cancer immunotherapy; therapeutic bacteria

1. Introduction

Almost a century since Dr. William Coley made his first attempt to use bacterial products as immunotherapy, the use of live, attenuated bacteria has become a promising alternative to combat cancer [1,2]. Ongoing progress in understanding various molecular and cellular immunology of bacterial physiology associated with host–pathogen interactions has helped in the design of attenuated bacteria as conventional vaccine vectors [3]. In recent years, with developments in technology and our ability to attenuate pathogenic strains, research has been mainly focused on biochemical and molecular techniques by which bacteria can be manipulated in the fight against cancer. Bacteria have been of particular interest due to their natural motile ability, which allows them to move away from the

vasculature and penetrate hypoxic regions of the tumor [4] and subsequently proliferate within tumor cells [5]. This solves the problem commonly faced by chemotherapeutics where they reach mainly the vascularized outside edges of the tumor but not the hypoxic core. Additionally, bacteria can be genetically modified to carry and express therapeutic proteins and tumor-associated antigens (TAAs), deliver genes, or transport chemotherapeutic molecules [6]. Direct delivery of drugs via bacteria to the tumor site enhances specific cancer-targeting therapies and limits the negative effects of treatment [7]. Alternatively, bacteria can also be harnessed to produce drugs within tumor cells, essentially manufacturing therapeutic molecules on site [8]. While our insights into cancer-specific treatments have vastly increased, there is still room for finding better targets for cancer therapies, possibly through the manipulation of known microorganisms [9]. Various bacterial species have proven useful in harnessing antitumoral immunity by initiating innate and adaptive immune responses in pre-clinical and clinical studies, which has increased the chances of tumor elimination without additional secondary side effects [10–12]. Microorganisms host a variety of mechanisms with potential in cancer therapy, many of which we have yet to be discovered and studied in detail. Recently, many bacterial therapeutics have been implemented in human clinical trials (phase I/II) [13–18]. In this article, we review recent advances in bacterial-mediated drug and delivery systems' discovery and discuss the benefits and current challenges in these serving as anti-cancer treatments. We also discuss how various pathogenic and non-pathogenic bacteria have been genetically manipulated to induce tumor regression and the prospects of BBCT.

2. Bacterial Components and Products Targeted via BBCT

Advancement of BBCT lies in focusing on cancer cells by different mechanisms that target a specific bacterial component or machinery. These mechanisms responsible for anti-cancer activity include targeting the tumor microenvironment, secretion of cytotoxic agents, manipulating bacterial virulence agents, and engineered bacterial vectors for the expression and release of tumoricidal proteins. Figure 1 depicts the overall mechanistic overview of BBCT.

2.1. Bacterial Targeting of the Tumor Microenvironment

One of the main driving factors for the use of bacterial-targeted drug delivery is related to the ability of anaerobic species to thrive in hypoxic tumor cores [19]. The tumor microenvironment is characterized by oxygen concentrations ≤ 10 mmHg [20]. Additionally, acidity primed by lactic acid results as a byproduct of metabolism of anaerobic bacteria because of decreased oxygen [21]. Further, the tumor microenvironment has increased tissue necrosis, resulting from tumor cell death due to the lack of nutrients and uncontrolled growth [4]. Hypoxia is a trademark of quickly proliferating solid tumors, a characteristic attributed to their expanding beyond the available blood supply [22]. The structure of blood vessel vasculature is functionally abnormal in tumors, resulting in irregular blood flow throughout the tissue, leading to oxygen deprivation [23]. The hypoxic condition forces tumors to develop adaptive genetic changes that withstand hypoxia-induced cell death and tissue necrosis [24]. The hypoxic tumor region is known to be associated with a higher expression of MDR1 (a multidrug-resistant gene) and P-glycoprotein genes, which are responsible for the development of multidrug resistance to various anticancer drugs [25].
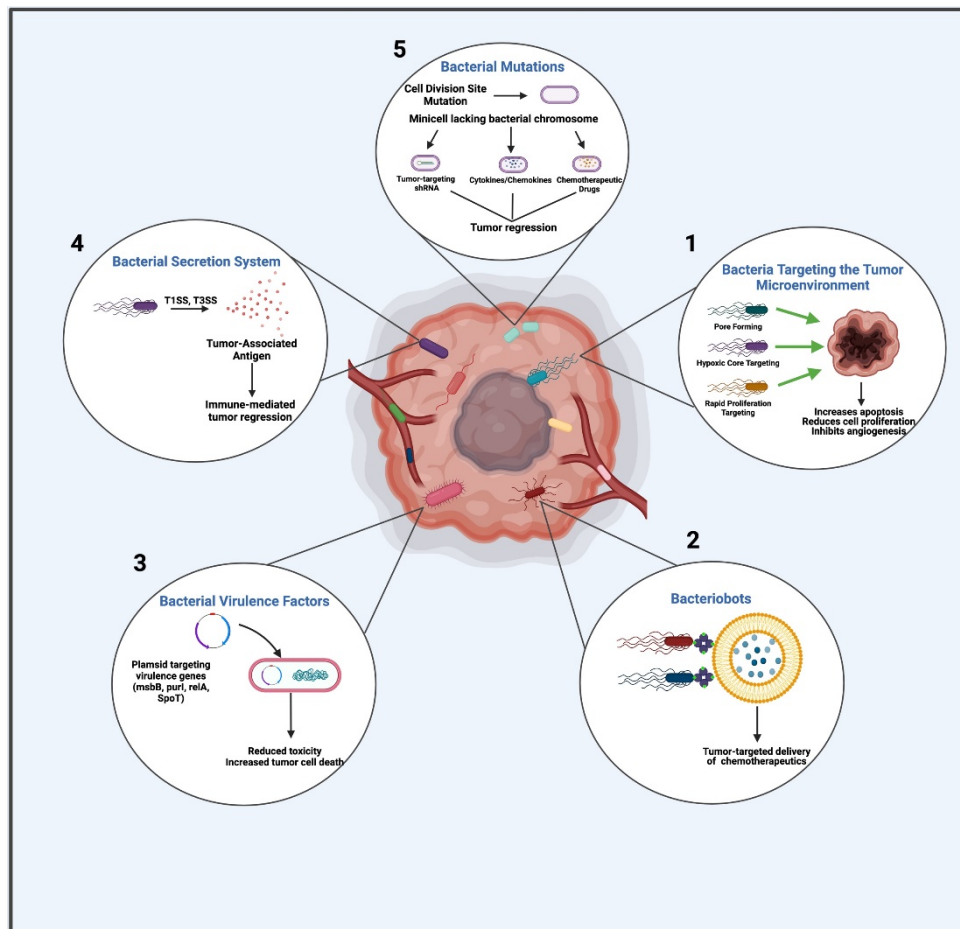
Figure 1. Schematic summary of the various bacterial mechanisms utilized in BBCT. (1) Anaerobic facultative bacteria specifically target the hypoxic environment of tumors initiating an inflammatory reaction resulting in tumor destruction. (2) Bacteriobots for cancer therapy, which involve targeting controlled drug release, improved cell adhesion, and improved penetration into the cell. (3) Bacterial virulence factors (e.g., msbB, purl, relA, SpoT) can be bioengineered to reduce toxicity and increase tumor cell death. (4) Bacterial toxins, such as the bacterial secretory system (T1SS and T3SS), can be used to inhibit the growth of solid tumors. (5) Bacterial mutations help delivery of immunomodulators such as cytokines, chemokines, and small molecules along with immune checkpoint antibodies, which can stimulate anti-tumor responses. This figure was created using Biorender.com.

However, the hypoxia caused by these poorly organized blood vessels creates a unique niche for anaerobic bacteria to flourish [9]. Therefore, areas of tumors that before were most resistant to chemotherapy can now be specifically targeted through the use of microorganisms as drug and gene delivery systems [26]. It has been shown that growth and survival of bacteria in tumors is dependent on their mechanisms for motility and survival, as well as their level of dependence on oxygen [27,28]. It has been previously demonstrated that *Salmonella* sp. and *Clostridia* sp. preferentially target and replicate in the core anaerobic zone of tumors [29] (the specific targets of these bacteria are discussed further in this review). Thus, bacteria pose a possible solution to the issue of specificity in drug and gene delivery of cancer therapy.

2.2. Bacteriobots

"Bacteriobots" are devices designed to use bacteria as microactuators and microsensors to deliver various types of chemotherapeutics and other therapeutic compounds to the inner and invasive layers of the tumor [30,31]. Bacteriobots are designed to regulate the speed and migration to direct the chemotaxis of bacteria towards the tumor site. The targeted tumor is attacked by bacteriobots that adhere to the cancer cells and are engineered

to secrete anti-tumor agents, further destroying the tumor [32]. Park et al. demonstrated motility of bacteriobots constructed by measuring the binding of biotin displayed on the outer membrane proteins of the bacterium *S. Typhimurium* and streptavidin, which was coated on the surface of drug-loaded liposomes [33]. Various other bacteria such as *S. marcescens E. coli*, magnetotactic bacteria, and *S. Typhimurium* have been employed to develop bacteria-based microbots. Their clinical applications, however, are limited due to high pathogenicity and acquired antibiotic resistance, as well as difficult expansion and specific nutritional requirements. However, soon we may expect to have bacteriobots designed with a tumor-targeting bacteria, for use as a biomedical and clinical microrobot for cancer diagnosis and therapy.

2.3. Bacterial Virulence Factors

Virulence factors are cellular structures, molecules, and regulatory systems that enable microbial pathogens to achieve colonization and growth within the host, immune evasion, and immunosuppression, as well as entry and exit out of cells and extraction of nutrition from cancer cells [34,35]. Thus, it is highly essential to normalize the bacterial virulence against the host immune system. However, some virulence factors can be responsible for the anti-tumor response, and, thus, deleting or manipulating these virulence factors can reduce anti-cancer effects of the bacteria. Thus, it is important to attenuate a strain without altering the anti-tumor activity. *Salmonella Typhimurium* strain VNP20009, which has been broadly studied for its anti-tumor specificity, is altered by deleting major virulence genes, including *msbB* and *purI* [36]. Deletion in *msbB* gene leads to myristoylation of the lipid A component of LPS, which induces TNF production and can reduce the risk of sepsis. Mutations in other genes like *rfaG* and *rfaD* result in the production of truncated LPS in the host, which in turn leads to the reduction of toxicity and generates a productive anti-tumor response [37]. Mutants made by deleting relA- and SpoT from *Salmonella* spp. are impaired in the synthesis of ppGpp, a signaling molecule known to be involved in gene expression stringent response in bacteria; however, the mutant strain exhibits less toxicity. The ΔppGpp strain is known to have anti-tumor responses and to activate inflammasome NLRP3 and IPAF as well as the expression of many pro-inflammatory cytokines [38].

Listeria monocytogenes' cytotoxicity can be altered by deleting genes that are involved in cell invasion. *Hyl* gene deletion can cause defects in phagolysosome release [39,40]; and mutation in gene *actA* or *ActA* PEST-like sequences abrogates intracellular diffusion [41,42], and mutant strains *inlA* and *inlB* lack properties of invasion [43,44]. *Clostridium* spp. infection induces a variety of secreted toxins, such as actin-specific ADP-ribosyltransferase, hemolysins, phospholipases, and other pore-forming toxins, which interfere with intracellular functions [45,46].

2.4. The Bacterial Secretion System

Bacteria employ secretion systems to transport virulence proteins, which can be manipulated and exploited for novel cancer treatments. Essentially, this involves signaling molecules that are necessary for delivery in a bacterial secretion system and then fusing therapeutic molecules to them for more efficient and targeted drug delivery [47]. A secretion system that is commonly taken advantage of in cancer therapy is the type III secretion system (T3SS), which acts by directly injecting bacterial proteins into the host cell cytoplasm [48]. The efficacy of T3SS for drug delivery has been the focus of several studies, genetically fusing T3SS with tumor-associated antigen, Survivin, resulting in complete tumor regression [49,50]. The expression and release of TAA/TSA through type 1 (T1SS) secretion systems of *Salmonella Typhimurium* have also been studied [51]. Fensterle et al. showed that mice immunized with an *S. Typhimurium* strain release prostate-specific antigen (PSA) via the HlyA (T1SS) system, activate CD8+ T lymphocyte-mediated immune response, and ultimately inhibit tumor development [51,52]. The release of peptides from *Listeria monocytogenes* p60 protein simulates the tumor antigen through T3SS of *S. Typhimurium* in a murine model of fibrosarcoma, demonstrating that 80% of mice immunized with p60

peptide were protected after a fibrosarcoma tumor cell challenge [53,54]. A live strain of *Pseudomonas aeruginosa* has been genetically engineered to transfer *Yersinia (T3SS)* YopE and YopH protein via the T3SS into mammalian cells [55]. This strain generates CTL responses against invading tumors in vivo [55].

2.5. Bacterial Mutations

A variety of rod-shaped bacteria, comprised of both the Gram-positive and Gram-negative groups, have been demonstrated to produce minicells through abnormal cell division. These minicells have the same properties of a normal cell membrane, ribosomes, RNA, and protein, but they lack a bacterial chromosome [50]. By creating mutations in cell division machinery in common rod-shaped bacteria, such as *Escherichia coli* or *Salmonella enterica*, genetically modified minicells have been loaded with chemotherapeutic drugs [56]. Minicells remain an important potential advancement to drug delivery, primarily because they are unable to proliferate, yet retain virulence properties essential for tumor targeting.

On the other hand, gene transfer properties of bacteria have played an important role in their potential for therapeutic drug delivery. Studies have shown that intracellular bacteria are known to transfer genes to mammalian cells in both in vitro as well as in vivo settings. Several different kinds of bacteria have been studied and manipulated for their potential as gene delivery vectors, such as invasive *E. coli*, *Shigella*, *Listeria*, *Pseudomonas*, and *Salmonella*. Gene transfer occurs when attenuated bacteria release plasmid DNA into the cytoplasm of the host cells, which then culminates in the expression of the transfected genes at the cellular level [57]. This can be further targeted for silencing of genes that favor tumor growth through the use of RNA interference. This entails the transfer of small hairpin RNAs (shRNAs) encoded into a plasmid, which are then transfected in the cytoplasm into small interfering RNAs (siRNAs), and finally act to promote degradation of target mRNA in tumors. This process has been studied to some degree in *Listeria monocytogenes* and *S. enterica* ssp. Typhimurium-expressing targets, such as CTNNB1, Stat3, or Bcl2, all of which are implicated in tumor survival [56].

3. Pathogenic and Non-Pathogenic Bacteria in BBCT

These bacterial-based mechanistic strategies have been extensively studied using various pathogenic and non-pathogenic bacteria-targeting BBCT (Table 1).

3.1. Pathogenic Bacteria in BBCT
3.1.1. *Salmonella* spp.

Salmonella enterica serovar Typhimurium (S. typhimurium) is a Gram-negative bacterium that is responsible for causing gastroenteritis in humans [58]. *S. typhimurium* is known to be one of the most promising bacterial mediators of cancer immunotherapy, which can be easily manipulated. Thus, it has been engineered and designed in many studies that explore the bacterium as a cancer-targeting therapeutic. *S. typhimurium* has been further investigated in combination with other classical treatments such as chemotherapy or radiotherapy as a synergistic treatment within the tumor microenvironment [59]. *S. typhimurium* is also a popular target due to its ability to grow in both aerobic and anaerobic environments, and, therefore, able to colonize in both non-hypoxic and hypoxic tumors [60]. Salmonella has shown promise due to its ability to specifically proliferate at tumor sites [9]. While Salmonella is often used in cancer therapy for its immunostimulant effects, applications for its use as a therapeutic delivery vehicle are plentiful. For example, Loeffler et al. genetically engineered *S. typhimurium* to express either the proapoptotic Fas ligand or CCL21, a chemokine with anti-tumor properties, and utilization of both proteins has shown inhibition of primary tumors and reduction in metastases in in vivo breast cancer models [61]. Further, *S. typhimurium* has been engineered to produce TNF-related, apoptosis-inducing ligand (TRAIL) controlled by a prokaryotic radiation-inducible promoter, recA. As a natural inducer of apoptosis and tumor cell death, TRAIL is a desirable cytokine to be secreted as a cancer therapy. In vivo results from this model have revealed inhibition of mammary

tumor growth and substantially increased rates of survival [56,62]. Other genes, such as cytolysin (HlyE), have been successfully expressed in *S. enterica sv. Typhimurium* under the regulation of a hypoxia-inducible promoter. As a pore-forming toxin, cytolysin has been shown to be effective in murine mammary tumors when specifically targeted to hypoxic regions [9,56]. Yoon et al. investigated the possible anti-tumor properties of TNF-α encased in a Salmonella capsule. TNF-α is a well-known inflammatory factor and promoter of cancer, and, in this study, exhibited anti-tumor effects when assessed in an in vivo model of triple-negative breast cancer (TNBC) [63]. The positive results of these earlier studies have led researchers to further investigate the treatment delivery potential of Salmonella more extensively in animal models.

In their study, Li et al. showed that plasmids co-expressing ENDO-VEGI151 and sur-vivin siRNA have successfully been transferred into an attenuated strain, *S. typhimurium* SL7207. Both genes show promise for use in cancer treatment, as survivin is an apoptosis inhibitor and ENDO-VEGI151 is a promoter of anti-angiogenesis. This treatment demonstrated an inhibition rate of over 90% in a mouse model with a xenografted human cancer tumor [64]. However, these are not the only genes that have been explored in animal experiments utilizing *S. typhimurium*. In the 4T1 TNBC mouse model, *S. typhimurium* was manipulated to express and secrete TGFα-PE38, a potent immunotoxin. There are several components to TGFα-PE38, including transforming growth factor alpha (TGFα) and epidermal growth factor receptor (EGFR). PE38 has been used for its general cytotoxic properties; however, EGFR is specific to treatment in cancer due to the finding that approximately half of TNBCs and inflammatory breast cancers (BCs) overexpress this gene, demonstrating inhibition in the growth of solid tumors [65]. In a recent publication, Mansour et al. used *Salmonella typhimurium* VNP-20009 (VNP) to deliver polypeptide Laz, which is inherent to the Neisserial group of bacteria. Polypeptide Laz crosses the blood–brain barrier during Neisserial infections. However, in this study authors limited their system to express the therapeutic protein Laz at the hypoxic tumor region under hypoxia-induced promoter, HIP-1, selecting Laz upstream for targeting hypoxic tumors [66].

A Salmonella strain (KST0650) was developed as an oxytolerant-attenuated variant from the parental strain KST0649 (ΔptsIΔcrr), via the application of radiation mutation technology (RMT). This newly developed strain was shown to have a 20-times-higher replication rate in cancer cells lines (CT26) and was comparatively less virulent than the parental strain KST0649 [67].

While Salmonella has a plethora of characteristics desirable for targeted cancer treatment delivery, there are many considerations to keep in mind due to the major role of Salmonella in food-borne illnesses, and, thus, the fear triggered by it. While technology has advanced to the point that we now understand bacteria enough to manipulate and attenuate them for alternative uses, there is still much left to be understood and uncovered about the role of Salmonella in cancer treatment before it can become a mainstay in therapy.

3.1.2. *Escherichia* spp.

Escherichia coli (*E. coli*) has been engineered and utilized for a variety of uses in science and medicine, with cancer treatment being no exception. Like Salmonella, intravenously administered *E. coli* has been proven to have the ability to target and colonize hypoxic regions of tumors. Genetically engineered *E. coli* strain K-12 secretes cytolysin A (ClyA) and has been administered as a single treatment intravenously in mice with CT26 colon carcinoma, 4T1 metastasizing TNBC, and B16 melanoma tumors [68]. *E. coli* and *S. enterica* are known to produce hemolytic protein ClyA, a 34 KD protein, which induces apoptosis through its pore-forming activity. In this study, administration of *E. coli K-12*-expressing ClyA significantly decreased tumor growth rates initially, but, later, tumor growth progressed. This outcome may be improved by providing subsequent doses of treatment or combining it with other therapies. *E. coli* was further used as a surrogate to produce another bacterial toxin, a pore-forming protein (α-hemolysin) gene from *Staphylococcus aureus* (SA). Within 24 h, α-hemolysin was released and resulted in 93% cell death, with 4T1 tumor volume reduced to only 9%

viable tissue [8]. While these studies are promising, research is still just brushing the surface on the implications of *E. coli* in drug delivery. Chiang et. al. have shown the role of bio butyrate in bacterial cancer therapy, by metabolically engineering Escherichia coli 1917 (EcN) to synthesize butyrate, resulting in the EcN-BUT strain [69].

More recently, *E. coli* has been reassessed in cancer treatment in a variety of breast and other cancer models. In 2018, Zhang et al. looked at the use of *E. coli* Nissle 1917 (EcN), because of its known ability to infiltrate the barrier of tumors and replicate in the tumor area, which is between necrotic and viable tissue [70], to produce minicells. Here, the minicells were loaded with doxorubicin, a common chemotherapy drug that inhibits cancer cell division by blocking the enzyme topoisomerase. Additionally, EcN was manipulated to display pHLIP, an insertion peptide used to deliver chemotherapeutic drugs without the need for further modification. Minicells were shown to effectively kill MCF7 and 4T1 cells in vitro and further were shown to be successful in the penetration of hypoxic and necrotic tumor tissue in mice challenged with 4T1 cells. In other work, *E. coli* has been engineered to release a single-domain antibody (nanobody) targeting CD47 within the tumor. CD47, alternatively known as integrin-associated protein (IAP), is a transmembrane protein with many functions, one of which is to help dispose of diseased or aged cells. This treatment was utilized in several in vivo cancer models, including 4T1 TNBC, B16 melanoma, and A20 murine lymphoma, and was shown to reduce rapid tumor progression and increase levels of tumor-infiltrating T cells [71].

3.1.3. *Listeria* spp.

One of the most popular vectors for cancer immunotherapy is Listeria monocytogenes, a Gram-positive, facultative anaerobic bacterium. Listeria is mostly known for its association to food-borne illness; however, many of the characteristics that make Listeria pathogenic are the same ones that are now being engineered for use as delivery systems in cancer therapy [72]. Listeria can hijack mechanisms of the host cell cytoskeleton to remain intracellularly mobile and spread from cell to cell [73]. It has been suggested that the use of Listeria may allow treatments to migrate deeper into tumors than with other microbial species, possibly due to their innate ability to evade the phagolysosome and assist in the delivery of plasmid DNA into the cytoplasm [56,74]. Listeria has been engineered in a variety of ways to achieve this end goal, including the early investigation of *L. monocytogenes* paired with nanoparticles, which were shown to effectively express GFP in solid human tumors [75]. Their tumor-targeting properties were demonstrated in in vivo tumors, where *L. monocytogenes* was shown to invade and proliferate in tumors, to ultimately deliver therapeutic genes [76]. Understanding this potential, *L. monocytogenes* was then paired with tumor-associated antigens (TAAs) for enhanced specificity, such as MAGE-B, which is of particular interest in breast cancer because of its frequency of expression in human breast cancer biopsies. This has also been assessed in 4T1 TNBC, where it is confirmed that MAGE-B treatment reduces metastases and promotes tumor cell death in vivo [77,78].

While Listeria possesses several characteristics with potential benefit, one of the most important to note is the presence of the pore-forming protein, listeriolysin O (LLO). LLO helps to ensure the transit of DNA molecules from endosomes into the cytoplasm of target cells. The effectiveness of LLO in relation to drug delivery has been examined in various manners. This has been used in a two-component system, where a neutral HER2-targeting liposome is attached to LLO, combined with condensed plasmid DNA with cationic polyethylene glycol (PEG) and modified polylysine (PL/DNA). When targeted to an endosome, LLO is able to disturb the integrity of the endosome, resulting in cytoplasmic delivery and expression of plasmid DNA. Ultimately, this culminates in increased expression within HER2-positive breast cancer cells lines [79]. Alternatively, polylacticcoglycolic acid (PLGA) microspheres have been incorporated with LLO to optimize cytosolic delivery to target cells and subsequent presentation to the immune system [80]. It has been shown that phagocytic cells readily take up the combination of microspheres with LLO, which consequently results in an increase in peptide-MHC-I expression on the

cell surface. Additionally, cytotoxic T cells have been stimulated through the activation of a T hybridoma cell line through treatment with microspheres and LLO.

Further, LLO has been used with specific anti-tumor therapies to evaluate efficacy in cancer treatment. In one study, LLO was linked to a luciferase-encoding PEGylated polylysine core disulfide in combination with the monoclonal antibody, trastuzumab. In this system, LLO was necessary to establish transit of DNA molecules into the cytoplasm while trastuzumab allowed targeting of HER2 receptors in breast cancer. Treatment in MCF7 and MCF7/Her18 breast cancer cell lines demonstrated increased expression of luciferase activity, indicating successful gene delivery into tumor cells [81]. More recently, Listeria has been investigated as a possible source for the generation of drug-delivering nanoparticles. Functional nanoparticles have been produced from self-assembling *Listeria innocua* DNA binding protein (LiDps) in starved cells, and then further manipulated with the addition of Gaussia princeps luciferase and Zinc(II)-protoporphyrin IX (ZnPP). Tumorigenic cells have shown effective uptake of Gluc-LiDps-ZnPP conjugate, which acts against tumors by producing reactive oxygen species through Bioluminescence Resonance Energy Transfer (BRET). Ultimately, this resulted in significant suppression in the migration of surviving SKBR3 breast cancer cells [82]. Through advancements in the manipulation of Listeria, this bacterium has become a favorite candidate in the quest for more effective treatment delivery systems.

3.1.4. *Clostridium* spp.

Clostridium is known to be one of the largest prokaryotic genera, comprised of anaerobic spore-forming bacteria. The Clostridium group of bacteria resist harsh environmental conditions such as high temperature and dehydration by producing endospores [83]. Clostridium also presents as a desirable delivery vehicle for therapeutic cancer drugs, having a natural ability to seek out and prosper in low-oxygen environments, such as those experienced in the core of the tumor microenvironment [9]. Clostridium is limited to tumor sites due to its inability to survive in other normal tissues that are rich with oxygen [70]. Clostridium and its related spores have been heavily implicated in cancer immunotherapy, with drug delivery potential taking lower precedence. The prevalence of clostridial spores in cancer therapies is well studied and has been reviewed in a number of scientific publications [84–88]. Various subtypes of Clostridium have been tested as anti-cancer agents including *C. butyricum*, *C. tetani*, *C. histolyticum* [89,90], *C. beijerinckii* [91], and *C. acetobutylicum* [92]. *Clostridium acetobutyicum* was one of the first to be investigated for its anti-cancer activity, as studies showed the ability to effectively engineer *C. acetobutyicum* to secrete mouse TNF-α. Similarly, *C. acetobutyicum* was also shown to be able to successfully secrete interleukin-2 (IL2), which, in humans, is known to stimulate immune cells by promoting the development of T cells [93].

Another Clostridium member, Clostridium novyi, has been genetically modified by removing a residential phage-carrying α-toxin to make the strain non-pathogenic. This major toxin was shown to be responsible for the toxicity of *C. novyi* by Vogelstein et al., who studied 26 anaerobes for their capability to divide and disseminate in a human colorectal cancer xenograft model [4]. Vogelstein et al. also developed the strain *C. novyi*-NT, which has been examined as an alternative for cancer immunotherapy and is undergoing a Phase I clinical study for the treatment of refractory tumors (NCT01924689). *C. novyi*-NT induces a vigorous inflammatory response engaging pro-inflammatory cytokines such as MIP-2, IL-6, G-CSF, and TIMP-1, which employ a significant number of immune cells to the site of infection and tend to increase long-lasting, adaptive anti-tumor immunity [94–96]. The precise mechanisms by which *C. novyi*-NT mediates tumor elimination are unknown; however, one of the major observations indicates that administration of *Clostridium difficile* (*C. diff.*) toxin B (TcdB)-treated CT26 colon cancer cells and B16–F10 melanoma cells in mice results in an extended tumor-specific immune response, providing insight into possible mechanisms for anti-tumor responses from *C. novyi*-NT [97].

With promising applications as a hypoxia-targeted delivery system, Clostridium deserves to be further investigated in this era of improved biotechnological methods. Clostridium-directed antibody therapy (CDAT) is another area of interest where Clostridium is mutated to induce production of high-specificity antibodies. By heterologous gene transfer, *C. novyi*-NT has been introduced with a heavy-chain subclass of antibodies (VHH), specifically VHH targeted against HIF1α [98]. These antibodies, when expressed in mammalian cells, inhibit HIF activity. Independent responses from *C. novyi*-NT are moderately rare; however, *C. novyi*-NT can be combined with other chemotherapeutic agents or radiation, a technique known as Combination Bacteriolytic Therapy (COBALT). The main reason for using *C. novyi* with other therapies is that *C. novyi*-NT can reach the necrotic and hypoxic region of the solid tumors, which are conventionally known to be resistant to other treatments such as radiation and chemotherapy. While *C. novyi*-NT has tremendous potential as a cancer therapeutic, numerous challenges still remain to be resolved before Clostridium-based BBCT can obtain essential regulatory approval and can be applied in the clinic.

3.1.5. *Corynebacterium* spp.

Corynebacterium diphtheriae is a group of Gram-positive bacteria and the causative agent of diphtheria. Corynebacterium can grow either as an aerobic or as a facultative anaerobic [99]. Diphtheria toxin (DT) is a highly robust toxin, and simply the entry of a single molecule into a cell can be toxic [99]. Due to this high toxicity, DT has been extensively studied to treat cancer cells by genetically deleting the cell receptor-binding domain and re-arranging the catalytic portion with the targeted proteins that collectively bind to the surface of the targeted cancer cells. DT-based immunotoxin (DTAT) can perform anti-tumor actions against different types of cancers, including glioblastoma and pancreatic cancer, through urokinase receptors (uPARs). A series of in vitro and in vivo studies have been used to show the ability and anti-tumor effects of immunotoxins (DTAT, DTAT13, and DTATEGF), which are directed by uPAR [100]. Although most of the pre-clinical work has shown positive responses, there remain no known clinical trials or possible clinical evaluations for any uPAR-based immunotoxins [100]. Not only the full length but also the truncated versions of DT have been used to establish recombinant immunotoxins against a series of cancers [101]. Various other DT-based immunotoxins have also been studied, which are specifically targeted to cancers of interest, including cell-penetrating peptide BR2 and receptor of Treg cells, CCR4 [102], DT386-BR2 [103], and DT-anti-CCR4 [104]. A genetically modified fusion protein, *Denileukin diftitox* (Ontak), was generated by fusing IL-2 and diphtheria toxin. This toxin introduces diphtheria toxin into the targeted cells that highly express IL-2 receptors, which hinders protein synthesis, thus causing cell death [105]. Ontak is also the first known immunotoxin approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma (CTCL) [106]. However, production of Ontak was suspended in early 2011 due to issues in preparation of Ontak and reports of the presence of contaminants, along with adverse events (AEs) [107].

3.1.6. *Pseudomonas* spp.

Pseudomonas aeruginosa is a Gram-negative, aerobic bacterium, which, under certain environmental conditions, can also grow as a facultative anaerobic bacterium [108]. Pseudomonas is known to have a plethora of virulence factors including toxins, which play a key role in its pathogenesis, including phytotoxic factor, pigments, hydrocyanic acid, proteolytic enzymes, endotoxins, and exotoxins [109]. Pseudomonas exotoxin A (PE) is known to be one of the major toxic virulence factors of pseudomonas [110] and has been extensively studied for its anti-tumor specificity [111,112] by inhibition of eukaryotic elongation factor 2 (Eef2) activity [111]. The ADP-ribosylation activity of pseudomonas affects the protein synthesis of the infected host cells. Various molecular strategies have been used by PE for effective killing of the host cell. Immunotoxins derived from PE have been tested in various pre-clinical as well as clinical studies against a variety of hematologic

malignancies and solid tumors with promising results. In a clinical trial, Moxetumomab pasudotox, an anti-CD22 immunotoxin agent, was used for the treatment of adults with relapsed or refractory hairy cell leukemia (HCL). Eighty patients were treated and 41% of the patients demonstrated complete remission [108].

In other studies, the immunotoxin, SS1P, targeting mesothelin has been administrated in combination with known immune-modulating chemotherapeutic agents, including pentostatin and cyclophosphamide to mesothelioma patients [112]. *Pseudomonas* spp. has also been genetically engineered to be used as delivery vehicle. *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PA-MSHA) has been engineered to attach mannose-sensitive fimbriae type 1 onto its surface. This strain has shown anti-cancer cytotoxic activities against breast, lung, cervical, hepatocellular, colon, and pancreatic cancer cell lines [113,114].

3.2. Non-Pathogenic Bacteria/Probiotics in BBCT

3.2.1. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are fastidious, Gram-positive, non-spore-forming cocci or rods, known to have high tolerance to low pH [115]. LAB are known to be beneficial not only for the balance of intestinal flora but also for their antimicrobial, antioxidant, anti-inflammatory, and anti-cancer effects [116]. Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Oenococcus, and Pediococcus [117] together form a heterogenous group of bacteria under the genera of LAB. LAB have proven to be a safe and appealing option in the realm of potential bacteria for use as drug delivery systems. LAB have an extensive history of being safe for human use in various areas of medicine and food, and now studies have implicated them in the distribution of drugs to solid tumors [118]. LAB inhabit the small and large intestines of humans and animals, and have been shown to have the capacity to travel after IV administration to solid tumors, where they can accumulate and proliferate [28]. The development of biotechnological tools has allowed progression to a point where these organisms can be engineered to secrete a protein of interest into the extracellular tumor environment to provide a more targeted therapeutic benefit. LAB strains show strong antioxidant properties due to their high catalase activity and α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activity. LAB also profoundly catalyze anti-inflammatory activity by the activation of anti-inflammatory cytokines (e.g., IL-10) and decreased expression of pro-inflammatory cytokines (e.g., IL-6) [119]. Numerous studies have depicted that probiotics reduce colorectal cancer-associated bacteria such as Fusobacterium and peptostreptococcus [120]. Regular intake of LAB has been shown to reduce breast cancer risk in women [121]. Fermented food containing L. acidophilus, L. bulgaricus, Streptococcus lactis, or Bifidobacteria have been shown to inhibit the proliferation of ER+ breast cancer in an animal model [122–124]. Thus far, the most common LAB currently used as drug delivery vehicles are Lactobacillus, Lactococcus, and Bifidobacterium species.

3.2.2. *Lactobacillus* spp.

Lactobacillus is a genus of Gram-positive, rod-shaped bacteria inhabiting the intestinal microbiome of humans and other mammals. As one of the major probiotic bacterium in the intestine, the key role of this bacterium is to share lactic acid fermentation with other bacteria and further strengthen the intestinal barrier [125]. *Lactobacillus plantarum* (L. *plantarum*) is being studied for various clinical applications, including cancer treatment. The L-14 form of *L. plantarum* extract has been shown to inhibit the viability and relocation of A375 cells, as well as regulating the expression of genes involved in migration in a human malignant melanoma model [126]. *Lactobacillus casei* harbors anti-tumor effects mediated by the downregulation of IL-22 and upregulation of caspases, inducing apoptosis [127]. Lactobacillus targets malignant cells by producing bacteriocins such as nisin that induce apoptosis and reduce cell proliferation by cell cycle arrest in the G2 phase [128]. Kim et al. reported using probiotic *Lactobacillus kimchicus* DCY51 for non-covalent loading of ginsenoside compound K (CK). CK is highly regarded in traditional Chinese herbal medicine due to its bioactive triterpenoid saponins, and it has been shown to inhibit hormone-independent breast cancer

by downregulating cyclin D1, an important part of the G1 phase of the cell cycle [78]. This study demonstrated that nanoparticle-bound DCY51 kills more A549 cells (human lung adenocarcinoma cell line) and HT29 cells (human colorectal adenocarcinoma cell line) compared to ginsenoside CK treatment alone [78]. Another study using a melanoma mouse model suggested that strain *L. reuteri* FLRE5K induces higher levels of the cytokines TNF-α and IFN-γ, which stimulate immunity and interfere with proliferation of melanoma cells [129]. Alternatively, Lactobacillus enriched with selenium has shown positive anti-tumor effects, as LAB can form elemental selenium nanoparticles (SeNPs) by reducing selenium ions and then proceeding to drop off the nanoparticles intracellularly. As an anti-carcinogenic essential micronutrient, selenium acts by preventing activation of oncogenes, and, therefore, preventing the transformation of normal cells into malignant ones. This was evaluated in mice bearing 4T1 breast cancer, where treatment with enriched Lactobacillus was shown to increase survival and decrease the number of tumor metastases to the liver [130]. In another study, fluorescent inorganic cadmium sulfide (CdS) nanoparticles were successfully transported into MCF-7 breast cancer cells using *Lactobacillus* spp. as a vector [131]. In a titration study, the authors showed that increasing concentrations of CdS NPs gradually decreases the metabolic activity of MCF-7 cells until a peak concentration of 5 ppm CdS NPs is reached, with 80% cell death at 24 h and complete cell death at 48 h [131].

3.2.3. *Lactococcus* spp.

Lactococcus was first employed as a delivery vehicle for drugs in diseases other than cancer, such as inflammatory bowel disease, where it has been genetically modified to secrete IL-10 [132]. Following this approach to other diseases, studies have followed suit by exploring Lactococcus as a rising form of drug and gene delivery in various types of cancer. *Lactococcus lactis* has been used as a KiSS1 peptide-producing factory, where *L. lactis* NZ9000-401 was constructed to express human KiSS1. KiSS1 peptide plays an important part as a tumor suppressor inhibiting cancer metastasis [70]. HT-29 cells displayed morphological changes and apoptosis when treated with *L. lactis*-expressing KiSS1. While this study looked at the effects in HT-29 human colon cancer cells, KiSS1 is expressed in human breast cancer, leading to the conclusion that this therapy poses an opportunity to benefit breast cancer treatment specifically. Alternatively, the usefulness of *L. lactis* in breast cancer has been proven by its success in secreting drugs already shown to be effective in reducing tumor size and slowing growth. An active form of *L. lactis* has demonstrated successful expression of Mig and IP-10 [133], both of which are chemokines and function to draw immune cells to the site of active infection. Both Mig and IP-10 have anti-angiogenic properties critical for tumor immunity. Additionally, *L. lactis* has been genetically engineered for induction of IL-12, a member of an interleukin family with immunoregulatory effects, including stimulation of IFN secretion and Th1 immune responses, as well as inhibition of Th2 responses. While Lactobacillus holds promise as a safe and effective delivery vehicle for breast cancer therapy, there is still much left to learn and assess before it will be deployed to the forefront of cancer treatment.

3.2.4. *Bifidobacterium* spp.

Bifidobacterium spp. is a branched, non-motile, obligate anaerobic bacteria. It is among the first bacteria to be colonized in the human gut [134]. Among 50 known species of Bifidobacterium spp. in various environments, only 10 are found in humans. Many studies have reported using Bifidobacterium spp. for its anti-tumor activities [135]. Although these microorganisms successfully colonize tumor cells, anti-tumor effects of Bifidobacterium have not yet been fully determined. However, preliminary observations have led to the investigation of *Bifidobacterium* spp. as a major delivery vehicle that can be bioengineered and modified to express genes of interest for cancer immunotherapy. Wang et al. showed that genetically engineered *B. breve,* modified to express IL-24 (*B. breve*-IL24), inhibits head and neck tumor growth by inducing apoptosis [136]. It has been demonstrated in mouse models that bioengineered Bifidobacterium spp. could deliver enterolactone, which converts fatty

acids into pectin oligosaccharides (POS), which significantly delay the development of leukemia [137]. The use of Bifidobacterium for gene delivery was first investigated by determining whether *B. longum* 105-A transformed with a spectinomycin-resistant gene effectively transfers resistance in mice, thereby indicating gene transfer [28]. In later years, this concept was applied to cancer-specific gene therapies such as the endostatin gene, an inhibitor of basic fibroblast growth factor (bFGF)-stimulated vessel endothelial cell proliferation. A dormant state in primary tumors can be achieved through systemic administration of endostatin in tumor-bearing mice. Endostatin-carrying *B. adolescentis* has demonstrated inhibition of hypoxic tumor growth and angiogenesis when administered intravenously to mice infected with Heps liver cancer [138].

Since it was established that Bifidobacterium could be used as a relatively safe and competent vehicle for treatment delivery, studies assessing specific cancer therapies have been conducted. The production of the enzyme, cytosine deaminase (CD), by *B. longum* has been combined with 5-fluorocytosine (5FC) in solid tumors, including breast cancer. A resultant high concentration of 5-fluorouracil (5FU) was found to be localized in the tumor, because of the reaction between 5FC and CD. This turned out to be beneficial for cancer therapy as 5FU is a more toxic prodrug than 5FC; therefore, it is possible to refrain from systemically distributing 5FU and instead using 5FC, which will only be converted to 5FU at the tumor site [118,139]. Bifidobacterium has also demonstrated efficacy in solid tumors when orally administered, making it of particular interest. *B. breve* has been shown to be able to successfully colonize solid B16 murine melanoma tumors after being orally administered and translocated to the gastrointestinal tract [140]. However, Bifidobacterium has primarily been evaluated through intravenous injection, with more common therapeutic breast cancer drugs making their way into the literature. Trastuzumab has become a mainstay treatment against HER2-positive breast cancer as a HER2-targeting monoclonal antibody. A genetically engineered version of *B. longum* has displayed significant repression of xenographed human HER2-positive tumors in mice [141]. Furthermore, effective delivery to solid tumors using Bifidobacterium microbots was demonstrated in a mouse model through fluorescent imaging of CdSeS quantum dots [131].

3.2.5. *Magnetococcus* spp.

In recent years, there has been an increased interest in exploring environmental microorganisms for properties with potential applications in cancer therapy. Found in the sediment in the depths of the water, a group of anaerobic bacteria known to align themselves with the planet's geomagnetic field, magnetotactic bacteria, have come to light as a possible beneficial tool for drug delivery [142]. These properties necessary to target tumors, as magnetotaxis, in addition to flagellar motors, are what allow the bacteria to migrate to and reside in low-oxygen areas [143]. The magnetic properties of these bacteria are also helpful in targeting tumors because they make it possible to magnetically guide them to the site of the tumor, in addition to their natural hypoxia-seeking state. *Magnetococcus marinus* MC1 is currently the most tested magnetotatic bacteria in the delivery of cancer therapeutics [7]. This Gram-negative coccus was found in the Atlantic Ocean and has been studied by covalently binding drug-containing nanoliposomes. Accordingly, MC-1 cells bearing nanoliposomes were injected near the tumor site and guided magnetically. This resulted in up to 55% of MC1 entering hypoxic tumor areas of SCID Beige mice within HCT116 colorectal xenographs [144]. Based on early successes, magnetotactic bacteria are a notable development agent in drug delivery using microorganisms and needs to be further explored and applied to more extensive in vivo tumor testing.

Table 1. Bacteria for cancer immunotherapy.

Bacteria	Strain	Mutated/Gene Modified	Cancer Type	Phenotypic Description	Ref
			Pathogenic bacteria-mediated cancer immunotherapy		
Salmonella typhimurium	A1-R	Δleu/Δarg	Prostate cancer	Auxotrophic strain defective in synthesis of leucine and arginine	[145]
	VNP20009	ΔmsbB/ΔpurI	Metastatic melanoma, Glioblastoma, Pancreatic cancer, Colon cancer, Breast cancer	Modification of Lipid A structure; reduced ability to induce TNF-α secretion; deficiency in adenine synthesis	[146–148]
	SHJ2037	ΔrelA/ΔspoT		ΔppGpp (global regulator); reduction in bacterial invasion	[149,150]
	SL3261, SL7207, BRD509, YB1	aro-	Prostate cancer, Melanoma, Breast cancer	Mutations in aromatic amino acid biosynthesis	[19,151–154]
	LH430; VNP (Pho/Q-)	ΔphoP/ΔphoQ	Colorectal cancer, Renal cancer	Reduced bacterial survival in macrophages	[37,155]
	MvP728	ΔpurD/ΔhtrA	Colon carcinoma, DBT glioblastoma, Melanoma	Defective in purine biosynthesis, produces heat-shock protein response to stress stimuli	[156]
	YB1; ST8	Δasd	Breast cancer, Colon cancer	Defective in diaminopimelic acid (DAP) synthesis	[19,157]
	X4550	Δcya/Δcrp	Osteosarcoma	Disabled production of cAMP (cyclic adenosine monophosphate) synthetase and cAMP receptor protein	[158]
	RE88	Δdam	Breast carcinoma	Defective in DNA adenine methylase production	[159]
	SB824	ΔsptP	Melanoma	Defective in pathogenicity island 1 (SPI-1)	[160]
	ST8	Δgmd	Colon cancer	Unable to replicate beyond the anaerobic regions of tumors	[137]
	SF100; SF200; S364	rfa-, ΔpagP/ΔpagL/ΔlpxR	Colorectal cancer, Fibrosarcoma	Highly truncated LPS and attenuated bacterial virulence	[161,162]
	MPO378	ΔpurD/Δupp	Breast Cancer cell line	Inefficient in purine biosynthesis and uracil phosphoribosyl transferase	[162]
	FlaB	*Vibrio vulnificus*flagellin B	Colon cancer	Engineered FlaB from Vibrio vulnificus-secreting bacteria	[150]
Listeria monocytogenes	rLM	Lm-LLO-E7	Cervical cancer, Leukemia, Ovarian cancer, Prostate cancer, Colon cancer, Breast cancer	Secretes a fusion protein comprised of nonfunctional LLO joined with HPV protein E7	[163]
	XFL7	Lm-LLO-PSA	Prostate cancer	Significantly higher number of IFN-γ-secreting cells	[164]
	DP-L4029	ΔactA	Colon cancer, Lung cancer	Defective surface-bound ActA polypeptide, constitutes LLO activity at physiologic pH	[44,165,166]
	DP-L4017	LLO L46IT, LLOD26	Lung cancer	Cytotoxic, defective cell-to-cell spreading and greater percentages of splenic- and tumor-infiltrating, antigen-specific CD8+ lymphocytes	[5,42,167]
	DP-L4042	ΔPEST	Colon cancer, Lung cancer	Cytotoxic, defective cell-to-cell spreading	[42,167]
	DP-L4405 DP-L4406	ΔinIA/ΔniB	Colon cancer	Impaired InIA-mediated infection	[168]
	CS-L0001	ΔactA/ΔinlB	Colon tumor lung metastases	Defective in cell-to-cell spreading	[44]

Table 1. *Cont.*

Bacteria	Strain	Mutated/Gene Modified	Cancer Type	Phenotypic Description	Ref
	CS-L0002	ΔactA/ΔlplA		*L. monocytogenes* vaccine vectors expressing influenza A nucleoprotein	[169]
	DP-L4038	ΔactA/L461T LLO		Inadequate surface-bound ActA polypeptide, constitutes LLO activity at physiologic pH	[165,166]
Mycobacterium bovis	BCG Pasteur	1137P2	Bladder cancer	Cancer cell phagocytosis by increasing proinflammatory cytokine activation and immune system	[47,170,171]
Clostridium novyi	NT	ΔtoxA/ΔtoxB	Glioblastomas neuroshphere, Colon cancer	Produces specific enzymes and toxins capable of destroying cancer cells	[172–174]
Escherichia coli	MG1655		4T1 breast cancer	Optimized physicochemical properties for bacterial attachment; Low cost for bioconjugation	[175]
Streptococcus pyogenes	OK-432		Lymphangioma intraoral ranula	Including TNF, IL-8, IL-6, IFN-γ, and VEGF; increase in WBCs	[176–179]
Pseudomonas aeruginosa	*F10*		Lung cancer, Breast cancer, Cervical cancer, and Colon cancer	Anti-tumor effects of 2,4-diacetylphloroglucinol (DAPG) extracted	[180]
	(PA-MSHA)		Pancreatic cancer	Anti-tumor effect of *P. aeruginosa*-MSHA (mannose-sensitive hemagglutinin) inducing apoptosis by the EGFRa pathway and caspase signaling	[181]
			Hepatocellular carcinoma	Anti-tumor effect of *P. aeruginosa*-MSHA (mannose-sensitive hemagglutinin) by EGFR/Akt/IκBβ/NF-κB pathway	[182]
Non-pathogenic bacteria-mediated cancer immunotherapy					
Lactobacillus reuteri	PTCC 1655	WT	Gastric cancer	Probiotic-based strategies: inhibition of cell proliferation by downregulation of uPA/uPA receptors (uPARs)	[183]
	FLRE5K1	WT	Melanoma	Preventive effect of *L. reuteri* on melanoma	[129]
Lactobacillus plantarum		WT	Colon cancer, Breast cancer, Oral cancer	Produces antioxidants, increases TNF-α, induces caspase-3 activity, inactivates Wnt/β-catenin signaling	[184,185]
Lactobacillus rhamnosus	SHA111; SHA112; SHA113	WT	Colorectal cancer, Cervical adenocarcinoma, Breast cancer	Apoptosis via up-regulation of BAD, BAX, Caspase3, Caspase8, and Caspase9, and down-regulation of BCL-2 genes	[183]
Lactococcus lactis			Head and neck tumor	Anti-tumor effect of nisin: by induction of apoptosis through a calpain-dependent pathway	[128]
Bifidobacterium bifidum			Lung cancer	Induction of immune responses, which leads to inhibition of tumor growth by activation of IL-12 and IFN-γ, lymphocyte proliferation, and CD8+ cytolytic induction	[186]
	CGMCC 15068		Colon cancer	*B. bifidum* growth in intestinal health by modulating dysbiosis and the gut metabolic profile	[187]
Bifidobacterium longum	NCC2705	WT	Colon adenocarcinoma	*B. longum* as a vector of tumstatin (Tum) inducing significant anti-tumor effect	[137]
	420 and 440	WT	Prostate cancer	*B. longum*-based vaccine inducing immune response against Wilms tumor 1 (WT1) antigen	[188]
Bifidobacterium breve	UCC2003	WT	Head and neck tumor	*Strain* expressing IL-24 gene: Apoptosis induction leads to anti-tumor activity	[136]

4. Clinical Trials Using Bacteria as Delivery Vehicles

In 1891, William B. Coley used live infections of a combination of *Streptococcus Pyogenes* and *Serratia marcescens* as an immunotherapy against sarcoma [1,189]. Since then, a multitude of bacterial strains have been studied and are now selected for testing in patients in clinical trials (Table 2).

Table 2. Clinical Trials.

Bacterial Strain	Type of Cancer	Clinical Phase	Identifier No.	Reference (All Links Were Accessed on 16 December 2021)
C. histolyticum	Lipoma	I	NCT01613313	https://clinicaltrials.gov/ct2/show/NCT01613313
	Lipoma	I	NCT02249052	https://clinicaltrials.gov/ct2/show/NCT02249052
C. butyricum M55	Vascular glioblastoma	I	-	[190]
C. novyi-NT	Solid tumor malignancies	I	NCT01924689	[173]
	Colorectal cancer	I	NCT00358397	https://clinicaltrials.gov/ct2/show/NCT00358397
	Solid tumor malignancies	I	NCT01118819	https://clinicaltrials.gov/ct2/show/NCT01118819
	Refractory advanced solid tumors	Ib	NCT03435952	https://clinicaltrials.gov/ct2/show/NCT03435952
L. monocytogenes	Cervical cancer	II	-	[191]
	Cervical cancer	III	NCT02853604	https://clinicaltrials.gov/ct2/show/NCT02853604
	Metastatic pancreatic tumors	II	-	[192]
	Malignant epithelial mesothelioma Adenocarcinoma of the pancreas, Non-small cell lung adenocarcinoma of the ovaries	I	NCT00585845	https://clinicaltrials.gov/ct2/show/NCT00585845
	HPV-16 +ve oropharyngeal carcinoma	I	NCT01598792	https://clinicaltrials.gov/ct2/show/NCT01598792
L. monocytogenes (LADD)	Prostatic neoplasms (castration resistant)	II	NCT01613313	https://clinicaltrials.gov/ct2/show/NCT02625857
	Non-small cell lung carcinoma	I	NCT02592967	https://clinicaltrials.gov/ct2/show/NCT02592967
S. typhimurium (χ4550)	Hepatocellular carcinoma	I	-	[193]
S. typhimurium VNP20009	Metastatic melanoma, metastatic renal cell carcinoma	I	-	[15]
S. typhimurium VNP20009	Melanoma	I	-	[17]
S. typhimurium VNP20009 expressing TAPET-CD (cytosine deaminase)	Head and neck, and esophageal adenocarcinoma	I	-	[[18]]

Table 2. *Cont.*

Bacterial Strain	Type of Cancer	Clinical Phase	Identifier No.	Reference (All Links Were Accessed on 16 December 2021)
S. typhimurium VNP20009	Advanced metastatic solid tumors	I	NCT00004216	https://clinicaltrials.gov/ct2/show/NCT00004216
	Solid tumors	I	NCT00006254	https://clinicaltrials.gov/ct2/show/NCT00006254
	Neoplasm metastatic tumor	I	NCT00004988	https://clinicaltrials.gov/ct2/show/NCT00004988
S. typhimurium expressing IL-2	Liver cancer	I	NCT01099631	https://clinicaltrials.gov/ct2/show/NCT01099631
S. typhimurium Ty21a VXM01	Pancreatic cancer	I	-	[194]
Mixed Bacterial Vaccine	Malignant tumors	I	NCT00623831	https://clinicaltrials.gov/ct2/show/NCT00623831

The information gathered in the clinical data shown in the table reveals many major obstacles as well as challenges that need to be addressed for further successful human application of bacteria as cancer immunotherapy in near future. While bacteria alone may not offer the best solution, broadening our knowledge and understanding as well as altering bacteria to have fewer side effects along with anti-tumor agents, immunogenic agents, and/or anti-oncogenes can prove to be beneficial.

5. Current Challenges

Due to the advancement of the microbiome as a major player in our search for remission of various human diseases, bacteria as a therapeutic prospect are gaining significant interest in many medical fields [195]. Tumor targeting-bacteria have peculiar distinctive features including unique gene packaging, targeting the hypoxic environment of tumor, and tumor selectivity, which make them an ideal vehicle for delivering therapeutic cargo specifically targeting cancers of various origins. However, although engineered bacteria have gained high therapeutic potential to target tumors, due to high heterogeneity of cancers at the molecular and histologic levels, a single anti-cancer agent may not be able to achieve cure by itself. Thus, a combinatorial approach may be required to develop a promising anti-cancer therapy.

One of the major concerns in the field of BBCT is the toxicity of bacteria due to associated toxins, which may lead to serious infections, considerable side effects, and even death. Researchers are, therefore, using attenuated and genetically modified strains to overcome these adverse outcomes. Reducing or removing specific virulence factors from bacteria by genetic modifications can also remedy the toxicity associated with using bacteriotherapy. However, it should be noted that there is a tradeoff of reducing virulence and removal of virulence factors and clinical outcomes, as removing virulence of a bacteria can reduce the potency of its anti-cancer affects. It is well documented that bacterial strains manipulated for cancer therapies are sensitive to changes in their virulence factors. Microbe-associated molecular patterns (MAMPs) need additional attention when they are adapting to bacterial strains during cancer therapy. However, it has been previously reported that structural changes in LPS can cause changes in the physiology of bacteria to transform from a virulent strain to a strain with anticancer properties. For example, a change in the structure of lipid A to hexa-acylated lipid A, has led to increased affinity for Toll-like receptor 4 (TLR4), which can induce anti-cancer responses [194]. Another major challenge in this field is the short half-life of the bacterial peptide of protein and unstable DNA.

One of the major caveats of BBCT is that it is not suitable for patients who have been on certain types of chemotherapy, as these may suppress the immune system to the extent that it cannot sufficiently respond to bacterial colonization. Additionally, live bacterial products

can colonize in foreign bodies like artificial heart valves, joint replacements, and implanted medical devices, which may serve as reservoirs for infection. Furthermore, recombinant plasmids carried by bacteria can be mutated, thus changing the fate of anti-tumor action before the cancer cells are penetrated. This can lead to various associated risks, including therapy failure, infection, or death [195]. A major public health concern is the development of multi-drug resistance of many of the bacteria used in BBCT.

6. Future Prospects

Careful manipulation of microbes may very well be the next necessary step to making them a routine part of cancer therapy. Conscientious exploitation of microbial mechanisms for their tumor-targeting properties also proposes major applications as a personalized therapy, as this new level of control can be utilized for each patient's unique tumor type. The ideal microbial therapy will theoretically combine a non-pathogenic but effective species that will consist of not one but multiple strains selected for their specific targets and then ultimately be combined with effective standard treatments for the best possible efficacy. The hypoxia-honing powers of microbes can be combined with other therapeutic methods to target the remaining tumor regions that are richer in oxygen. The genetic flexibly of microorganisms may truly be their greatest strength, allowing for precise tuning of individualized therapy for maximum cytotoxic effects.

The idea of treating cancer with microbes as delivery vehicles has a long way to go before rising to the popularity of current mainstay therapies. Toxicity issues and cultural stigmas must be addressed before microorganisms will be trusted in the realm of cancer therapy. The field of BBCT is still considered to be quite novel and more scientifically sound studies need to be conducted to overcome the ongoing limitations and side effects associated with bacteriotherapy. However, the potential that BBCT holds is impossible to overlook, with a plethora of promising mechanisms that may be manipulated to target tumors and improve patient outcomes. Despite encouraging in vitro and in vivo results of BBCT, very few studies have led to clinical trials. Therefore, it is obligatory that the scientific and clinical communities begin to design additional clinical trials to investigate and harness the efficacy of BBCT.

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