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Optimization of a Lyophilized BCG-Mannan Vaccine Formulation: HPLC Validation and Stability Assessment

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ABSTRACT

Background: Tuberculosis (TB) remains a cause of infectious disease mortality, despite the availability of the Bacillus Calmette–Guérin (BCG) vaccine, which is effective for severe childhood TB but less effective for adult pulmonary TB. Therefore, improving the physicochemical stability and immunogenicity of the BCG vaccine is important in TB vaccine development. This study aimed to develop a lyophilized BCG-mannan formulation, hypothesizing that purified mannan will enhance its properties and immune responses compared to standard BCG.

Methods: Mannan, a polysaccharide from *Mycobacterium bovis*, was assessed for its potential stabilizing and immunomodulatory effects in a lyophilized BCG-mannan formulation. A design of experiments approach optimized the formulation. A validated high-performance liquid chromatography (HPLC) method was developed to analyze purified mannan, evaluating its physicochemical properties and long-term stability. The formulation immunogenicity was tested in male BALB/c mice using an enzyme-linked immunospot assay to measure antigen-specific IFN-γ-producing splenocytes.

Results: The reverse-phase HPLC method exhibited excellent performance, with a linearity of 80–120 µg/mL (R^2 = 0.999), an accuracy range of 98.66-103.34%, and a coefficient of variation below 2%. The optimal formulation consisted of 2.5% mannan, 7.5% trehalose, and 4% mannitol, retaining over 90% bacterial viability post-lyophilization and showing 24 months of stability. Mice immunized with this formulation indicated a significantly higher frequency of IFN-γ-producing splenocytes compared to those receiving the conventional BCG ($p < 0.01$)

Conclusion: The optimized lyophilized BCG-mannan formulation demonstrated significant immunogenicity and stability, recommending further preclinical investigations. The validated HPLC method proves reliable for quality control of purified mannan in vaccine formulations.

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1. INTRODUCTION

Tuberculosis (TB) is a leading cause of death from a single infectious agent worldwide, even with the extensive use of the Bacillus Calmette–Guérin (BCG) vaccine. According to the World Health Organization (WHO), TB poses a substantial global health burden, particularly in low- and middle-income countries. In 2024, an estimated 10.7 million people developed TB, and 1.23 million died from the disease, underscoring the continuing need for improved preventive strategies[1]. Although BCG effectively protects against severe childhood TB, its efficacy in adults is inconsistent, highlighting the need for improved vaccine formulations capable of eliciting stronger and more durable immune responses[2,3].

The limitations of BCG have stimulated interest in complementary strategies to improve its immunological performance[3]. Incorporating immunomodulatory components into vaccine formulations may enhance antigen uptake and processing, as well as its presentation by antigen-presenting cells[4,5]. Likewise, appropriate formulation and stabilization strategies are crucial for preserving vaccine quality during storage[6,7]. Thus, developing improved BCG formulations requires attention to both immunological efficacy and quality attributes.

Recent advances in vaccine formulation have demonstrated that the rational selection of stabilizing excipients can improve vaccine stability and immunogenicity. Among carbohydrate-based biomaterials, mannan is noteworthy for its interactions with mannose receptors and C-type lectin receptors found on antigen-presenting cells, including dendritic cells and macrophages. This interaction can enhance antigen uptake and stimulate immune activation, rendering carbohydrate-based adjuvants particularly relevant for vaccine design[4,5,8].

Freeze-drying (lyophilization) is frequently used to improve the long-term stability of live attenuated vaccines by reducing residual moisture, thus preserving microbial viability during storage and transportation[7]. However, optimizing lyophilized BCG formulations is challenging because inappropriate excipient compositions or processing conditions may adversely affect bacterial survival, cake integrity, and reconstitution characteristics[6,9]. Research has demonstrated that formulation composition and lyophilization conditions substantially influence the physical characteristics and viability of BCG preparations. For instance, optimizing a recombinant BCG formulation and its freeze-drying cycle has improved cake integrity and bacterial survival during accelerated stability testing[6]. Studies applying the Taguchi method have demonstrated that particular stabilizers could improve the thermal stability and viability of lyophilized BCG[9]. This method, in particular, is valuable for screening and optimizing formulations when multiple factors need evaluation with a limited number of experimental runs[9]. Nonetheless, successful formulation optimization does not ensure the suitability of a vaccine; the final formulation must also maintain a consistent composition and acceptable stability throughout its shelf life.

Long-term stability is critical in vaccine development because changes in vaccine characteristics during storage may affect the quality, potency, and suitability of the product. The WHO guidelines on stability evaluation of vaccines emphasize the importance of establishing vaccine stability through empirical methods, detailed in WHO Technical Report Series No. 962, Annex 3[2,7]. Hence, assessing the long-term refrigerated stability of lyophilized BCG formulations is important, as preserving bacterial viability and formulation integrity during storage is vital[6,7]. Reliable analytical methods are essential for quality control during vaccine development. High-performance liquid chromatography (HPLC) remains a robust analytical technique for quantitatively determining and characterizing carbohydrate components, particularly when appropriate derivatization or detection strategies are employed[10,11]. In particular, precise quantitative analytical characterization is necessary for purified mannan preparation intended for incorporation into a vaccine formulation to ensure consistency and proper dosing. The development and validation of an analytical procedure are distinct stages in the analytical lifecycle. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q14 guideline provides a science- and risk-based framework for developing analytical procedures, whereas ICH Q2(R2) offers the validation process and necessary performance characteristics. These guidelines collectively provide a solid basis for developing and validating an HPLC method for quantitative analysis[12] (ICH Q2(R2): https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline).

Despite evidence supporting mannan as an immunomodulatory carbohydrate and studies on BCG formulations, an important knowledge gap persists. Previous investigations have primarily focused on aspects such as the immunogenicity of mannan-BCG combinations[8], optimization of BCG stabilizers and lyophilization conditions[6,9], or analytical characterization of carbohydrate preparations[10,11]. However, there is a lack of integrated studies that combine quantitative analytical control of purified mannan derived from *Mycobacterium bovis* BCG, systematic formulation optimization using a Taguchi L16 DoE approach, long-term stability assessment, and

evaluation of immune responses in the same development program. This integration is important because analytical quality, formulation performance, storage stability, and biological activity are interconnected factors affecting vaccine quality.

The present study aimed to establish an integrated development and characterization program for a lyophilized BCG-mannan formulation. Our objectives included validating an HPLC method for mannan quantification, optimizing the BCG-mannan composition, evaluating long-term stability, and measuring purified protein derivative (PPD)-specific cellular immune responses using an enzyme-linked immunospot (ELISpot) assay. By combining these elements, the study seeks to evaluate the quality, stability, and immunological performance of the optimized BCG-mannan formulation, hypothesizing that incorporating purified BCG-derived mannan will improve physicochemical stability and cellular immunogenicity compared to conventional BCG formulations.

2. MATERIALS AND METHODS

2.1. Study design

This study comprised five consecutive experimental phases: (i) purification of mannan from *M. bovis* BCG Pasteur strain 1173P2, following a previously validated purification procedure[8]; (ii) development and validation of an HPLC analytical method for the quantitative determination of purified mannan in accordance with the ICH Q2(R2) guideline(ICH Q2(R2) (https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline); (iii) optimization of a lyophilized BCG-mannan formulation using a Taguchi L16 orthogonal array design based on Quality by Design principles[12]; (iv) physicochemical characterization and long-term stability evaluation of the optimized formulation, according to WHO recommendations for freeze-dried BCG vaccines[7]; and (v) assessment of antigen-specific cellular immune responses in BALB/c mice using an IFN-γ ELISpot assay[13,14].

2.2. Materials and mannan purification

All chemicals used for chromatographic analysis and vaccine formulation were of analytical or HPLC grade. Purified mannan was isolated from *M. bovis* BCG Pasteur strain 1173P2 supplied by the Pasteur Institute of Paris (France) using a previously validated extraction and purification method[8]. The purification process included sequential removal of lipids and enzymatic digestion of glucans, proteins, DNA, and RNA, followed by dialysis and chromatographic purification. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to check for protein contamination in the purified mannan. No molecular-weight or monosaccharide composition values were assigned to avoid introducing unsupported characterization data. The purified mannan was stored at 2–8°C until further analysis.

2.3. Preparation of BCG vaccine bulk

The BCG Pasteur strain 1173P2 was obtained from the Pasteur Institute of Iran (Tehran, Iran). The vaccine bulk was prepared under aseptic conditions using validated manufacturing procedures. Briefly, the working seed culture was grown in Sauton medium at 37 °C and harvested during the logarithmic growth phase. The biomass was filtered to obtain semi-dry bacterial mass and suspended in a sterile 1.5% sodium L-glutamate solution (Merck, Germany) to achieve the desired concentration for formulation. Before lyophilization, each batch was evaluated for identity, purity, sterility, and viable bacterial count according to WHO recommendations for BCG vaccine production and the quality control specifications of the institution[2,13].

2.4. Instrumentation

Chromatographic analyses were performed using an Agilent HPLC system equipped with a refractive index (RI) detector. Lyophilization was carried out using a laboratory-scale freeze dryer (Martin Christ, Germany). Analytical weighing was conducted using a Sartorius analytical balance.

2.5. HPLC method development and validation

An HPLC method was developed to quantitatively determine purified mannan isolated from *M. bovis* BCG. Mannan (0.7 mg/mL) was dissolved in ultrapure water and filtered through a 0.22-µm membrane filter. Chromatographic separation utilized an Econo-Pac C18 column at 40 °C, employing a mobile phase of ultrapure water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.05% trifluoroacetic acid (solvent B), at a flow rate of 1.0 mL/min and an injection volume of 20 µL. The gradient program included 0–5 min (95% A and 5% B), 5–15 min (linear gradient to 50% A and 50% B), 15–18 min (isocratic at 50% A and 50% B), and 18–20 min return to initial conditions (95% A and 5% B). The total run time was 20 min, with column equilibration at 1200–1400 psi. Due to the absence of a suitable ultraviolet chromophore, mannan was detected using an RI detector. Method validation followed ICH Q2(R2) guidelines, assessing specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and system suitability. Specificity was confirmed by comparing chromatograms of blank solutions, reference standards, and vaccine matrix samples. Linearity was evaluated using five calibration

standards (80–120 µg/mL)(ICH Q2(R2) (https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline). Accuracy was determined via recovery experiments at 80%, 100%, and 120% of the target concentration. Method precision was evaluated as both repeatability (intra-day precision) and intermediate precision (inter-day precision) using six independent replicate injections under identical conditions, with precision expressed as the percentage coefficient of variation (CV). LOD and LOQ were estimated based on the standard deviation and calibration curve slope. System suitability was assessed before each analytical sequence, evaluating retention time consistency, theoretical plate number, tailing factor, chromatographic resolution, and injection repeatability. Resolution was calculated using the formula Rs = 2(tR2 - tR1)/(W1 + W2). Acceptance criteria were established in accordance with the ICH Q2(R2) guideline, and all analytical parameters met the predefined specifications (ICH Q2(R2): https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline).

2.6. Formulation optimization using Taguchi L16 DoE

Optimization of the lyophilized BCG formulation was performed using a Taguchi L16 DoE approach. This method allowed for efficient evaluation of multiple formulation variables while minimizing experimental runs, focusing on purified mannan, trehalose, and mannitol, which enhance stability during lyophilization and storage[4,14,15]. The Taguchi L16 orthogonal array included three factors at four levels: purified mannan (0, 1.5, 2.5, and 4.0%), trehalose (0, 2.5, 5.0, and 7.5%), and mannitol (0, 2, 4, and 6%). Sixteen experimental combinations were evaluated for bacterial viability, residual moisture content, cake appearance, and reconstitution time. Cake appearance was scored from 1 to 10, with higher scores indicating better cake integrity. Quality attributes followed WHO recommendations for freeze-dried BCG vaccines[7,16]. For Taguchi optimization, the signal-to-noise (S/N) ratio was calculated for each response characteristic, with the "larger-the-better" criterion applied for bacterial viability versus the "smaller-the-better" criterion applied for moisture content and reconstitution time. The final formulation was selected through simultaneous assessment of quality attributes, rather than a single response, with further experiments confirming its efficacy. Each of the 16 Taguchi runs was performed as a single, unreplicated preparation and measurement (n = 1 per run); no within-run replicate measurements were obtained for the L16 screening matrix.

2.7. Lyophilization procedure

Optimized vaccine formulations were prepared by mixing the BCG bulk suspension with the selected stabilizing excipients based on the Taguchi method. After gentle homogenization, aliquots were aseptically dispensed into sterile vials for freeze-drying. Lyophilization was performed using a laboratory-scale freeze dryer. Initially, the shelf temperature was reduced to -40 °C at a cooling rate of ~1 °C/min, maintained for 2 h for solidification. Primary drying occurred at ~0.1 mbar pressure, gradually increasing the shelf temperature from -40°C to -10°C to facilitate ice sublimation. The endpoint of primary drying was indicated by convergence of product and shelf temperature, confirming complete free-ice sublimation. Secondary drying was performed at +25 °C for 4–6 h under reduced pressure to eliminate residual bound water, verified by a steady pressure rise test. After the cycle, vials were vacuum-sealed and stored at 2–8 °C for long-term stability studies. Quality of each lyophilized batch was assessed through visual inspection of cake appearance, residual moisture determination by Karl Fischer titration, reconstitution time, and viable bacterial count, following WHO guidelines for freeze-dried BCG vaccines[2,16]. These specific parameters were suitable for laboratory-scale formulation but need further optimization for industrial production.

2.8. Vaccine characterization and stability evaluation

2.8.1. Vaccine quality characterization

Before lyophilization, the BCG bulk suspension underwent comprehensive quality-control testing to confirm its suitability for vaccine formulation. The identity of the vaccine was verified using validated microbiological procedures, and sterility testing was performed following pharmacopeial requirements for biological products. *Mycoplasma* contamination was assessed using a culture-based method, and all production batches were confirmed to be free of detectable contamination before formulation. The concentration of viable bacteria in the bulk suspension was determined by counting colony-forming units (CFUs) on Löwenstein-Jensen medium, and adjustments were made to meet the predefined manufacturing specifications. Only batches that met all quality-control criteria were used for formulation optimization and subsequent biological evaluation[2].

2.8.2. Determination of bacterial viability

The viability of *M. bovis* BCG was assessed immediately after lyophilization and throughout the stability study. Lyophilized vaccine vials were reconstituted with Sauton medium to achieve the required bacterial concentration, followed by preparation of serial ten-fold dilutions. Aliquots from each dilution were

inoculated onto Löwenstein-Jensen medium and incubated at 37 °C for 21–35 days. CFUs were counted weekly, and bacterial viability was expressed as CFU per vial. As per WHO recommendations, the preservation of viable bacteria was used as a measure of the biological stability of freeze-dried BCG vaccines[2].

2.8.3. Physicochemical evaluation

The physicochemical quality of the lyophilized product was immediately assessed after lyophilization and throughout the storage period. Residual moisture content was determined by Karl Fischer titration, with values below 3% considered acceptable. The appearance of the lyophilized cake was evaluated visually for uniformity, structural integrity, and the absence of collapse, shrinkage, cracking, or discoloration. Reconstitution time was measured after adding the recommended diluent and considered acceptable when complete dissolution occurred within one minute. The pH of the reconstituted vaccine was measured using a calibrated digital pH meter, and visual clarity was assessed immediately after reconstitution[2].

2.8.4. Stability study

Long-term stability studies were conducted following the WHO recommendations and ICH stability guidelines. The optimized lyophilized formulation was stored under recommended refrigerated conditions (2–8 °C) for 24 months. Samples were analyzed at predetermined intervals (0, 3, 6, 12, 18, and 24 months)[15]. At each time point, bacterial viability, residual moisture, pH, cake appearance, sterility, clarity after reconstitution, and reconstitution time were evaluated. The vaccine stability was considered acceptable when all predefined critical quality attributes remained within their established acceptance criteria throughout the study period[2].

2.8.5. Assessment of local reactogenicity

The local reactogenicity of the optimized lyophilized BCG-mannan formulation was assessed following WHO recommendations for evaluating excessive dermal reactivity to BCG vaccines[17]. This assessment was performed at the initial time point (0 month) and after 24 months of refrigerated storage. Six healthy guinea pigs, each weighing at least 250 g and having received no prior treatment that could interfere with the test, were included in the study. The vaccine was administered via the intradermal route using a randomized design. Each animal received 0.1 mL of the reconstituted vaccine, along with its 1:10 and 1:100 dilutions, corresponding to concentrations of 1, 0.1, and 0.01 mg/mL, respectively, at randomly selected skin sites. The corresponding dilutions of a reference preparation were also administered at randomly selected sites on the same animals. The pigs were monitored throughout the observation period for their general clinical condition and local reactions. No overt signs of toxicity, mortality, weight loss, or abnormal local reactions at the injection sites were observed following administration of the BCG-mannan formulation. The diameter of the local lesions at each injection site was measured in millimeters 28 days after administration using a millimeter ruler. These measurements were used descriptively to characterize the local biological response associated with the formulation at the initial and 24-month storage time points. Because this assessment was performed as a descriptive evaluation of local reactogenicity and was not designed or validated as a quantitative comparative potency assay, the lesion diameter was not assigned a predefined potency acceptance criterion[17].

2.9. Immunogenicity assessment

2.9.1. Animals and experimental design

Thirty male BALB/c mice (6–8 weeks old; body weight 17–24 g) were obtained from the laboratory animal center of the Pasteur Institute of Iran. The animals were acclimatized for one week before experimentation and housed under standard laboratory conditions (22 ± 2 °C, relative humidity 50–60%, and a 12-h light/12-h dark cycle) with free access to food and water. The mice were randomly allocated to six groups (n = 5 per group): (i) negative control, (ii) conventional BCG, (iii) lyophilized BCG, (iv) optimized lyophilized BCG-mannan formulation, (v) purified mannan alone, and (vi) excipient control[18]. The immunogenicity experiment was designed as an exploratory study to obtain preliminary estimates of treatment-related PPD-specific IFN-γ responses. Five animals were used per group, considering the 3Rs to reduce animal use. No formal statistical power calculation was performed due to the lack of reliable estimates for the expected effect size and variability for this specific formulation and immunological endpoints. Hence, the immunological findings were considered preliminary and should be interpreted with caution because of the limited sample size. Animals in the BCG-containing groups (conventional and lyophilized BCG and an optimized lyophilized BCG-mannan formulation) received a single intraperitoneal injection of 3×10^5 CFU of *M. bovis* BCG Pasteur strain 1173P2 per mouse. The purified mannan group received 100 µg of purified mannan per mouse. The excipient and negative-control groups received the corresponding control preparations. All injections were administered in a final volume of 100 µL. The doses of viable BCG, purified mannan, and excipients were consistent with the composition of the respective formulations used in the experimental groups. Twenty-eight days after immunization, mice were euthanized, and samples were collected for evaluating the PPD-specific IFN-γ immune response.

2.9.2. IFN-γ ELISpot assay

Splenocytes were isolated through mechanical disruption of spleen tissue under sterile conditions. Following erythrocyte lysis and washing, the cells were resuspended in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (Merck), penicillin (100 U/Ml), streptomycin (100 µg/mL; NovinPharma, Iran), and 2 mM L-glutamine (Merck). For the ELISpot analysis, 2×10^5 splenocytes were seeded into each well of anti-mouse IFN-γ ELISpot plates and stimulated with PPD (10 µg/mL). Culture medium alone and concanavalin A (5 µg/mL) served as negative and positive controls, respectively. After incubation in a humidified atmosphere containing 5% CO2 at 37 °C for 18–20 h, the plates were processed according to the manufacturer's instructions. The number of spot-forming units (SFU) was quantified using an automated ELISpot reader (AID GmbH, Germany). Background spot counts obtained from unstimulated wells were subtracted from those of antigen-stimulated wells, and results were expressed as SFU per 10^6 splenocytes[19]. This assay was selected as the primary immunogenicity readout to evaluate PPD-specific cellular responses induced by the optimized formulation. While IFN-γ is an important component of antimycobacterial cellular immunity, it alone is not a validated correlate of protection for TB vaccines.

2.10. Statistical analysis

Experimental design and optimization were performed using Minitab version 17, while statistical analyses were done with GraphPad Prism version 9.0. The Taguchi L16 orthogonal array (three factors, four levels each) comprised 16 experimental runs, and each run was performed once as a single-run, unreplicated design; consequently, the response values reported for the L16 matrix (Table S1) represent individual single measurements rather than means of replicate measurements. Because no within-run replicates were available, the resulting ANOVA error term (Table S2) reflects unexplained residual variation in the fitted model, including higher-order interactions and experimental noise not assigned to the three main factors, rather than variance estimated from independent experimental or biological replicates. Therefore, the reported *p* values were interpreted as model-based statistical indicators and were not considered as evidence of independent biological or technical replicate variability. Accordingly, no replicate-based pure-error estimate or replicate-based inferential ANOVA was performed for the L16 Taguchi screening matrix; the ANOVA and associated *p* values were used solely to rank the relative influence of the formulation variables within the screening design, and not as confirmatory tests of statistical significance. ANOVA evaluated the effects of formulation factors on bacterial viability, moisture content, and reconstitution time, accounting for total variation. The Taguchi S/N ratio was used to identify favorable factor levels, while cake appearance was analyzed separately. The final formulation was confirmed through three independent confirmation experiments (n = 3), for which results are presented as mean ± SD, distinct from the single-run L16 optimization matrix described above. For immunogenicity, one-way ANOVA and Tukey's test were used, with a $p < 0.05$ considered statistically significant.

3. RESULTS

3.1. Validation of the HPLC method for quantitative determination of purified mannan

The developed HPLC method successfully separated purified mannan from formulation-related components, producing a sharp and symmetrical chromatographic peak with a mean retention time of 8.0 ± 0.2 min. No interfering peaks were observed in blank samples, excipient solutions, or vaccine matrix preparations, confirming the high specificity of the analytical procedure. Excellent linearity was obtained over a concentration range of 80–120 µg/mL, with a correlation coefficient of 0.999, demonstrating a strong linear relationship between peak area and analyte concentration (Fig. S1). Method accuracy, determined by recovery experiments at three concentration levels, ranged from 98.66% to 103.34%, indicating excellent agreement between measured and theoretical concentrations. Both intra- and inter-day precision demonstrated coefficients of variation below 2%, confirming the high repeatability and intermediate precision of the analytical method. The calculated LOD and LOQ were 1.25 µg/mL and 3.78 µg/mL, respectively, indicating adequate analytical sensitivity for routine quality control applications. All system suitability parameters fulfilled predefined acceptance criteria. The theoretical plate number exceeded 4000, the tailing factor remained below 2.0, chromatographic resolution was >2, and injection repeatability showed a CV <1%, confirming the reliability and consistency of the developed chromatographic method. The resolution between adjacent peaks was calculated as 4.59, which exceeds the acceptance criterion of >2. Overall, the validated HPLC method satisfied all analytical performance requirements specified in the ICH Q2(R2) guideline and was considered suitable for routine quantitative analysis of purified mannan during vaccine formulation and quality control testing (Table 1).

Table 1. Validation results of the HPLC method for quantitative determination of purified mannan and system suitability parameters

Parameter	Result	Acceptance criteria	Compliance
Retention time (min)	8.0 ± 0.2	Consistent	Pass
Linearity (µg/mL)	80–120	-	Pass
Correlation coefficient (R^2)	0.999	≥0.99	Pass
Accuracy (%)	98.66–103.34	95–105	Pass
Intra-day precision (% CV)	0.42–1.87	≤2	Pass
Inter-day precision (% CV)	1.80–1.97	≤2	Pass
LOD (µg/mL)	1.25	-	Pass
LOQ (µg/mL)	3.78	-	Pass
Theoretical plates	4015	>2000	Pass
Tailing factor	0.87	<2	Pass
Resolution	4.59	>2	Pass

Chromatographic conditions included injection volume: 20 µL; flow rate: 1.0 mL/min; column temperature: 40°C; total run time: 20 min; equilibration time: ≥30 min; gradient program including 0–5 min (95% A and 5% B), 5–15 min (linear gradient to 50% A and 50% B), 15–18 min (isocratic at 50% A and 50% B), and 18–20 min (return to initial conditions); column pressure: 1200–1400 psi; detection method: RI detector; specificity: no interfering peaks were observed at the retention time (RT) of mannan; acceptance criterion: no interference from the blank/placebo. Resolution was calculated as Rs = 2(tR2 – tR1)/(W1 + W2).

3.2. Optimization of the lyophilized BCG-mannan formulation

The Taguchi L16 orthogonal array was used to evaluate the effects of purified mannan, trehalose, and mannitol on the selected quality attributes of the lyophilized BCG formulation. The experimental matrix and response results for all 16 formulation runs are presented in Table S1. Bacterial viability varied across the experimental runs, with intermediate-to-high levels of mannan and trehalose generally yielding better outcomes. Residual moisture and reconstitution time are also dependent on these factors, indicating unique contributions to the physicochemical characteristics of the lyophilized product. Figure 1 illustrates the main effects of the three formulation components on the critical quality attributes.

The screening ANOVA (based on the unreplicated L16 design; see Section 2.10) indicated that trehalose predominantly affected bacterial viability (50.24%), residual moisture (52.99%), and reconstitution time (55.43%), while mannan and mannitol had smaller relative contributions. For bacterial viability, trehalose showed the largest model-based effect (p = 0.026), whereas mannan (p = 0.109) and mannitol (p = 0.371) showed smaller model-based effects at the 0.05 threshold. Conversely, all three components showed strong model-based effects on residual moisture and reconstitution time (p < 0.001). The complete ANOVA results are summarized in Table S2. As the L16 matrix was an unreplicated screening design (Section 2.10), these ANOVA outcomes are reported as indicators of relative factor influence rather than as confirmatory tests of statistical significance. The Taguchi S/N analysis was used to identify the factor-level combinations associated with the most favorable response characteristics (Table S3). The best predicted S/N ratios were 34.87 dB for bacterial viability, -7.37 dB for residual moisture, and -1.19 dB for reconstitution time. For all three parameters, the optimal combination of factors was determined to be 2.5% for purified mannan, 7.5% for trehalose, and 4% for mannitol. The final formulation was selected by considering bacterial viability, residual moisture, cake appearance, and reconstitution time. Confirmation experiments using the predicted optimal formulation demonstrated excellent reproducibility (n = 3, CV <2%), supporting the reproducibility of the optimization process. The optimized formulation resulted in a uniform lyophilized cake with excellent appearance and physical integrity, exhibiting no evidence of collapse, shrinkage, cracking, or discoloration. As shown in Table 2, the optimized formulation had a residual moisture content of 1.15 ± 0.05%, compared to 2.35 ± 0.09% for conventional BCG. It maintained a viable bacterial content of 91.7 ± 1.8%, while conventional BCG had 82.4 ± 2.1%. The reconstitution time was reduced to 0.65 ± 0.08 min compared to 1.8 ± 0.2 min for conventional BCG. The optimized formulation met all predefined acceptance criteria for residual moisture (<3%), viable bacteria (>80%), cake appearance, physical integrity, and reconstitution time (<1 min). Collectively, these findings demonstrate that the Taguchi DoE approach successfully identified an optimized excipient composition that met the specified biological and physicochemical quality attributes of the lyophilized BCG-mannan formulation compared to conventional BCG (Table 2).

3.3. Stability evaluation of the optimized lyophilized BCG-mannan formulation

The long-term stability of the optimized lyophilized BCG-mannan formulation was evaluated over a 24-month storage period at refrigerated conditions (2–8°C).

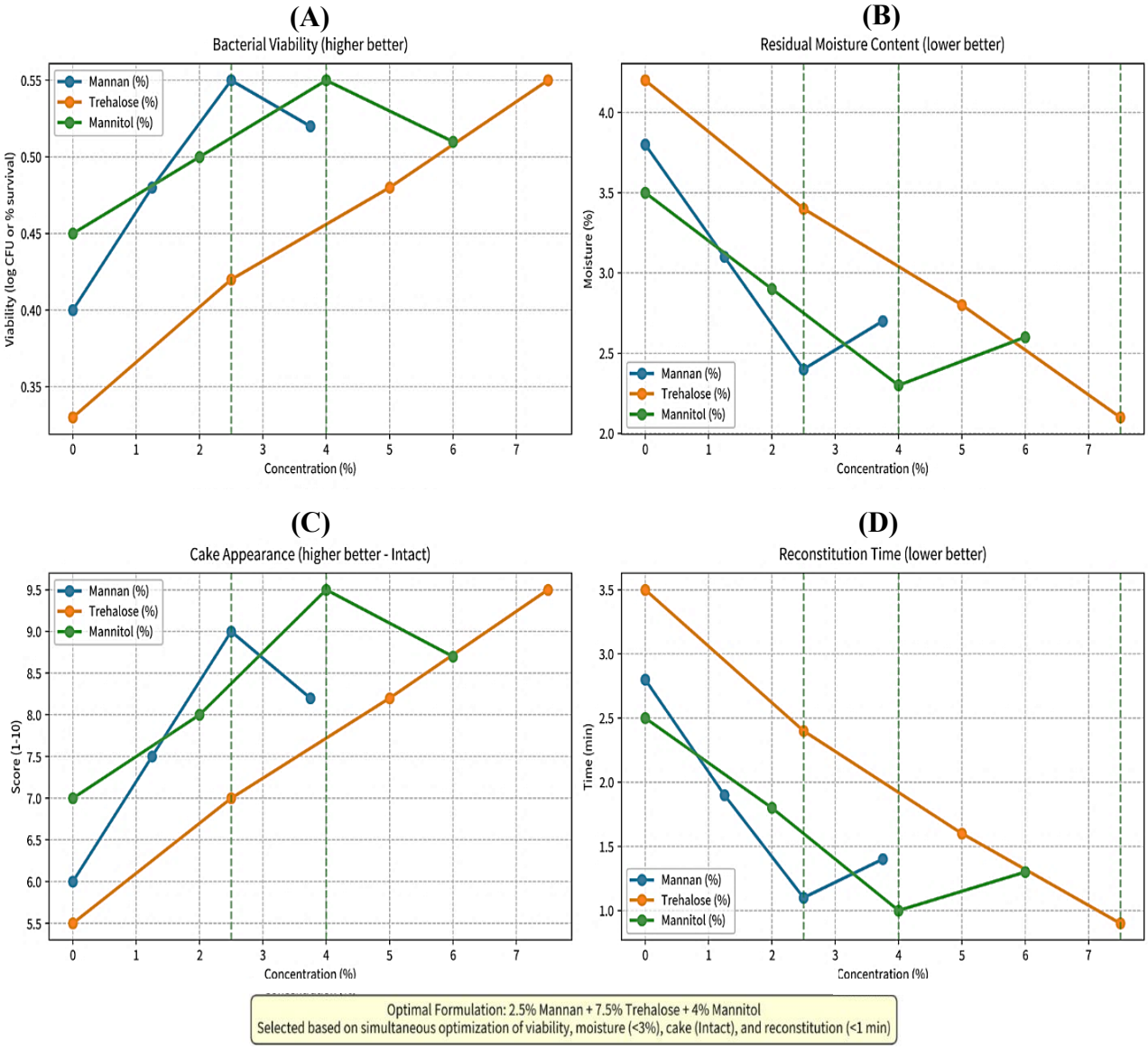

Fig. 1. Main-effects plots obtained from the Taguchi L16 orthogonal array, showing the effects of purified mannan, trehalose, and mannitol concentrations on the critical quality attributes of the lyophilized BCG-mannan formulation. These attributes include bacterial viability, residual moisture content, cake appearance, and reconstitution time. (A) Bacterial viability: increasing mannan concentration up to 2.5% improved bacterial viability, while higher concentrations led to a slight reduction. (B) Residual moisture: formulation factors showed different effects on moisture content, with trehalose contributing to reduced residual moisture across all the tested concentrations. (C) Cake appearance: all formulations showed acceptable cake integrity, with variations among different factor levels. (D) Reconstitution time: concentrations of mannan, trehalose, and mannitol influenced the reconstitution behavior of the formulation. The optimized formulation (2.5% purified mannan, 7.5% trehalose, and 4% mannitol) was selected based on the overall combined quality attributes.

Vaccine quality was assessed at predetermined intervals (0, 3, 6, 12, 18, and 24 months) following the WHO recommendations for freeze-dried BCG vaccines. Figure 2 illustrates the long-term stability profile of the formulation during this period. Throughout the storage period, the optimized formulation maintained stable physicochemical and microbiological characteristics. Viable bacterial counts ($\log_{10}$ CFU/dose) were determined at each time point to monitor BCG viability during storage. The results for viable bacterial counts and viability retention relative to baseline are presented in Table 3. The residual moisture content remained below the acceptance limit of 3%, increasing slightly from 1.15% post-lyophilization to 1.42% after 24 months of storage. While viable bacterial counts showed a gradual decline over time, they consistently remained within the acceptance specifications throughout the 24-month period (Table 3). A visual examination demonstrated that the lyophilized cake retained its original appearance, showing no signs of deterioration. Vaccine sterility was maintained throughout the storage period, with no microbial contamination detected.

Table 2. Comparison of selected quality attributes between the conventional BCG vaccine and the optimized lyophilized BCG-mannan formulation

Quality attribute	Conventional BCG	Optimized lyophilized BCG-mannan formulation	Acceptance criterion
Residual moisture (%)	2.35 ± 0.09	1.15 ± 0.05	<3
Viable bacteria (%)	82.4 ± 2.1	91.7 ± 1.8	>80
Cake appearance	Acceptable	Excellent	Pass
Reconstitution time (min)	1.8 ± 0.2	0.65 ± 0.08	<1
Physical integrity	Acceptable	Excellent	Pass

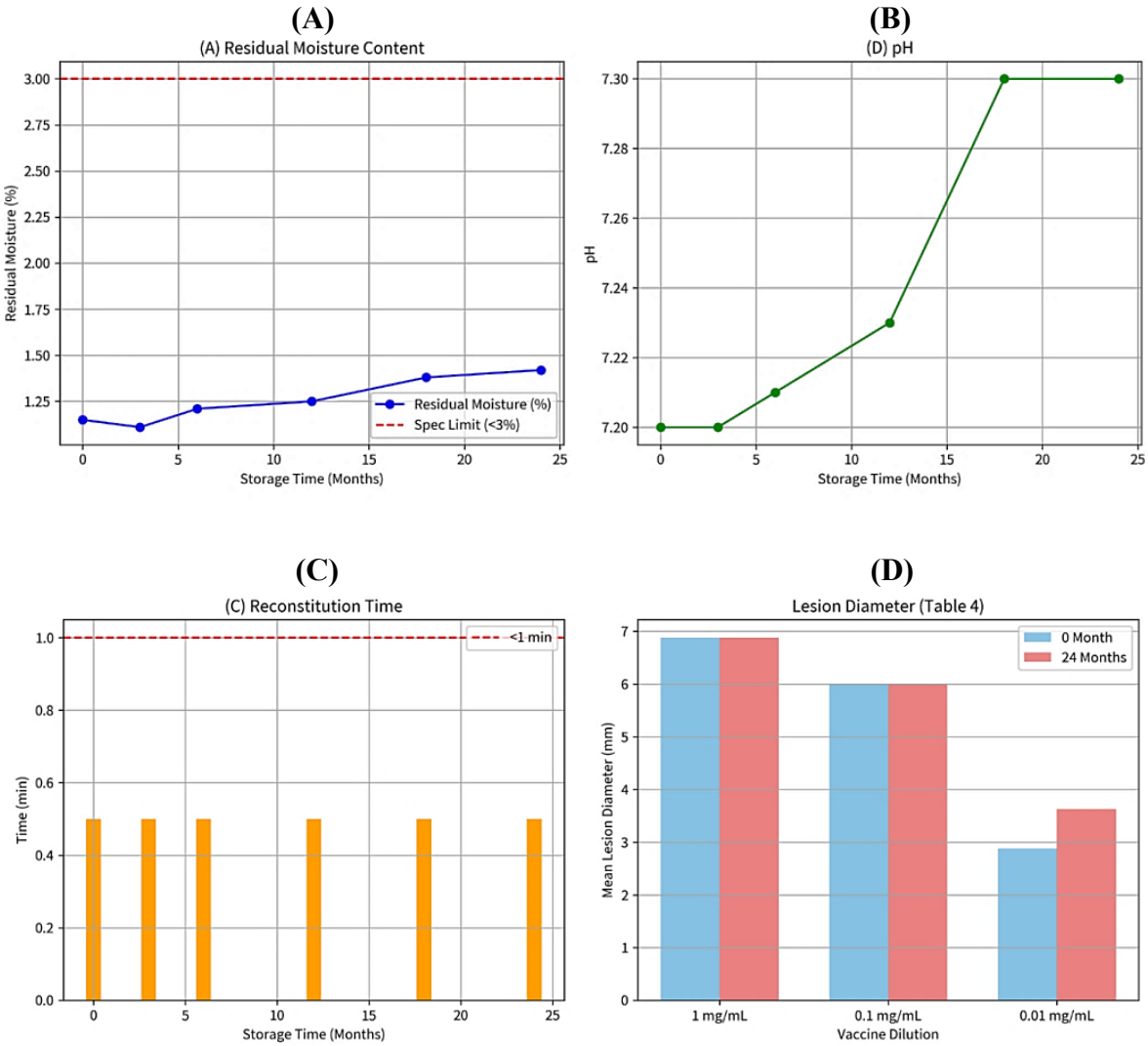

Fig. 2. Long-term stability profile of the optimized lyophilized BCG-mannan formulation during 24 months of refrigerated storage at 2–8 °C. (A) Residual moisture content remained below the predefined acceptance limit of 3% throughout the storage period, increasing from 1.15% at baseline to 1.42% after 24 months. (B) The pH of the reconstituted vaccine remained relatively stable during storage, changing from 7.20 at baseline to 7.30 after 24 months. (C) Reconstitution time remained below 1 min throughout the storage period, indicating the preservation of the rapid reconstitution characteristics of the lyophilized formulation. (D) The bar chart shows the mean lesion diameter following local reaction assessment at baseline (0 month) and after 24 months of refrigerated storage across the tested vaccine dilutions (1, 0.1, and 0.01 mg/mL). All data are presented as mean ± SD, where applicable. Quantitative viable bacterial counts and their corresponding 95% confidence intervals are presented separately in Table 3.

Table 3. Long-term stability results of the optimized lyophilized BCG–mannan formulation during 24 months of refrigerated storage (2–8°C)

Parameter	Acceptance criteria	Month					
		0	3	6	12	18	24
Viable bacterial count (log_{10} CFU/dose)	Within specification	6.516	6.507	6.505	6.502	6.488	6.474
Viability retention (%)	≥predefined limit	100	97.9	97.6	97.0	93.9	90.9
Residual moisture (%)	<3%	1.15	1.11	1.21	1.25	1.38	1.42
pH	Stable	7.20	7.20	7.21	7.23	7.30	7.30
Cake appearance	Intact	Pass	Pass	Pass	Pass	Pass	Pass
Sterility	Sterile	Pass	Pass	Pass	Pass	Pass	Pass
Reconstitution time	<1 min	Pass	Pass	Pass	Pass	Pass	Pass

Following reconstitution, the vaccine formed a homogeneous suspension within 1 min, and pH stability was confirmed, increasing from 7.20 at baseline to 7.30 at month 24. Local reaction measurements were also performed at baseline and after 24 months of refrigerated storage using vaccine dilutions of 1, 0.1, and 0.01 mg/mL. At baseline, the mean lesion diameters were 6.88, 6.00, and 2.88 mm, respectively. After 24 months, the corresponding values were 6.88, 6.00, and 3.63 mm (Table 4). These results demonstrate that the optimized lyophilized BCG-mannan formulation maintained acceptable physicochemical and microbiological characteristics throughout the 24-month refrigerated storage period, according to predefined criteria (Tables 3 and 4).

3.4. Immunogenicity results

3.4.1. IFN-γ ELISpot responses

The immunogenicity of the optimized lyophilized BCG-mannan formulation was evaluated by measuring antigen-specific IFN-γ secretion using the ELISpot assay. Splenocytes isolated from immunized BALB/c mice were stimulated ex vivo with PPD, and the frequency of IFN-γ-producing cells was expressed as SFU per 10^6 splenocytes. As shown in Table 5 and Figure 3, mice immunized with the optimized lyophilized BCG-mannan formulation exhibited a significantly higher frequency of PPD-specific IFN-γ-producing cells than those vaccinated with the conventional BCG formulation. The optimized formulation induced 62 ± 4 SFU/10^6 splenocytes, whereas the conventional BCG vaccine generated 45 ± 3 SFU/10^6 splenocytes, representing an approximately 38% increase in PPD-specific IFN-γ-producing cellular responses ($p < 0.01$). The lyophilized BCG group also demonstrated improved cellular responses compared to the conventional formulation; however, the magnitude of the response was lower than that observed in the optimized lyophilized BCG-mannan formulation group. As expected, animals receiving purified mannan alone, the excipient control, or the negative control showed only minimal background responses. The increased PPD-specific IFN-γ cellular response observed with the optimized formulation may be associated with the presence of purified mannan and the preservation of bacterial viability during the freeze-drying process; however, our study did not directly establish a mechanistic relationship between mannan incorporation and the observed cellular response. Although IFN-γ has an important role in cellular immunity against mycobacterial infections, it is not by itself a validated correlate of protection for TB vaccines. Therefore, any observed increase in IFN-γ response should be interpreted as a sign of antigen-specific cellular immunogenicity, rather than as evidence of protective immunity. The optimized formulation increased PPD-specific IFN-γ cellular responses under the conditions tested; however, protective efficacy was not assessed.

4. DISCUSSION

This study optimized a lyophilized BCG-mannan vaccine formulation and validated an HPLC method for quantitative analysis of purified mannan, while also examining the physicochemical stability of the vaccine during refrigerated storage. Incorporation of purified mannan into the vaccine formulation increased the frequency of PPD-specific IFN-γ-producing splenocytes under the tested conditions. However, these findings do not provide definitive evidence of enhanced protection against TB. To our knowledge, few studies have integrated analytical validation, formulation optimization, stability assessment, and immunological evaluation for mannan-containing lyophilized BCG formulations within a single workflow.

Table 4. Local reaction measurements of the optimized lyophilized BCG-mannan formulation at baseline and after 24 months of refrigerated storage

Storage time (month)	Vaccine dilution (mg/mL)	Mean lesion diameter (mm)
0	1	6.88
0	0.1	6.00
0	0.01	2.88
24	1	6.88
24	0.1	6.00
24	0.01	3.63

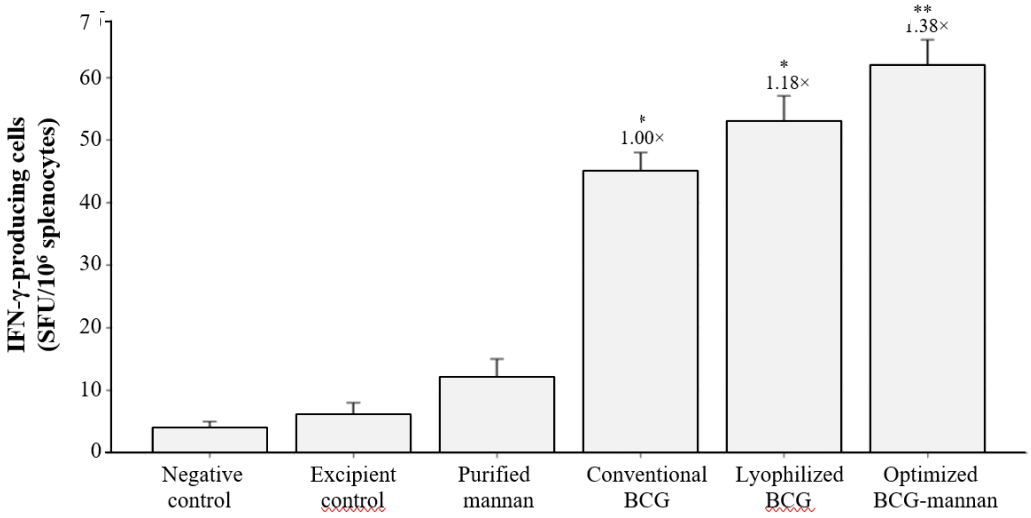

Fig. 3. Antigen-specific IFN-γ ELISpot responses in BALB/c mice immunized with different vaccine formulations. Splenocytes were stimulated ex vivo with purified PPD, and IFN-γ-producing cells were quantified as SFU per 10^6 splenocytes. Data are presented as mean ± SD (n = 5). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Mice immunized with the optimized lyophilized BCG-mannan formulation exhibited the highest frequency of IFN-γ-producing cells (62 ± 4 SFU/10^6 splenocytes), compared to conventional BCG (45 ± 3 SFU/10^6 splenocytes), lyophilized BCG (53 ± 4 SFU/10^6 splenocytes), purified mannan (12 ± 3 SFU/10^6 splenocytes), excipient control (6 ± 2 SFU/10^6 splenocytes), and negative control (4 ± 1 SFU/10^6 splenocytes). The optimized formulation induced an approximately 38% increase (1.38-fold) in PPD-specific IFN-γ-producing cells compared to conventional BCG ($^{*}p < 0.05$ and $^{**}p < 0.01$).

Reliable analytical methods are crucial for the quality control of biological products. The HPLC method developed in this study demonstrated satisfactory specificity, linearity, precision, sensitivity, and compliance with ICH Q2(R2) guidelines (https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline). The use of refractive index detection was appropriate for mannan, despite the absence of strong ultraviolet chromophores and inherent heterogeneity of carbohydrate molecules[10,11]. Thus, the validated method provides a suitable analytical platform for characterizing purified mannan during formulation development and quality control.

The Taguchi DoE approach provided an efficient approach for optimizing purified mannan, trehalose, and mannitol while reducing the number of experimental runs[2,15]. The optimized formulation showed improved bacterial viability, reduced residual moisture, better cake integrity, and shorter reconstitution time. These observations support earlier research on the protective effects of trehalose and mannitol in live bacterial vaccines[2,20]. Previous BCG stabilization studies have shown that lactose, dextran, and monosodium glutamate can improve thermal stability and viability, although their optimal compositions differed from that identified herein[9]. Similarly, Jin et al. have reported improved cake appearance and stability in recombinant BCG using mannitol, trehalose, sucrose, and sodium glutamate[6]. Such differences emphasize that excipient performance depends on concentration, interactions with bacterial matrix, strain characteristics, and the specific lyophilization cycle.

Table 5. Comparison of IFN-γ-producing cell responses across different experimental groups

Experimental group	IFN-γ-producing cells (SFU/10^6 splenocytes)	Fold Change vs. conventional BCG	*p* value vs. conventional BCG
Negative Control	4 ± 1	—	—
Excipient Control	6 ± 2	—	NS
Purified Mannan	12 ± 3	0.27	NS
Conventional BCG	45 ± 3	Reference	—
Lyophilized BCG	53 ± 4	1.18	< 0.05
Optimized lyophilized BCG-mannan formulation	62 ± 4	1.38	< 0.01

Data are presented as mean ± SD (n = 5). *P* values were calculated using one-way ANOVA followed by Tukey's multiple-comparison test. The conventional BCG group was used as the reference group for pairwise comparisons. NS indicates no statistically significant difference ($p \geq 0.05$).

Trehalose has been widely investigated as a protective excipient for BCG. Although previous work demonstrated improved resistance to freeze-drying, substantial viability loss during prolonged storage was also reported[21]. Our optimized formulation; however, maintained viable bacterial counts within the predefined acceptance limits over 24 months of refrigerated storage. Tan et al. have similarly demonstrated that the viability of freeze-dried *M. bovis* is strongly influenced by formulation composition and storage temperature, with the highest viability observed at 4 °C. Their observation of cake collapse under unfavorable glass-transition conditions further highlights the need to optimize both bacterial viability and physical stability[22]. Our formulation maintained an intact cake and residual moisture below 3% during refrigerated storage. Evidence from alternative drying approaches underscores the importance of formulation-process interactions. Price et al. developed a spray-dried BCG formulation containing mannitol and trehalose that retained viability and immunogenicity during prolonged storage[23]. Although spray drying differs from lyophilization, these findings independently support the contribution of optimized excipient systems to the stability of live BCG preparations. Collectively, available evidence indicates that trehalose and mannitol are frequently beneficial, but no universally optimal stabilizer composition exists. The optimal formulation likely depends on the BCG strain, excipient concentrations, drying conditions, target moisture content, and storage temperature.

An important feature of the present formulation was the potential dual role of purified mannan as a stabilizing and immunologically active component. These findings extend the observations of Mardani et al., who reported immunostimulatory activity of the purified mannan isolated from *M. bovis* BCG[8]. In our study, mannan incorporation was associated with the preservation of bacterial viability during freeze-drying and an increased PPD-responsive IFN-γ response. However, these results do not confirm that mannan directly causes the immunological effect or improved protection against M. tuberculosis. Therefore, rather than describing mannan as a confirmed multifunctional excipient/adjuvant, it is more appropriate to consider it a formulation component with observed stabilizing and immunomodulatory effects, which requires further investigation[8,24,25].

The optimized formulation maintained residual moisture below the predefined acceptance limit and viable bacterial counts within specifications throughout 24 months of refrigerated storage. These results are consistent with the recognized importance of stabilizing excipients and controlled moisture levels for maintaining the quality of freeze-dried BCG vaccines[13,20]. Low residual moisture may reduce degradation processes that compromise the viability of live attenuated vaccines during storage. However, the present study did not independently establish a direct causal relationship between specific formulation attributes and long-term viability[26]. Furthermore, numerical comparisons with previous studies should be made cautiously because of differences in formulations, storage conditions, and drying technologies. The reported stability therefore demonstrates successful preservation under the intended refrigerated conditions rather than temperature-independent stability or superiority over alternative dry-powder BCG platforms[22,23].

A major finding of our study was the significantly increased frequency of PPD-specific IFN-γ-producing splenocytes following the administration of the optimized BCG-mannan formulation. While IFN-γ is important for antimycobacterial cellular immunity, it is not a validated correlate of protection for TB vaccines[14,27,28,29]. Therefore, increased IFN-γ production alone cannot be interpreted as evidence of improved vaccine efficacy[27,30]. Because PPD contains multiple mycobacterial antigens, the ELISpot response likely reflects recognition of a broad antigenic repertoire[28]. The enhanced response may involve altered antigen presentation or cellular activation following mannan incorporation, but these mechanisms were not directly investigated. Mannose-containing structures can interact with mannose receptors and other C-type lectin receptors on dendritic cells and macrophages, potentially enhancing antigen presentation and Th1-associated responses[29,31-34]. The biological activity of mannan may also depend on molecular weight, branching pattern, and administration route[29]. However, receptor binding and downstream signaling were not assessed in this study; consequently, these proposed mechanisms remain hypothetical. The present findings are therefore complementary to, rather than directly comparable with, previous work demonstrating the immunostimulatory properties of BCG-derived mannan[8].

Several limitations should be considered. Immunogenicity was assessed using only the IFN-γ ELISpot without assessment of cytokine profiles, T-cell subsets, or functional antimycobacterial activity. PPD stimulation provides a broad antigenic response and does not identify the specific responding cell populations. The exploratory mouse study included only five animals per group, limiting statistical precision and generalizability. In addition, intraperitoneal administration differs from conventional BCG vaccination routes and may influence immune

responses. Most importantly, no challenge with virulent *M. tuberculosis* was performed; therefore, no direct evidence of reduced bacterial burden or disease protection was obtained. Future studies should evaluate the immunological effects of the optimized formulation using multiparameter flow cytometry, broader cytokine profiling, T-cell phenotyping, and functional assays such as mycobacterial growth inhibition. Virulent *M. tuberculosis* challenge models will also be necessary to determine whether the observed immunological differences translate into meaningful protection. Structural characterization of purified mannan and investigation of its interactions with relevant innate immune receptors are likewise warranted.

Overall, this study presents an integrated analytical, pharmaceutical, stability, and preliminary immunological framework for developing a mannan-containing lyophilized BCG formulation. The validated HPLC method, optimized excipient composition, 24-month refrigerated stability, and increased PPD-specific IFN-γ responses support further investigation of purified mannan as a potential stabilizing and immunomodulatory formulation component. However, the immunological findings remain preliminary and should not be interpreted as evidence of enhanced protective efficacy against TB.

5. CONCLUSION

The validated HPLC method provides a robust and reproducible approach for quantitative determination of purified mannan during formulation development. The optimized mannan-containing formulation showed favorable physicochemical attributes and maintained bacterial viability and stability during refrigerated storage. The optimized formulation increased PPD-specific IFN-γ ELISpot responses under the conditions tested; protective efficacy was not assessed. Overall, the findings support further evaluation of purified mannan as a formulation component showing stabilizing and immunomodulatory effects in this experimental system, while additional immunological, functional, safety, and challenge studies are required to determine its relevance to protective immunity.

DECLARATION

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Generative AI and AI-assisted technologies

In this study, AI-assisted tools were used exclusively for grammar correction and language polishing using Grammarly. No generative AI was employed for scientific content generation, data analysis, interpretation of results, or any other substantive aspect of this manuscript.

Ethical approval

All animal procedures were approved by the Institutional Animal Ethics Committee of the Falavarjan Branch, Islamic Azad University, Isfahan, Iran (Approval No. IR.IAU.FALA.REC.1402.056) and were conducted in accordance with the ARRIVE 2.0 guidelines and the National Research Council Guide for the Care and Use of Laboratory Animals.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SZ: conceptualization, literature review, formal analysis, wrote the original draft, conducted the investigation, data curation, and visualized the data; MM and DD: conceptualization, designed the methodology, supervision, project administration, and writing–review and editing; SMH and MMS: formal analysis, visualization, data curation, resources, and writing –review & editing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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