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Review

Harnessing *Bacillus subtilis* Spore Surface Display (BSSD) Technology for Mucosal Vaccines and Drug Delivery: Innovations in Respiratory Virus Immunization

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Abstract: Respiratory viruses present significant global health challenges due to their rapid evolution, efficient transmission, and zoonotic potential. These viruses primarily spread through aerosols and droplets, infecting respiratory epithelial cells and causing diseases of varying severity. While traditional intramuscular vaccines are effective in reducing severe illness and mortality, they often fail to induce sufficient mucosal immunity, thereby limiting their capacity to prevent viral transmission. Mucosal vaccines, which specifically target the respiratory tract's mucosal surfaces, enhance the production of secretory IgA (sIgA) antibodies, neutralize pathogens, and promote the activation of tissue-resident memory B cells (BrMs) and local T cell responses, leading to more effective pathogen clearance and reduced disease severity. *Bacillus subtilis* spore surface display (BSSD) technology is emerging as a promising platform for the development of mucosal vaccines. By harnessing the stability and robustness of *Bacillus subtilis* spores to present antigens on their surface, BSSD technology offers several advantages, including enhanced stability, cost-effectiveness, and the ability to induce strong local immune responses. Furthermore, the application of BSSD technology in drug delivery systems opens new avenues for improving patient compliance and therapeutic efficacy in treating respiratory infections by directly targeting mucosal sites. This review examines the potential of BSSD technology in advancing mucosal vaccine development and explores its applications as a versatile drug delivery platform for combating respiratory viral infections.

Keywords: respiratory viruses; mucosal vaccines; *Bacillus subtilis* spore surface display (BSSD); oral vaccine delivery; drug delivery systems

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1. Introduction

Respiratory viruses, encompassing small non-enveloped viruses like rhinoviruses and adenoviruses, as well as more complex enveloped viruses such as influenza virus (IAV), respiratory syncytial virus (RSV), human parainfluenza viruses (HPIV), human metapneumovirus (HMPV), and coronaviruses, circulate globally and impose a substantial health burden across all age groups. This is largely due to their rapid evolution, efficient transmission, and zoonotic potential. The primary mode of transmission for these viruses is through aerosols and droplets, which complicates prevention and control efforts. Once inhaled, these viruses enter the body via the airway, infect respiratory epithelial cells, and cause diseases of varying severity depending on the infection site within the respiratory tract [1,2]. Upon entry, the first barrier these viruses encounter is a layer of mucus composed of hydrated glycoproteins. After breaching this barrier, the viruses reach the epithelial cell membrane and initiate their replication cycle. Viral surface proteins bind to cellular receptors, establishing a virus–cell interface that triggers responses from both entities, such as spike protein cleavage or membrane fusion in the case of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or endocytosis in the case of IAV. As obligate

intracellular parasites, viruses rely on, and often remodel, cellular protein networks to facilitate their replication [3,4].

Traditional vaccines, typically administered intramuscularly, have been effective in reducing severe illness and mortality. However, they often fail to induce sufficient mucosal immunity, which is crucial for preventing viral transmission and spread [5]. Mucosal vaccines, which target the respiratory tract's mucosal surfaces, present a promising alternative by stimulating immune responses directly at the site of viral entry. These vaccines enhance the production of sIgA antibodies, which serve as a frontline defense at mucosal surfaces by neutralizing pathogens and preventing their dissemination. Additionally, mucosal vaccines promote the activation of BrMs and local T cell responses, leading to more effective pathogen clearance and reduced disease severity [6].

The rapid evolution, efficient transmission, and zoonotic potential of respiratory viruses, coupled with the shortcomings of traditional vaccines, underscore the need for innovative approaches such as BSSD technology in developing effective mucosal vaccines. BSSD technology is emerging as a powerful platform for developing mucosal vaccines. This innovative approach harnesses the stability and robustness of *Bacillus subtilis* spores to present antigens on their surfaces. BSSD technology offers several advantages for mucosal vaccination, including enhanced stability, cost-effectiveness, and the ability to induce robust local and systemic immune responses. Furthermore, the cost-effectiveness of mass-producing BSSD technology, combined with its inherent adjuvant properties that enhance the therapeutic effects of delivered drugs, underscores its practicality for drug delivery systems, particularly for oral administration, as this technology can effectively deliver drugs directly to mucosal sites and target respiratory diseases [7]. This review takes a look at the potential of BSSD technology in the development of mucosal vaccines and drug delivery systems against respiratory viruses. It aims to demonstrate how BSSD technology can enhance local and systemic immune responses, improve vaccine efficacy, and facilitate targeted drug delivery, thereby addressing the limitations of traditional approaches and ultimately contributing to reducing the global health burden posed by these pathogens.

2. Overview of Infectious Respiratory Viruses

Viral respiratory tract infections represent a significant global health challenge, contributing to substantial morbidity, mortality, and economic burdens. These infections are caused by a range of viruses, including rhinoviruses and enteroviruses (Picornaviridae), influenza viruses (Orthomyxoviridae), parainfluenza viruses, metapneumoviruses, respiratory syncytial viruses (Paramyxoviridae), coronaviruses (Coronaviridae), and several adenoviruses. These pathogens replicate within the respiratory tract and are transmitted through direct or indirect contact, droplets, and aerosols [8,9]. The dynamics of transmission are influenced by environmental conditions, population density, and host factors, complicating efforts to study and control these infections. The clinical manifestations of these viruses vary from mild to severe, with young children, the elderly, and immunocompromised individuals being particularly susceptible. These pathogens are associated with a wide spectrum of clinical syndromes, including the common cold, pneumonia, and exacerbations of chronic respiratory conditions.

Despite the widespread impact of these viruses, effective antiviral therapies and vaccines remain limited. Preventive strategies primarily focus on risk mitigation and vaccination, when available. The potential for rapid, extensive transmission, as exemplified by SARS-CoV, highlights the urgent need for effective treatments and vaccines to alleviate the global burden of these infections [9,10]. Table 1 offers a comparative analysis of the key features of various respiratory viruses.

Table 1. Overview of genome types and structures of and diseases caused by respiratory viruses [10,11].

Virus	Viral Family	Genome Type	Genome Length	Virion Characteristics	Characteristic Clinical Conditions
Influenza Virus	*Orthomyxoviridae*	Negative-sense, segmented ssRNA	13.5 kb	80–120 nm, with an envelope	Acute respiratory infections (commonly known as the flu). Can lead to complications like pneumonia, bronchitis, or worsening of chronic conditions like asthma or heart disease, especially in vulnerable populations like the elderly and immunocompromised.
RSV	Pneumoviridae	Negative-sense RNA	15.2 kb	120–200 nm, with an envelope	Bronchiolitis, wheezing
Human Metapneumovirus (HMPV)	Paramyxoviridae	Negative-sense RNA	13 kb	150–200 nm, enveloped	Upper respiratory tract issues
HPIV	Paramyxoviridae	Negative-sense RNA	15 kb	150–200 nm, enveloped	Croup, bronchiolitis
Human Rhinovirus (HRV)	Picornaviridae	Positive-sense RNA	6–7 kb	30 nm, non-enveloped	Upper respiratory tract symptoms
Low-Pathogenic Coronaviruses (LP-HCoV)	Coronaviridae	Positive-sense RNA	27–32 kb	120 nm, enveloped	Upper respiratory tract symptoms
SARS-CoV & Middle East respiratory syndrome coronavirus (MERS-CoV)	Coronaviridae	Positive-sense RNA	27–32 kb	120 nm, enveloped	Lower respiratory tract infections, ARDS
Adenovirus (AdV)	Adenoviridae	Double-stranded DNA	35 kb	90 nm, non-enveloped	Upper respiratory tract infections, conjunctivitis, gastroenteritis, myocarditis

Limitations of Traditional Vaccines for Respiratory Infections

Traditional vaccines, typically administered intramuscularly or subcutaneously, have been pivotal in reducing severe illness and mortality associated with various viral infections. However, they have several limitations, particularly in the context of respiratory viral infections. These vaccines primarily induce systemic immunity, generating circulating IgG antibodies and systemic T cell responses. Unfortunately, they often fail to elicit robust mucosal immunity, which is crucial for protecting against respiratory viruses that enter and replicate within the mucosal surfaces of the respiratory tract. The absence of significant mucosal IgA production leaves these mucosal surfaces—the primary entry points for respiratory viruses—vulnerable, thereby permitting viral transmission and reinfection [12].

Although traditional vaccines are effective in mitigating disease severity and preventing complications, their inability to induce strong mucosal immune responses limits their effectiveness in preventing viral transmission. This limitation is particularly problematic for highly transmissible respiratory viruses, wherein halting the spread of infection is vital for controlling outbreaks. Additionally, the systemic immunity conferred by traditional vaccines can diminish over time, necessitating booster doses. Respiratory viruses such as influenza and SARS-CoV-2 are known for their rapid mutation rates, leading to the emergence of variants that may escape the immunity provided by existing vaccines. The lack of localized mucosal immunity exacerbates this issue, as it allows variant viruses to establish infections at mucosal surfaces even when systemic immunity is present [12,13].

Furthermore, traditional vaccines often struggle to induce strong tissue-resident memory T (TRM) cell responses, which are essential for long-term protection and rapid responses to viral infections at mucosal surfaces. These T cells are critical for clearing infected cells and providing lasting immunity at the site of viral entry. Certain populations, such as the elderly and immunocompromised individuals, may also exhibit reduced responses to traditional vaccines. A diminished ability to mount a strong systemic immune response can result in lower vaccine efficacy, particularly against rapidly evolving respiratory viruses [14]. These limitations highlight the need for alternative vaccine strategies, such as mucosal vaccines, which are designed to directly target mucosal surfaces and induce both local and systemic immune responses.

From an economic perspective, spore-based vaccine platforms offer several advantages over traditional vaccine technologies. The ease of production associated with *B. subtilis* spores allows for rapid scaling during outbreaks, which is crucial for mass vaccination efforts. This flexibility can mitigate economic limitations typically encountered in conventional vaccine production, where slow and costly manufacturing processes can hinder timely responses to public health emergencies [15].

Additionally, as needle-free vaccines that are easy to store and transport under critical conditions, spore-based vaccines not only reduce logistical challenges but also lower healthcare costs associated with needle-based immunization, such as the need for trained personnel and disposal of medical waste. Furthermore, the production and storage of these vaccines are generally more cost-effective, as they do not require a cold chain, which can significantly reduce transportation and storage costs. Traditional vaccine technologies often involve complex manufacturing processes and stringent temperature control, leading to higher overall costs. These characteristics highlight the potential of spore-based vaccine platforms to not only improve public health outcomes but also provide a more economically viable solution for large-scale vaccination during widespread disease outbreaks [16].

3. Mechanisms of Mucosal Immunity: A Critical Defense

Each component of mucosal immunity contributes uniquely to the defense of mucosal surfaces, including the respiratory tract, against pathogens. The following section provides a brief overview of key mechanisms, with further analysis provided later in the text to expand on their roles in immune protection.

The respiratory tract's essential role in oxygen exchange renders its extensive mucosal surface highly susceptible to exposure and infection by respiratory pathogens. Viruses

such as influenza virus, RSV, HIPV, and SARS-CoV-2 exploit this vulnerability, spreading rapidly through populations via exhaled droplets or aerosols. This highlights the need to reconsider traditional vaccination strategies, which have primarily aimed to induce strong systemic antibody and cellular immunity. As an alternative, there is an increasing focus on establishing immunity closer to the at-risk mucosal surfaces.

"Mucosal immunity" strategies, typically involving the delivery of replicating viral vectors to the respiratory mucosa, have been employed for many years. In the following sections, we discuss the structure and function of mucosal surfaces, immune surveillance mechanisms, and the mucosal immune system, including both humoral and cellular responses [17,18].

3.1. Structure and Function of Mucosal Surfaces

The mucosal surfaces of the body are safeguarded against pathogens and environmental antigens by the mucosal immune system, a distinct and extensive component of the immune system. Functionally separate from the circulatory immune system, the mucosal immune system constitutes the largest part of the body's immune defense, comprising both innate and adaptive immune cells and molecules. The respiratory tract, a primary entry point for pathogens, is anatomically divided into the upper respiratory tract (URT), which includes the nasal cavity, pharynx, and larynx, and the lower respiratory tract (LRT), which includes the trachea, primary bronchi, and lungs, encompassing the conducting airways and the respiratory zone. The nasal cavity mucosa, covered by a thin mucus layer, serves as a critical protective barrier. Epithelial cells, which line the entire respiratory tract, play an essential role in host defense by directly interfacing with the external environment [19].

Beneath the mucosal surface lies a network of immune tissues, including components of the mucosa-associated lymphoid tissues (MALTs). These are highly organized secondary lymphoid structures where antigen-specific immune responses are initiated. MALTs include several specialized tissues such as gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT), conjunctiva-associated lymphoid tissue (CALT), and vaginal-associated lymphoid tissue (VALT). These structures are analogous to lymph nodes, featuring B-cell-rich follicles, T-cell-rich interfollicular areas, antigen-presenting dendritic cells (DCs), and microfold (M) cells within the epithelial layer. High endothelial venules (HEVs) facilitate the movement of lymphocytes between MALTs and other lymphoid tissues, thereby enhancing systemic immunity [20].

In addition to these organized lymphoid structures, the mucosal immune system includes innate immune cells such as natural killer (NK) cells, innate lymphoid cells (ILCs), mucosal-associated invariant T (MAIT) cells, and γδ-T cells. Antimicrobial molecules like defensins and mucins also play a crucial role in maintaining mucosal immunity. It is important to note species-specific variations in the structures of MALTs; for instance, rats possess anatomically distinct NALT and BALT, while humans and mice have oropharyngeal and bronchoalveolar lymphoid tissues that respond to pulmonary infections [21,22].

3.2. Barrier and Defense Mechanisms at Mucosal Surfaces

Mucosal surfaces in the respiratory, gastrointestinal, and urogenital tracts are protected by delicate epithelial barriers. The innate immune response depends on the recognition of evolutionarily conserved features on pathogens, known as pathogen-associated molecular patterns (PAMPs), through pattern-recognition receptors like toll-like receptors (TLRs). Upon detecting these patterns, TLRs trigger the release of cytokines and chemokines, signaling underlying immune cells, such as DCs and macrophages, to initiate innate defenses and support adaptive immunity [23].

3.3. Mucosal Adaptive Immune Responses

Adaptive immunity at mucosal surfaces is designed to provide targeted protection while maintaining tolerance to non-threatening antigens. This specialized immune system involves secretory immunoglobulin A (IgA), mucosal cytotoxic T lymphocytes (CTLs), and mucosal IgG, which can be locally produced or derived from the serum [24].

Secretory IgA (sIgA): sIgA is a critical component of mucosal immunity, acting as the first line of defense by preventing pathogens from penetrating mucosal surfaces [25]. The induction of IgA immunity against mucosal pathogens primarily relies on the activity of T helper cells. However, it is important to note that IgA responses to commensal flora may occur independently of the thymus and are typically characterized by lower affinity. In humans, cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10, in combination with IL-4, play a crucial role in promoting B-cell class switching to IgA and facilitating the differentiation of these cells into IgA-secreting plasma cells. In this context, mucosal T cells are significant contributors as they produce high levels of IL-4, IL-10, and TGF-β, which are vital for IgA class switching. Additionally, human mucoepithelial cells serve as a major source of TGF-β and IL-10, indicating that the interaction and cooperation between neighboring lymphocytes and epithelial cells within the mucosal microenvironment are essential for guiding the preferential maturation of IgA-committed B cells [26,27].

Mucosal IgG: IgG, the most prevalent antibody in the bloodstream, is also present in type I mucosal tissues (e.g., the respiratory and gastrointestinal tracts) and increases in abundance after antigen exposure or vaccination. While sIgA predominates in type I mucosae, IgG is more common in type II mucosae, such as the corneal, oral, esophageal, lower respiratory, and lower female reproductive tracts, due to the expression of the neonatal Fc receptor (FcRn) rather than the polymeric immunoglobulin receptor (pIgR) [28]. Intact IgG in mucosal tissues can neutralize pathogens and prevent systemic infection. Recent findings suggest that IgG transport across epithelial barriers may involve receptor-mediated pathways, with FcRn facilitating bidirectional transport across epithelial barriers, potentially aiding in antigen uptake into the mucosa [25]. The regulation of IgG production in mucosal tissues involves complex interactions between local immune cells and the systemic immune system, with cytokines and chemokines modulating IgG production.

Cytotoxic T Lymphocytes (CTLs): Mucosal CTLs play a crucial role in controlling and clearing mucosal viral infections, even though they cannot block pathogen entry. For example, CD8(+) CTLs localized at the mucosal site of exposure provide long-lasting resistance to mucosal viral transmission, as demonstrated in studies using HIV peptide immunogens and recombinant vaccinia viruses [29]. This resistance is lost with CD8(+) cell depletion, emphasizing the importance of mucosal CTLs for protection against mucosal challenges. Enhancing mucosal CTL responses and resistance can be achieved through the local delivery of IL-12 with a vaccine, underscoring the need for local CTL induction for effective vaccines against mucosally transmitted viruses (Figure 1).

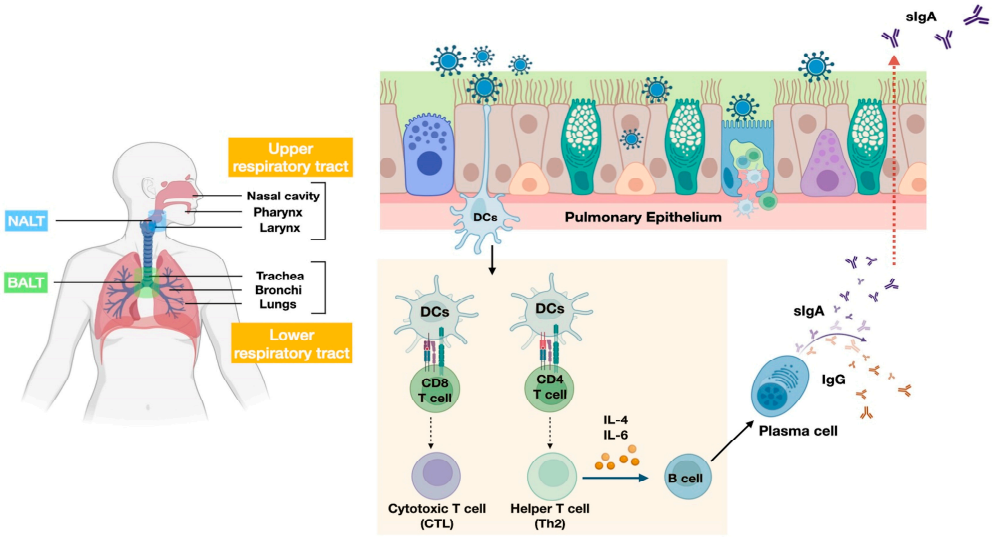

Figure 1. Schematic representation highlighting the role of NALT and BALT as the first line of defense against respiratory infections. These tissues act as inductive sites for initiating immune responses to inhaled pathogens, contributing to mucosal immunity in the respiratory tract.

3.4. Strategic Immunization Routes for Modulating Mucosal Immune Responses

The primary rationale for utilizing mucosal routes for vaccination stems from the fact that most infections originate in or impact mucosal surfaces. For respiratory infections, directly applying the vaccine to mucosal tissues is often essential for eliciting a protective immune response [30]. Different mucosal immunization routes produce variations in the strength and duration of the immune response. Ideally, vaccination at a single site would confer both local and systemic protection. For respiratory viruses, nasal and oral vaccination routes are critical strategies for inducing effective mucosal and systemic immune responses.

Intranasal and inhaled delivery methods target the upper respiratory tract, the initial site of entry for many respiratory viruses such as influenza and SARS-CoV-2. These routes induce robust mucosal IgA responses not only in the respiratory tract but also in other mucosal sites, including the salivary glands, NALT, BALT, and the lower respiratory tracts. Additionally, nasal vaccination can stimulate systemic immunity, generating circulating antibodies (IgG) and CTLs that offer protection throughout the body [31]. A unique feature of intranasal or inhaled immunization is its ability to induce the activity of Th17 effector cells and IL-17-producing TRM cells. Studies have shown that Th17 responses are triggered irrespective of the adjuvant type, although adjuvants can enhance the strength and effectiveness of this response. Previously, Th17 responses were associated with adverse immune effects; however, research on nasal immunization and Peyer's patches has demonstrated their crucial role in immune defense and pathogen clearance [32].

In contrast, oral vaccination primarily targets the GALT and is particularly effective against pathogens that enter or replicate in the gastrointestinal tract. The production of sIgA in the GALT is a central aspect of an orally induced immune response. sIgA neutralizes pathogens on mucosal surfaces, particularly in the gut, by releasing secretory components into the gut lumen. M cells and other immune cells in the GALT facilitate antigen uptake and enhance the immune response [33]. Although oral vaccination may not be as effective as nasal vaccination for respiratory-specific protection, it can still contribute to mucosal immunity due to the common mucosal immune system. Oral vaccines can induce systemic antibody production and CTL responses, albeit typically to a lesser extent than nasal vaccines for respiratory pathogens. Thus, while oral vaccination provides broader mucosal immunity, including in the gastrointestinal tract, nasal vaccination is generally more effective for generating targeted mucosal and systemic immunity in the respiratory tract. Oral vaccination may serve as a complementary approach, especially when broad mucosal immunity is desired [31].

Table 2 provides an overview of the different mucosal immunization routes, the specific mucosal areas they target, the characteristics of the immune responses they induce, and additional notes on their effectiveness.

Table 2. Impact of mucosal immunization routes on mucosal immune responses [34].

Immunization Route	Targeted Mucosal Areas	Immune Response Characteristics	Features
Oral	Gastrointestinal tract, oral mucosa, NALT, mammary glands	Activates immune responses in GI tract and associated mucosal sites	Effective for mucosal pathogens and certain tumors
Intranasal	Salivary glands, NALT, BALT, lower respiratory tract	Induces activity of Th17 effector cells and IL-17-producing TRM cells, strong immune response in lung mucosa	Can reduce pathogen transmission, adjuvants enhance response
Inhaled	Lung mucosa, lower respiratory tract	Similar to intranasal, robust immune response in lung mucosa	Effective for respiratory pathogens
Intravaginal	Vaginal mucosa	Less effective due to tissue stratification and hormonal fluctuations	Suboptimal for local mucosal immune response
Sublingual	Oral mucosa, potentially systemic and local immune sites	Higher IFNγ-secreting CD8 T cell levels in lungs compared to intramuscular	Lower levels of neutralizing antibodies in genital tract compared to intramuscular
Intramuscular	Systemic and localized immune responses	Generally effective but less focused on mucosal immune responses	Traditional route for many vaccines

4. Overview of Mucosal Vaccines for Respiratory Viruses

Mucosal vaccines have garnered significant attention, particularly during the coronavirus disease 2019 (COVID-19) pandemic, due to their ability to elicit both local antibody responses and tissue-resident T cell responses. This dual mechanism provides a robust defense against infection and transmission at the entry points of respiratory pathogens. Unlike intramuscular vaccines, which primarily stimulate systemic immunity, mucosal vaccines directly target mucosal surfaces, offering a more localized and specific immune response. Despite their potential advantages, the availability of mucosal vaccines for respiratory diseases remains limited compared to the extensive range of established injectable vaccines. Developing effective mucosal vaccines presents unique challenges due to the distinct characteristics of mucosal surfaces, which necessitate specialized adjuvants and delivery systems not required for injectable vaccines [35]. Table 3 provides an overview of the current status and key features of mucosal vaccines for three major respiratory viruses: SARS-CoV-2, influenza, and RSV. These vaccines, administered intranasally, are designed to induce localized immune responses in the respiratory tract, potentially offering superior protection against virus transmission and infection compared to traditional injectable vaccines.

Table 3. Mucosal vaccine developments for respiratory viruses.

Virus	Mucosal Vaccine	Status	Principal Features	Ref.
SARS-CoV-2	- Convidecia Air™ (CanSinoBio Biologics) - iNCOVACC (Bharat Biotech) - Sputnik V (Russia) - CA4-dNS1-nCoV-RBD - RAZI-COV PARS	- EUA or conditional approval for some vaccines	- Injection vaccines limit upper respiratory immune response, allowing transmission. - Future vaccines may use computational forecasting for broad-spectrum protection.	[36]
Influenza	- FluMist® - FluMist Quadrivalent	- FluMist® approved in 2003 - Excluded in 2017–18 season	- Intranasal vaccines induce strong immune responses and are important for children. - Exclusion due to low efficacy against influenza A in some seasons.	[37,38]
RSV	- None available yet	- Injectable vaccines approved by Pfizer (Abrysvo™) and GSK (Arexvy™)	- Severe reactions in initial trials with inactivated vaccines. - New recombinant injectable vaccines show promise for older adults. - Nasal vaccines still in development.	[39,40]

5. Overview of *Bacillus subtilis* Spore Surface Display (BSSD) Technology

Surface display is a molecular technique that involves anchoring peptides and proteins on the surfaces of bacteriophages, cells, or spores. This technique utilizes natural surface proteins as anchors to target and display desired passenger proteins. Common approaches for surface display include the use of yeast or prokaryotic cells, phages, and bacterial spores. The applications of surface display technology are diverse, encompassing live vaccine development, peptide library screening, antibody production, bio-adsorbent creation for removing harmful chemicals, whole-cell biocatalyst formation, and biosensor development [41,42].

Phage display, pioneered by George Smith in 1985, involves presenting foreign peptides or proteins on the surface of a phage particle. In Smith's technique, the M13 phage is used to generate fusion with the capsid protein p3. Bacterial cell surface display, first reported in 1986, employs the OmpA and LamB proteins of *E. coli*. In yeast surface display, introduced by Boder and Wittrup in 1997, *Saccharomyces cerevisiae* is commonly used. A significant challenge with regard to these systems is the need for chimeric proteins to traverse the cytoplasmic membrane, a feat not achievable by all proteins [43]. This limitation

can be addressed by employing bacterial endospores, leading to the development of BSSD technology [42].

Spore surface display technology leverages spore-forming microorganisms, particularly from the genus *Bacillus*, to anchor functional exogenous proteins onto spore surfaces. *Bacillus*, an aerobic bacterium classified as Generally Recognized As Safe (GRAS), is favored for industrial protein production due to its low nutritional requirements and status as a model Gram-positive microorganism. *Bacillus* spores, formed within the mother cell during sporulation, provide notable advantages for surface display, including the presence of molecular chaperones such as DnaK, GroEL, and small heat shock proteins (sHSPs) like YocM, which assist in the proper folding of heterologous proteins [42]. These chaperones, particularly upregulated under stress conditions like heat shock, play crucial roles in refolding denatured proteins and preventing aggregation within the spore core, thereby ensuring the stability and functionality of displayed proteins [42,44].

This technology is promising for expressing heterologous proteins with high activity and stability. Spores are resistant to harsh environmental conditions, which enhances the stability and utility of exogenous proteins in challenging environments. Furthermore, because spores develop within the cytoplasm of the mother cell, heterologous proteins intended for surface display are localized directly in the spore's structure without the need to cross the cytoplasmic membrane. This direct incorporation simplifies the display process and enhances the stability of the displayed proteins. The applications of spore surface display include enzyme production, oral vaccines, drug delivery, multimeric protein synthesis, and environmental contamination control. Among *Bacillus* species, *B. subtilis* is particularly significant due to its well-characterized spore structure, advanced genetic tools, and extensive genomic data. *B. subtilis* is a Gram-positive, rod-shaped bacterium with low G + C content in its genome. It is commonly found in soil, plant roots, and the gastrointestinal tracts of animals. Its spores' resistance to harsh conditions makes them ideal for surface display technology [45].

5.1. Formation and Structure of B. subtilis Spores

To effectively leverage *B. subtilis* spores in spore surface display technology, understanding their formation and structural characteristics is essential. Spores are critical for this bacterium's survival under adverse conditions and constitute a key component in various biotechnological applications.

5.1.1. Spore Formation

B. subtilis forms spores to endure harsh environmental conditions, a process initiated by nutrient depletion. This triggers histidine kinase sensors (KinA, KinB, KinC) that phosphorylate the transcription factor Spo0A, leading to the regulation of genes associated with asymmetric cell division and spore-specific sigma factors [46,47]. The sporulation process unfolds as follows:

- **Asymmetric Cell Division**: The bacterium undergoes asymmetric division to produce a larger mother cell and a smaller forespore. The forespore develops into the mature spore, while the mother cell supports and protects it throughout development.
- **Sigma Factor Activation**: Sigma factors are proteins that regulate gene expression during sporulation. Specifically, σE is activated in the mother cell, and σF in the forespore, under the control of Spo0A. These factors orchestrate various stages of spore formation.
- **Engulfment**: The mother cell engulfs the forespore, forming a double membrane structure that provides a protective boundary.
- **Protective Layer Synthesis**: Additional sigma factors, σG and σK, facilitate the synthesis of protective layers around the forespore, including the spore crust, cortex, and coat, enhancing resistance to environmental stressors.
- **Dipicolinic Acid Accumulation**: The mature spore accumulates dipicolinic acid, which dehydrates the forespore and increases its resistance to heat, radiation, and chemicals.

- **Maturation and Release**: The mother cell lyses, releasing the mature spore, which can withstand extreme conditions until conditions become favorable for germination.

These steps ensure the production of resilient spores capable of surviving harsh environments and germinating under suitable conditions [7,41,44].

5.1.2. Structure of *B. subtilis* Spores

B. subtilis spores possess a complex structure with multiple protective layers, as described below:

- **Spore Coat**: Composed of over 70 proteins organized into an inner coat, an outer coat, and a crust, this layer protects against chemicals and lysozyme. Key morphogenetic proteins involved in coat formation include SpoIVA, SpoVM, SpoVID, SafA, and CotE.
- **Spore Cortex**: Mainly made of peptidoglycan, the cortex maintains spore resistance and dormancy. Its loose structure enhances resilience, and modifications like O-acetylation reduce sensitivity to lysozyme.
- **Spore Core**: Enclosed by the inner forespore membrane, the core contains essential enzymes, DNA, ribosomes, tRNA, and dipicolinic acid complexed with calcium (CaDPA). This composition dehydrates the core, enhancing heat resistance. Small acid-soluble proteins (SASPs) protect DNA from UV radiation, desiccation, and high temperatures [48,49].

These structural features allow *B. subtilis* spores to endure extreme conditions and remain dormant for extended periods, with the ability to rapidly germinate when conditions improve. Understanding these mechanisms is crucial for optimizing spore surface display technology.

5.2. Critical Elements and Anchor Proteins in the B. subtilis Spore Surface Display System

The BSSD system is a versatile tool used in molecular biology for anchoring exogenous proteins onto spore surfaces. This technique utilizes fusion vectors that include genes encoding both anchor and target proteins. Following transformation of the vector into the *B. subtilis* host strain, sporulation induces the display of heterologous proteins on spore surfaces, enabling them to function in harsh environments. The efficiency of BSSD systems is influenced by several factors, including anchor proteins, target proteins, linkers, expression vectors, and other experimental parameters [50,51]. The following are critical considerations for optimizing BSSD:

Anchor Proteins: Anchor proteins are integral to the BSSD system as they attach exogenous proteins to spore surfaces. These proteins can be linked to exogenous proteins at either the C or N termini. Successful display of exogenous proteins requires selecting appropriate anchor proteins that meet the following criteria.

1. **Strong Anchoring**: The anchor protein should have a robust anchoring domain to securely attach and display the exogenous proteins on the spore surface.
2. **Compatibility**: The anchor protein must be compatible with the exogenous proteins, enabling the formation of functional fusion proteins without adverse interactions.
3. **Protease Resistance**: The fused proteins should be resistant to protease hydrolysis in the extracellular or periplasmic space. Protease resistance can be assessed using protease accessibility tests [44,52].

Several spore coat proteins have been employed as anchor proteins in BSSD, including CotB, CotC, CotE, CotG, CotX, CotY, CotZ, CgeA, and OxdD. Among these, CotB, CotC, CotG, and CotX are particularly notable for their applications in displaying enzymes or antigens for spore-based vaccines, as they are located in the outermost layer of the spore coat. These proteins are characterized by their coiled-coil motifs, which facilitate oligomerization and assembly into a protective layer around the spore, ensuring the stability and resilience of *Bacillus subtilis* spores (Figure 2). CotB was the first spore coat protein utilized for this purpose, with various lengths of CotB successfully anchoring exogenous proteins. CotC and CotG, on the other hand, are versatile proteins that can accommodate

any amino acid sequence as an anchor motif. When selecting anchor proteins for spore-based vaccine applications, the location and abundance of these proteins on the spore surface are crucial aspects with respect to ensuring optimal interaction with the external environment. For more detailed structural information or specific references, please consult the authors of this work [7,41].

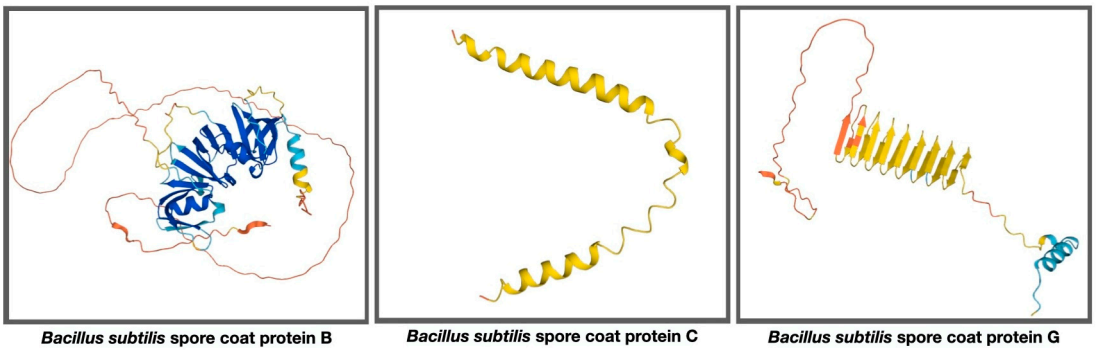

Figure 2. Schematic representations of the 3D structures of *Bacillus subtilis* spore coat proteins B, C, and G.

Table 4 provides a summary of the key characteristics, regulatory factors, assembly and interaction details, and the efficiency and applications of CotB, CotC, and CotG as anchor proteins in the BSSD system.

Linker Peptides: Flexible linker peptides are crucial for addressing the rigidity between anchor proteins and target proteins in spore surface display systems. These peptides can adopt stable helical structures, facilitating effective protein fusion and function. Research has demonstrated that incorporating flexible linker peptides into fusion vectors is an effective strategy for optimizing the functionality of fusion proteins. Commonly used linker peptides include GGGEAAAKGGG, GGGGS, EAAAK, and AAAAAAAAAA. The first two linkers, GGGEAAAKGGG and GGGGS, have been extensively employed in previous studies. The AAAAAAAAAA linker has been specifically utilized to enhance the expression of phytase and β-glucuronidase [53].

Table 4. Protein display on *B. subtilis* spore surface using CotB, CotC, and CotG as anchor proteins.

Anchor Protein	Regulatory Factors	Assembly/Interaction Details	Heterologous Protein Display	Efficiency/Applications	Ref.
CotB	Regulated by σK, GerE, and GerR.	• Highly hydrophilic C-terminal with serine-rich repeats. • Involves CotG and CotH. • Mutation of CotG leads to accumulation of 46 kDa CotB. • CotB exists in 46 kDa (CotB-46) and 66 kDa (CotB-66) forms, likely as a homodimer. • CotG and CotB interact directly, essential for CotB-66 formation.	Urease and other proteins displayed.	Enhances stability and resistance in external environments.	[44,54]
CotC	Regulated by σK, GerE, and SpoIIID.	• Assembles into 32 and 36 kDa forms. • Five distinct protein forms (12–30 kDa) post translationally. • Forms CotC-CotU heterodimer with CotE.	Heat-labile enterotoxin B subunit, urease, ethanol dehydrogenase, beta-galactosidase, proline, enolase, thermostable synthase displayed.	• Nearly ten times more efficient than CotB for internal carrier proteins. • Less efficient than CotB for external exposure (e.g., urease).	[54]

Table 4. *Cont.*

Anchor Protein	Regulatory Factors	Assembly/Interaction Details	Heterologous Protein Display	Efficiency/Applications	Ref.
CotG	Regulated by σK and GerR.	• Assembles primarily into 32 kDa homodimers. • May form 36 kDa homodimers with itself or another CotG protein.	Details of heterologous protein display not specified.	CotG interaction with CotB essential for CotB-66 formation.	[44,54]

5.3. Strategies of B. subtilis Spore Surface Display Systems

The use of *B. subtilis* spores for protein surface display was first reported by Isticato et al. in 2001 [55]. This technique involves constructing a recombinant expression vector containing both a target gene and a spore coat protein gene, with the expression of the vector being driven by its respective promoter. The vector is then introduced into the host strain. During sporulation in a challenging culture medium, the exogenous protein is expressed on the spore surface and acquires the spores' inherent stress resistance [7].

There are two primary methods for displaying antigens on *B. subtilis* spore surfaces.

1. **Recombinant Approach:** This method involves genetically modifying the bacterial genome to express a target protein fused with a spore coat protein. During spore formation, this fusion protein becomes integrated into the spore coat, allowing for efficient presentation of the heterologous proteins using standard molecular biology techniques. The recombinant approach offers significant advantages for vaccine development, including the following:

- **Specificity:** It enables precise incorporation of target antigens into the spore coat, ensuring that the vaccine presents the desired immunogenic proteins.
- **Stability:** Antigens remain intact and functional within the spore, which is critical for maintaining vaccine efficacy during storage and administration.
- **Consistency:** It guarantees uniform antigen presentation, essential for generating reliable and effective immune responses against respiratory pathogens.

This approach is particularly beneficial for creating vaccines that require precise and stable antigen delivery [56,57]. Common fusion strategies in the recombinant approach include fusions at the N-terminus, C-terminus, or within the Cot protein (sandwich fusions) [57].

2. **Non-Recombinant Approach:** This method involves adsorbing a purified protein directly onto the surface of unmodified spores. It accommodates larger quantities of protein compared to the recombinant method and avoids the use of genetically modified organisms, potentially simplifying applications in animal or human settings [52].

Table 5 compares the advantages of the recombinant approach over the non-recombinant approach, highlighting its superior precision, stability, consistency, and versatility in spore surface display technology [56,57].

Table 5. Comparison of recombinant and non-recombinant approaches for spore surface display [56,57].

Aspect	Recombinant Approach	Non-Recombinant Approach
Specificity and Precision	High precision in gene insertion and protein expression	Lower precision; relies on external attachment methods
Stability and Integration	Long-term genetic stability and integration	Often temporary; relies on physical or chemical attachment
Protein Folding and Functionality	Enhanced by host's molecular chaperones	May not benefit from cellular machinery for proper folding
Consistency and Reproducibility	Consistent and reproducible protein display on spores	Variability in protein attachment and display

Table 5. *Cont.*

Aspect	Recombinant Approach	Non-Recombinant Approach
High Throughput Potential	Scalable for mass production with uniform protein expression	Potentially lower throughput due to inconsistent attachment
Avoidance of Cross-Linking Agents	No need for cross-linking agents, reducing toxicity risk	Often requires chemical agents, which can introduce modifications
Regulatory Advantages	Easier to meet regulatory standards for safety and efficacy	May face challenges with regulatory compliance
Versatility and Customization	High versatility for genetic modifications and customization	Limited customization; relies on external protein characteristics

6. BSSD Technology in Mucosal Vaccine Development

Mucosal infections, including those affecting the respiratory tract, represent a significant global health challenge. Ideally, mucosal vaccination strategies that can block infections at their entry points are preferable to other prevention methods. Recombinant bacterial spores displaying foreign antigens have shown considerable promise in inducing protective immune responses and are emerging as effective delivery systems for mucosal vaccines. Specifically, *Bacillus subtilis* spores, with their ability to maintain antigen stability on their surfaces, coupled with their proven safety record and resistance to harsh conditions, are well-suited for oral and nasal vaccine delivery. These spores can traverse the gastrointestinal tract (GIT) barrier, making them excellent candidates for targeting the mucosal surfaces of the respiratory tract [58].

Table 6 highlights the advantages of employing BSSD technology for developing mucosal vaccines against respiratory viruses.

Table 6. Advantages of the spore surface display platform for respiratory virus vaccines [56,57].

Advantage	Description
Targeted Immune Response	**Mucosal Immunity:** Induces local immune responses in the respiratory tract, the primary site of infection. **Systemic Immunity:** Provides strong systemic immune responses for comprehensive protection.
Enhanced Stability	**Thermal Stability:** Spores are resistant to extreme temperatures, eliminating the need for cold chain storage. **Extended Shelf Life:** Vaccines remain viable for long periods, facilitating stockpiling and availability.
Ease of Administration	**Oral Delivery:** Non-invasive oral vaccines are more acceptable and easier to administer, ideal for mass-vaccination campaigns. **Intranasal Delivery:** Effective for inducing mucosal immunity directly at the infection site.
Scalability and Cost-Effectiveness	**Scalable Production:** Simple and cost-effective production processes can be easily scaled up during pandemics. **Low Production Costs:** Reduces overall vaccine costs, making it accessible for low- and middle-income countries.
Broad-Spectrum and Long-Lasting Protection	**Durable Immune Response:** Induces long-lasting immunity, reducing the need for frequent boosters. **Cross-Protective Antigens:** Can be engineered to present multiple antigens for broad protection against various strains.
Safety Profile	**Non-Pathogenic Nature:** Uses safe, non-pathogenic spores such as *Bacillus subtilis*. **Low Risk of Reversion:** Unlike live-attenuated vaccines, spores do not revert to virulence, enhancing safety.

Table 6. *Cont.*

Advantage	Description
Versatility and Customizability	**Platform Technology:** Versatile platform adaptable to different pathogens. **Combination Vaccines:** Offer potential to develop combination vaccines for multiple respiratory pathogens.
Adjuvant Properties	**Self-Adjuvating:** Inherent adjuvant properties of spores enhance immune response without additional adjuvants.
Innovative Delivery Systems	**Microneedle Patches:** Can be integrated with microneedle patches for painless and easy administration.
Adaptability	**Rapid Modification:** Can be quickly modified to respond to emerging viral strains or new respiratory viruses.

Numerous studies have demonstrated that *B. subtilis* spores are highly effective carriers for vaccines administered both nasally and orally (see Table 7). These spores can display various antigens on their surfaces or within their cells, effectively inducing both systemic and mucosal immune responses in animal models [58,59]. For example, spore coat proteins like CotB and CotC, among others, can significantly enhance immune responses. Additionally, these spores can stimulate a range of responses, including systemic IgG, mucosal sIgA, and cytokines such as IFN-gamma and TNF-alpha, which are critical for robust immune protection [60,61] (Figure 3).

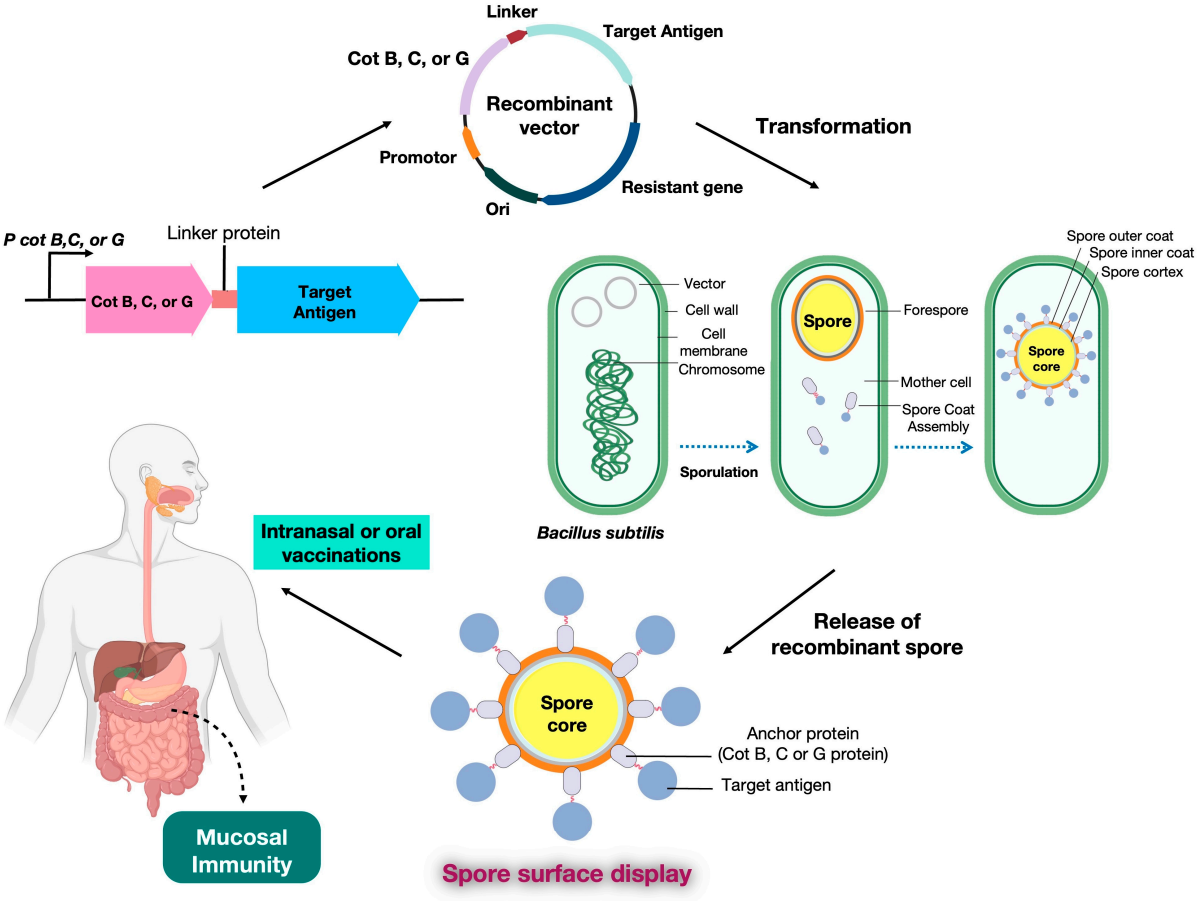

Figure 3. Schematic representation of BSSD technology for vaccine development. The recombinant vector is transformed into *Bacillus subtilis*, enabling antigen expression on the spore surface during sporulation. These spore-based vaccines can be administered orally or nasally, targeting mucosal immunity and offering a stable, immunogenic platform for vaccine delivery.

Table 7. Summary of studies using bacterial spores as vaccine carriers.

Vaccine Target	Bacterial Species	Spore Surface Protein	Expression Strategy	Immunization Route	Immunological Response	Ref.
Tetanus toxin fragment C (TTFC)	*Bacillus subtilis*	CotB	Surface display	Oral	Systemic and mucosal immune responses; specific IgG antibodies	[60]
Heat-labile toxin B subunit (LTB)	*Bacillus subtilis*	CotC	Surface display	Oral	Systemic and mucosal immune responses	[61]
Various antigens (e.g., LTB, anthrax PA)	*Bacillus subtilis*	CotB, CotC	Spore surface and germinated spore	Oral	Systemic IgG, mucosal sIgA, Th1 cytokine responses	[62]
Alpha toxin of Clostridium perfringens	*Bacillus subtilis*	-	Intracellular expression	Oral, inhalation	IgG antibodies; Th1 cytokines; protection against toxin challenge	[63]
C. difficile toxins A and B	*Bacillus subtilis*	-	Surface display	Oral	IgA secretion; protection against C. difficile infection	[64]
Influenza clade 1 viruses	*Bacillus subtilis*	-	Surface display	Intranasal	Systemic and mucosal immune responses; cross-protection	[65]
MPT64 antigen (TB)	*Bacillus subtilis*	CotB	Surface display	Intranasal, booster doses	Th1 immune responses; reduction in bacterial load	[66]
M2e antigen (influenza A)	*Bacillus subtilis*	Spore coat proteins	Surface display	Oral	Specific antibody responses; low immunogenicity	[67]
Plasmodium falciparum CSP	*Bacillus subtilis*	CotC	Surface display	Intranasal	Increased IgG levels; potential malaria vaccine candidate	[68]
Salmonella Pullorum OmpC	*Bacillus subtilis*	CotC	Surface display	Oral	IgG and IgA antibodies; cross-protection against Salmonella	[69]
SARS-CoV-2 Spike Protein (RBD)	*Bacillus subtilis*	CotA, CotB, and CotC	Surface display	Oral	Significant levels of IgM, IgG, and IgA antibodies; increased cytokine levels	[70]

7. *B. subtilis* Spore-Based Oral Carriers: A Novel Approach in Drug Delivery Systems

Recent advancements in drug delivery have highlighted the use of small particle sizes and biodegradable materials to allow enhanced drug uptake, targeted delivery, and improved bioavailability, particularly for poorly soluble drugs [71]. Among these innovations, bacterial minicells, especially those derived from *B. subtilis* spores, have emerged as promising drug delivery systems [72]. The cost-effectiveness of mass-producing *B. subtilis* spores, combined with their inherent adjuvant properties that enhance the therapeutic effects of delivered drugs, underscores their practicality for drug delivery applications [73].

The oral route is preferred for drug administration due to its convenience and high patient compliance. However, creating an effective oral drug delivery system is challenging due to the complex environment of the GIT. An optimal oral delivery system must withstand the acidic and enzymatic conditions of the stomach and navigate biological barriers like mucus and the epithelial lining, which can impede drug absorption. Conventional methodologies for oral drug delivery, such as solid dosage forms (tablets and capsules), liquid formulations, and traditional nanoparticles, face significant limitations. Many of these systems struggle to maintain drug stability in the harsh GIT environment, often leading to reduced bioavailability. Furthermore, the presence of mucus can trap particles, preventing them from reaching the epithelial surface for absorption. Traditional approaches may also lack targeted delivery capabilities, resulting in systemic side effects and a decrease in therapeutic efficacy. Despite these challenges, oral delivery remains desirable for achieving both systemic and localized therapeutic effects [73,74].

Probiotic *B. subtilis* spores offer unique structural advantages as drug carriers. The spore capsid (SC), the outermost layer, exhibits exceptional resistance and stability in the GIT [75]. This resilience is due to the diverse protein composition of the SC, which provides a "muco-inert" property that prevents entrapment in the mucus layer. Additionally, the SC facilitates multi-receptor-mediated endocytosis, promoting efficient drug transport across the epithelial barrier and into the bloodstream. Notably, the presence of cysteine residues and sulfhydryl groups in the SC enhances its muco-inert property by cleaving disulfide bonds in mucus glycoproteins. These distinctive properties have inspired the development of biomimetic spore nanoplatforms designed to overcome mucosal and epithelial barriers, enabling more efficient drug delivery [73]. For example, researchers have successfully reconstituted artificial spore-like particles from the *B. subtilis* spore coat layer. These particles, which can be easily modified to carry drugs or other therapeutic molecules, represent a new frontier in targeted drug delivery and other medical applications [76].

In conclusion, *B. subtilis* spores offer significant promise as a platform for drug delivery. Their inherent stability, adjuvant properties, and ability to overcome biological barriers in the GIT position them as an attractive option for oral drug delivery systems. Moreover, the development of biomimetic spore nanoplatforms expands the potential of Bacillus spores in targeted drug delivery and other therapeutic applications.

7.1. Utilizing Nonrecombinant and Recombinant Spore Display Systems for Drug Delivery Applications

B. subtilis spores have emerged as highly effective drug delivery vehicles due to their intrinsic negative charge and hydrophobic characteristics, which allow them to efficiently adsorb and bind protein antigens, such as the alpha toxin of *Clostridium perfringens* and the tetanus toxin of *C. tetani* [77]. These spores also demonstrate the ability to adhere to viral particles; for example, intact H5N1 virions have been successfully adsorbed onto killed *B. subtilis* spores. When used in the nasal vaccination of mice, this approach provided complete protection against H5N1 challenges [65].

In 2018, researchers developed an innovative *Bacillus*-spore-based oral drug delivery system for colon cancer treatment, where curcumin was covalently linked to the spore's outer coat along with folate. This system exhibited colon-specific drug release, improved curcumin bioavailability, and significant anti-tumor activity, further highlighting the potential of spores for targeted drug delivery in colon cancer therapy [78].

More recently, a clinical trial evaluated the efficacy of nasal-spraying probiotics containing *Bacillus* spores for treating acute respiratory symptoms in children infected with RSV. The probiotic treatment resulted in rapid symptomatic relief, a reduction in viral load and co-infecting bacteria, and the modulation of cytokine release. These findings suggest that nasally sprayed probiotics could serve as an effective treatment for pediatric acute respiratory tract infections (ARTIs), particularly in the absence of specific vaccines or pharmacological therapies. Nasally sprayed *Bacillus* spores (LiveSpo Navax) have shown promise as a safe and effective treatment for RSV-induced ARTIs in children [79].

Overall, both nonrecombinant and recombinant display systems utilizing *B. subtilis* spores demonstrate significant potential for various drug delivery applications, particularly in treating respiratory infections. Their versatility and safety and the ability to engineer *Bacillus* spores for targeted drug delivery underscore their promise as a platform for developing innovative therapeutic approaches, including vaccines, cancer therapies, and treatments for infections.

7.2. The Safety Aspects of B. subtilis Spores for Drug Delivery

The safety profile of Bacillus spores, particularly those of *B. subtilis*, is well-established, making them a highly attractive candidate for drug delivery systems. *B. subtilis* is classified as GRAS and is non-toxic, ensuring it does not cause disease in humans or animals [80]. Additionally, *B. subtilis* spores are highly resilient, capable of withstanding environmental stresses such as heat, radiation, and desiccation. This robustness ensures that they retain functionality under harsh conditions, thereby maintaining safety across various applications [81]. Once their therapeutic function is fulfilled, *B. subtilis* spores are naturally degraded by the body, minimizing any long-term impact on the host [82]. Their interaction with the immune system is particularly advantageous for vaccine delivery, as it effectively stimulates a beneficial immune response without provoking adverse reactions, inflammation, or other harmful effects [83].

A critical safety feature of engineered *B. subtilis* spores is their genetic stability, which ensures that inserted genes remain intact without mutating or transferring to other organisms. This stability is essential for maintaining safety in therapeutic applications [84]. Moreover, numerous studies and clinical trials have confirmed the safety of *B. subtilis* spores in a range of applications, from nasal sprays for respiratory infections to oral delivery systems for cancer therapy. These trials have shown minimal side effects and good tolerability in patients [85]. Overall, the combination of safety, versatility, and effectiveness underscores the potential of *B. subtilis* spores as a platform for innovative drug delivery systems.

8. Future Perspectives and Challenges

The development of BSSD-based vaccines and drug delivery systems presents several challenges and opportunities for innovation. A critical aspect is ensuring effective and stable antigen expression on the spore surface. This requires optimizing genetic constructs and employing molecular chaperones to enhance expression levels. Stability studies are crucial for maintaining antigen integrity over time, which is vital for the efficacy of both vaccines and drug delivery systems [86,87].

Achieving an efficient and uniform display of antigens or therapeutic molecules on the spore surface is another significant challenge. To address this, researchers utilize appropriate anchoring motifs and optimized fusion proteins. Techniques such as electron microscopy are employed to confirm successful surface display. For drug delivery, it is equally important to ensure that the therapeutic agent is uniformly distributed and retains its activity on the spore surface [88].

Another difficulty lies in ensuring consistent immune responses across different individuals or animal models. To mitigate this issue, well-characterized animal models are employed to evaluate immune responses, and adjuvants are included to enhance immunogenicity. Standardizing vaccine formulations is essential to reduce variability in immune responses. In drug delivery, the challenge is to maintain consistent therapeutic efficacy

across diverse populations, which may necessitate personalized approaches or adjustments to formulations [89].

Scaling up production while maintaining quality and consistency poses additional challenges. This requires the development of robust fermentation and purification protocols, coupled with stringent quality control measures and process validation [90]. For drug delivery, scalability also involves ensuring that the production processes can accommodate the diverse range of therapeutic agents that BSSD technology may deliver.

A critical concern is developing a cost-effective manufacturing process while ensuring the long-term stability of vaccines or drugs during storage and distribution. Optimizing manufacturing processes can reduce costs and enhance efficiency. Stabilization methods, such as lyophilization, are crucial for maintaining the product's effectiveness during storage [91]. In drug delivery, cost-effectiveness is essential to make advanced therapies accessible, while long-term stability ensures that therapeutic agents remain potent until they reach the patient. Given these benefits, freeze-drying is considered a viable option for pharmaceuticals, biopharmaceuticals, nanomedicines, and novel drug delivery systems [92].

Innovations in targeting specific tissues or cells, such as using nanoparticle delivery systems, could enhance the precision of drug delivery, particularly for respiratory infections where targeting lung tissues may improve treatment outcomes [93,94]. Integrating BSSD technology with personalized medicine approaches could enable the customization of vaccines and drug delivery systems based on individual patient profiles, thereby improving efficacy and reducing side effects [95]. The development of novel formulation technologies, such as nanotechnology or microencapsulation, could further enhance the stability, efficacy, and delivery of both vaccines and therapeutic agents [96]. As BSSD technology continues to evolve, addressing regulatory and ethical considerations will be crucial to ensure the safe and equitable deployment of these advanced therapies in global healthcare settings. By overcoming these challenges and embracing future innovations, BSSD technology holds the potential to revolutionize vaccine development and drug delivery, offering novel therapeutic options for respiratory infections and beyond.

9. Conclusions

BSSD technology represents a cutting-edge advancement in vaccine development, offering a unique blend of stability, resilience, and efficient antigen presentation. The intrinsic durability of bacterial spores facilitates the creation of robust and long-lasting mucosal vaccines, particularly for respiratory infections, which are notoriously challenging to control due to variability in immune responses and the diversity of pathogens. While the potential applications of BSSD are promising, significant challenges remain, including the optimization of antigen stability, the assurance of safe and effective delivery, and the scaling up of production processes. Future research should prioritize overcoming these obstacles, refining spore display systems, and identifying new antigen targets to fully harness the potential of BSSD technology in combating respiratory infections.

Beyond vaccine development, BSSD technology also shows considerable promise in drug delivery systems. The stability and resistance of bacterial spores can be exploited to create innovative drug delivery platforms, particularly for oral vaccines and therapeutics, where controlled release and targeted delivery are crucial. Integrating BSSD with advanced drug delivery strategies could enhance the bioavailability and efficacy of therapeutics, making treatments more accessible and effective for patients.

Overall, BSSD technology offers a promising avenue for the development of next-generation vaccines and therapeutics, leveraging the unique properties of bacterial spores to enhance both efficacy and stability. As research continues to advance, BSSD has the potential to become a cornerstone in advanced vaccine and drug delivery strategies, providing innovative solutions to some of the most pressing public health challenges related to respiratory diseases and beyond.

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