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Repurposing bacterial lysates: Engineering inhalable locust bean gum microparticles for respiratory infection prevention

Joana Pinto da Silva [a], Melibea Berzosa [b], Lucas Chiarentin [c,d], Alberto Delgado-López [e], Maria Patrícia Almeida [f], Carla Vitorino [c,d], Ana Figueiras [c,g], Ana M. Rosa da Costa [f,h], Carlos Gamazo [e], Ana Grenha [a,f,*]

[a] Centre for Marine Sciences (CIMAR-LA), Universidade do Algarve, Faro, 8005-139, Portugal
[b] Wellcome Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, BT9 5AL, United Kingdom
[c] Faculty of Pharmacy, Universidade de Coimbra, Coimbra, 3000-548, Portugal
[d] Coimbra Chemistry Centre, Institute of Molecular Sciences – IMS, Department of Chemistry, Universidade de Coimbra, Coimbra, 3000-535, Portugal
[e] Department of Microbiology, University of Navarra, Pamplona, 31008, Spain
[f] Faculty of Sciences and Technology, Universidade do Algarve, Faro, 8005-139, Portugal
[g] REQUIMTE/LAQV, Drug Development and Technologies Laboratory, Faculty of Pharmacy, Universidade de Coimbra, Coimbra, 3000-548, Portugal
[h] Centre for Electronics, Optoelectronics and Telecommunications, Universidade do Algarve, Faro, 8005-139, Portugal

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ABSTRACT

This study explores the development of inhalable microparticles containing bacterial lysates (BL) from multiple relevant bacterial species that include *Staphylococcus aureus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*, and locust bean gum (LBG) for pulmonary immunization against respiratory infections. LBG, a galactomannan, was chosen for its mucoadhesive properties and affinity for antigen-presenting cells. Microparticles were prepared by spray-drying, testing different LBG:BL ratios. The morphological analysis revealed convoluted microparticles, and BL association efficiency up to 81 % was determined. Antigenic assays confirmed that bacterial antigens of *S. pyogenes,* used as reference, remained preserved on the microparticles despite the applied processing conditions. The aerodynamic analysis showed that lower BL content (LBG:BL = 10:0.2, w/w) produced more suitable particles for pulmonary delivery, with 4.6 μm mass median aerodynamic diameter (MMAD) and 29 % fine particle fraction (FPF). Fluorescence microscopy confirmed uniform distribution of BL within the LBG matrix and LBG microparticles demonstrated superior adhesive properties compared to controls. Sustained release of BL from microparticles was observed *in vitro*, reaching around 80 % after 6 h, which increased to around 85 % after 24 h. Cytotoxicity studies showed appropriate cell viability at physiologically relevant concentrations (around 70 % or more). These findings suggest that LBG-based microparticles loaded with BL have potential to be explored as inhalable formulation for prevention of respiratory infections, offering targeted delivery and prolonged antigen presentation to enhance immune responses.

1. Introduction

Respiratory infections represent a significant global health challenge, imposing a substantial economic burden on healthcare systems. Bacterial infections on the basis of conditions such as pneumonia, bronchitis, and pharyngitis contribute heavily to this issue. For some conditions there are (parenteral) vaccines available, which display limitations related with injection discomfort and the induction of limited mucosal immune response that results in incomplete protection of the lung [1]. Despite ongoing research efforts, effective preventive strategies for these diseases remain insufficient. Bacterial lysates (BL) are used in clinical practice as alternative approach to prevent respiratory tract infections by leveraging the immunomodulatory components, in this case being administered orally or, less typically, *via* intranasal route [2]. BL are extracts derived from pathogenic bacteria frequently implicated in respiratory diseases, containing antigenic components from multiple relevant bacterial species. OM-85 and LW-50020 are two examples of BL, comprising extracts of bacterial species that include

* Corresponding author at: Centre for Marine Sciences (CIMAR-LA), Universidade do Algarve, Campus de Gambelas, 8005-139, Faro, Portugal.
E-mail address: amgrenha@ualg.pt (A. Grenha).

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Staphylococcus aureus, *Streptococcus pyogenes*, and *Klebsiella pneumoniae* [2]. Despite the availability and long-term use of various BL formulations, their efficacy data is inconsistent and often contradictory [2,3]. Due to the absence of robust clinical evidence, the European Medicines Agency has restricted the use of BL therapies solely to the prophylaxis of recurrent respiratory tract infections, adding that in no case can they be used to treat pneumonia [3]. Emphasis is now placed on the need for novel strategies to enhance BL effectiveness in preventing respiratory infections [2,3].

An emerging approach involves the administration of probiotic bacteria or their derivatives to the respiratory tract. Recent research has demonstrated that certain bacterial species within the airways correlate with reduced respiratory disease complications and exacerbations [4,5]. This has led to exploration of delivering orally safe probiotics, directly to the airways to exploit their beneficial interactions with resident microflora [4]. The COVID-19 pandemic has further highlighted the potential of pulmonary probiotic delivery as both therapeutic and prophylactic strategies against respiratory infections [5]. Alternatively, non-live formulations such as paraprobiotics (inactivated probiotics) or postbiotics (bacterial fragments or metabolites) offer potentially improved formulation stability, though their duration and extent of action may be more limited compared to live probiotics [4]. In fact, postbiotics are more stable and have a longer shelf life than probiotics because they lack living microorganisms. By contrast, probiotic formulations are challenging to develop since microbial viability must be preserved during manufacturing, storage, and delivery [4].

BL can be considered a specific type of postbiotic, consisting of microbial components with potent immunomodulatory properties. Unlike inactivated commensal-derived probiotics, which may support microbiome–host interactions, or metabolite-based postbiotics, which primarily act through host signaling pathways, BL derived from pathogenic species present complex antigenic profiles that engage innate immune receptors and promote broad-spectrum immunological training [2,4]. These preparations have been shown to stimulate both innate and adaptive immune responses, contributing to enhanced mucosal and systemic defense against infections [2].

Pulmonary delivery of BL *via* inhalation offers a promising alternative to induce more robust immune response in the lung in the sequence of the direct delivery of antigens to the primary site of infection, thus potentially addressing the limitations of oral administration. BL inhalation may facilitate the interaction between antigens and the bronchial-associated lymphoid tissue (BALT), which is rich in antigen-presenting cells (APC), namely dendritic cells (DC), responsible for initiating the immune response [6]. As referred above, the current routes of BL delivery appear less effective, potentially due to an inadequate mucosal response marked by low secretory IgA levels in the places where the infection is established [7]. Secretory IgA is essential for neutralizing pathogens through opsonization and phagocytosis and an inadequate IgA response may explain the reduced protection against recurrent infections [7]. Targeting the lung may stimulate both a local mucosal response *via* secretory IgA production and a systemic response through IgG production.

Effective BL delivery to the lung requires, however, that the formulation being developed exhibits suitable aerodynamic properties (diameter < 5 μm) to reach the lung. For immunomodulation applications, particles in the 2–5 μm range are particularly advantageous as they target intermediate respiratory regions where immune cells are highly active and diverse immune responses can be initiated [4,8]. This size range is strategically relevant for BL formulations containing antigens from multiple pathogens with varying colonization patterns throughout the respiratory tract [4,8]. While particles exceeding 5 μm would predominantly deposit in upper airways where some pathogens colonize, and particles smaller than 3 μm would reach deep alveolar regions, the intermediate size enables simultaneous targeting of multiple respiratory compartments for broad-spectrum immune stimulation [4,8]. This deposition could facilitate interaction with BALT and promote both mucosal and systemic immune responses [9,10].

The strategy of generating local lung protection through increased IgA production may be further increased through the enhancement of the targeting of APCs. In fact, these cells are known to exhibit specific surface receptors that can be directly targeted through the use of ligands present in the delivered BL formulation. The mannose receptor, a C-type lectin, is one of the most common targets for such interactions [11]. Our research group has previously reported the high affinity of a set of polysaccharide-based microparticles for phagocytic cells. One of the polysaccharides exhibiting the higher affinity is locust bean gum (LBG), a galactomannan obtained from the seeds of *Ceratonia siliqua*, comprising a linear chain of mannose with side units of galactose [12,13]. This property makes LBG an attractive candidate for developing carriers of BL, in the form of microparticles endowed with the capacity to mediate active interactions with APC, leveraging the ability of mannose residues as targeting ligands. Reported as a mucoadhesive polymer [14], LBG could further extend the residence time of the microparticles in the lungs upon inhalation, allowing for prolonged interaction with immune cells with potential improvement of the generated immune response.

This work represents a drug repurposing approach, wherein BL act as immunomodulatory agents similar to existing marketing options. A novel delivery strategy involving inhalation of BL is proposed, which benefits from the direct targeting of the primary infection site in respiratory infections (the lungs) and the specific cells involved in the immune response (APC). The selected formulation approach comprises dry powders (microparticles) in line with current considerations of reducing the use of propellants in inhalation devices, which are environmentally harmful. Inhalable microparticles were thus developed by spray-drying with adjusted LBG:BL ratios to maximize association efficiency and loading capacity. Thorough characterization was provided through morphological, physicochemical, aerodynamic, and *in vitro* evaluation that included assessment of particle morphology and spatial distribution of components, moisture content, mucoadhesion, release kinetics, storage stability and cytotoxicity. Overall, this study is centred on the development of aerodynamically suitable LBG microparticles that effectively associate BL to enable efficient pulmonary delivery.

2. Materials and methods

2.1. Materials

LBG was a kind gift from Industrial Farense (Portugal). BL correspond to the OM-85 preparation (Broncho-Vaxom® capsules, OM Pharma, Switzerland), containing lysates from eight bacterial species: *Haemophilus influenzae, Diplococcus pneumoniae, Klebsiella pneumoniae* and *ozaenae, Staphylococcus aureus, Streptococcus pyogenes* and *viridans,* and *Neisseria catarrhalis*. Bovine serum albumin (BSA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), fluorescein sodium salt (FL), rhodamine B isothiocyanate (RITC), 2-bromoethylamine hydrobromide-formaldehyde (BEI-FA), Freund's incomplete adjuvant, chitosan and porcine stomach mucin (type III) were purchased from Sigma-Aldrich (Germany). Bradford reagent was supplied by Bio-Rad (USA), phosphate-buffered saline (PBS) tablets pH 7.4 were acquired from VWR (USA), ethanol 70 % and 96 % (v/v) from Laborspirit (Portugal), dimethyl sulfoxide (DMSO) from Riedel-de Haën (Germany) and D(+)-Lactose monohydrate from Scharlab (Spain). Ultrapure water (Milli-Q, Millipore, UK) was used throughout.

2.2. Bacteria and culture media

Streptococcus pyogenes used in this study as a representative strain was obtained from the Spanish Type Culture Collection (CECT 189). Agar-blood plaques (BD Agar Columbia BBL™ with 5 % sheep blood) and Brain Heart Infusion (BHI) medium (BD BBL™) were obtained from BD Biosciences (USA).

2.3. Cell culture

Human lung epithelial adenocarcinoma cells (A549) were obtained from the American Type Culture Collection (ATCC, Middlesex, UK) and used between passages 96 and 135. Dulbecco's Modified Eagle's Medium (DMEM) and penicillin/streptomycin were obtained from PAN-Biotech (Germany). Fetal bovine serum (FBS) was supplied by Bio-Concept (Switzerland), L-glutamine from Thermo Fisher Scientific (USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from VWR (USA). Non-essential amino acids and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Germany).

2.4. LBG purification

To purify LBG and remove potential impurities that could trigger adverse reactions, an ethanol precipitation method was carried out based on an established protocol [15]. Briefly, LBG was dispersed in deionized water at a concentration of 1 % (w/v) and heated to 85 °C with constant stirring for 2 h to ensure complete solubilization. The homogenized mixture was centrifuged (22,000*g*, 1 h, 20 °C; Avanti J30I, Beckman Coulter, USA) to separate insoluble protein components. The supernatant containing solubilized LBG was collected and ethanol 96 % (v/v) added to precipitate the LBG polysaccharide (galactomannan). The mixture was centrifuged under identical conditions. The resulting LBG precipitate was filtered through a sieve to remove any remaining insoluble material. After separating the precipitate into small threads, the purified LBG was dried in a vacuum oven at 30 °C for 72 h and stored in a desiccator until further use.

2.5. Preparation of BL-loaded LBG microparticles

Purified LBG was ground to a fine powder using a mortar and pestle. The powdered LBG was dispersed in ultrapure water preheated to 85 °C (1 % w/v) and stirred for 2 h at that temperature, followed by overnight stirring at room temperature. The microparticles were prepared from LBG dispersions by spray-drying (Büchi Mini Spray-Dryer B-290, Büchi Labortechnik AG, Switzerland). Both unloaded and BL-loaded microparticles were produced. When BL were associated, varying mass ratios of LBG to BL (from 10:0.2 to 10:1.2) were tested. The corresponding dispersions were prepared by mixing the whole content of Broncho-Vaxom® capsules necessary to obtain the required amounts of BL, which could not be isolated, with the previously elaborated LBG dispersion, under magnetic stirring. The final dispersions had a solid content ranging from 1.7 to 4.3 % (*w/v*) and were kept under stirring at approximately 60 °C during the spray-drying process to prevent the increase of viscosity. The spray-dryer was operated in open mode configuration and the parameters were set as follows: inlet temperature of 130 °C, compressed air flow rate of 473 L/h, aspirator at 100 %, and feed flow rate at 1 mL/min. The resulting microparticles were collected from the collecting vessel and stored in a desiccator until further use.

The yield of the spray-drying process was determined gravimetrically by comparing the initial total solids with the final weight of the produced microparticles.

2.6. Physical and morphological characterization of microparticles

Microparticle morphology was observed using field-emission scanning electron microscopy (FESEM; FESEM Ultra Plus, Zeiss, Germany). Before imaging, microparticles were coated with iridium using a sputter coater (Q150T S/E/ES, Quorum Technologies, UK). The Feret diameter was estimated from the FESEM images, based on the measurement of at least 500 particles using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

The Morphologi 4® particle imaging system (Malvern Panalytical, Malvern, UK) was further used to characterize particle size and shape parameters. Samples were dispersed onto glass slides using compressed air (6 bar) and images of over 15,000 individual particles (ISO 13322-1, 2014) between 0.5 and 50 μm were captured using a 18 MP detector camera and Nikon CFI 0 optical system. Measurements of circle equivalent diameter, circularity, elongation, and convexity were performed by the software and averaged for each formulation ($n = 3$).

2.7. Spatial characterization of microparticles: distribution of components assessed by confocal laser scanning microscopy (CLSM)

The components of microparticles, LBG and BL, were separately labelled with fluorescent markers. LBG (1 g) was dispersed in 1×10^{-4} M HCl (pH 4) to obtain a 1 % (w/v) dispersion. This was magnetically stirred for 30 min at room temperature, then heated to 85 °C with slow stirring for 30 min. After overnight stirring at room temperature, FL (45.5 mg) was dissolved in 96 % ethanol and added to the LBG dispersion. EDAC (51.8 mg) was dissolved in ultrapure water and added after that. The mixture was then kept under stirring, protected from light, for 72 h. For BL labelling, the equivalent to 30 mg of OM-85 were dissolved in 10 mL of 0.1 M bicarbonate buffer (pH 9.5) with stirring for 4 h. The mixture was filtered through a 1 μm membrane, then 12.5 mg RITC, previously dissolved in 5 mL DMSO, were added. The reaction was maintained under stirring at room temperature and protected from light, for 40 h.

Both mixtures were dialyzed against deionized water using a 3.5 kDa membrane (SnakeSkinTM, Thermo Scientific). Water was changed twice daily for 1 week. Samples were frozen at −20 °C followed by 3 days at −80 °C, and finally freeze-dried for 48 h (LyoAlfa 15, Telstar, Spain). Freeze-dried products were weighed and stored in a desiccator protected from light. To produce fluorescently-labelled microparticles, LBG-FL was dispersed at 1 % (w/v) with addition of BL and BL-RITC (LBG:BL: BL-RITC = 10:0.2:0.3, w/w), following the microparticle preparation method described above.

The distribution of components in the BL-loaded microparticles was examined using CLSM (Zeiss LSM710 microscope, Zeiss, Germany). Dry powder samples were mounted on glass slides with a drop of immersion oil to ensure sample stability during microscopic examination. The samples were illuminated with dual laser excitation at wavelengths of 488 nm and 561 nm. Fluorescent emissions were collected through separate channels: LBG-FL (λ = 490–560 nm) and BL-RITC (λ = 575–685 nm). Imaging was performed at 100× magnification using an oil immersion objective (alpha Plan-Apochromat 100×/1.46 Oil DIC M27). The resulting grayscale images from individual channels were pseudo-colored green for LBG-FL and red for BL-RITC and subsequently merged using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to produce composite multicolor images.

2.8. Determination of association efficiency and loading capacity

To determine the association efficiency (AE) of BL and loading capacity (LC) of microparticles, the dry powders were dispersed in ultrapure water (10 mg/mL) by magnetic stirring for 1 h at 60 °C. The amount of BL was estimated by establishing a correlation with the protein content, which was determined using the Bradford protein assay [16]. To do so, the samples were mixed with Bradford reagent and absorbances measured at 595 nm. A BSA calibration curve in ultrapure water (8–80 μg/mL) was elaborated and used as standard for the estimation of BL protein content. AE (%) and LC (%) were calculated using the following equations ($n \geq 3$):

$$\text{AE}\ (\%) = \frac{\text{loaded BL mass}}{\text{theoretical BL mass}} \times 100 \quad (1)$$

$$\text{LC}\ (\%) = \frac{\text{loaded BL mass}}{\text{total MP mass}} \times 100 \quad (2)$$

2.9. Aerodynamic particle size distribution analysis

The aerodynamic properties of the dry powder formulations were evaluated using an Andersen cascade impactor (ACI, Copley Scientific Ltd., UK) equipped with an induction port (IP) and featuring cut-off diameters ranging from 8.60 to 0.26 μm. The aerodynamic particle size distribution (APSD) was assessed following the European Pharmacopoeia guidelines (Apparatus D, European Pharmacopoeia 10.0), using a protein detection method for quantification of BL amount. Collection plates were pre-coated with a solution of ethanol containing 1 % (v/v) glycerol to minimize particle bounce, and glass microfiber filters (grade 934-AH, Whatman) were placed in the final stage (F). The impactor flow rate was calibrated to 60 L/min using a flow meter (model DFM4, Copley Scientific Ltd., UK). Powder samples (30 mg) were loaded into hydroxypropyl methylcellulose (HPMC) size 3 capsules (Quali-V-I, Qualicaps, Spain) and discharged using a high resistance RS01® inhaler (Plastiape Spa, Italy) over 4 s. The content of 10 capsules was discharged in each aerodynamic test, with each formulation tested at least in duplicate. The ACI components were rinsed with warm ultrapure water and microparticle deposition on each stage was assessed by quantifying the protein content as previously described (Section 2.8 - Determination of Association Efficiency and Loading Capacity). Owing to the absence of a component to quantify, the unloaded LBG microparticles were assessed by performing a gravimetric analysis of the powder deposited in each stage (3 capsules of 30 mg/each were used in each test).

Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated by plotting the cumulative percentage of protein content deposited against the ACI stage cut-off diameters on a log-probability scale. Emitted dose (ED) was calculated as the proportion of BL mass delivered from the capsules and device. Fine particle analysis involved measuring fine particle dose (FPD), representing the mass of BL in particles smaller than 5 μm, and fine particle fraction (FPF), determined as the percentage of total BL mass in particles with aerodynamic diameter below 5 μm.

2.10. Evaluation of antigenicity preservation

Antibodies against *Streptococcus pyogenes* were generated to evaluate bacterial antigen preservation. A freeze-dried aliquot of *S. pyogenes* (CECT 189) was subcultured by incubating in agar-blood at 37 °C for 24 h. This was then used to inoculate 50 mL of BHI medium at 37 °C for 3 h. This culture was scaled up by inoculating 20 mL into 3 L of BHI and incubating at 37 °C, 125 rpm for 24 h. Bacteria were finally inactivated *via* treatment with BEI-FA [17].

New Zealand White rabbits (Granja San Bernardo, Spain) were immunized intramuscularly on days 1 and 14 with the inactivated bacteria using Freund's incomplete adjuvant (1:1, v/v). Serum samples were collected and used in a microplate agglutination test (MAT). Microparticle dispersions in water (1 mg/mL) of unloaded LBG or BL-loaded LBG (LBG:BL = 10:0.2, 10:0.7 and 10:1.2, w/w) microparticles were incubated with immune rabbit serum (1:20, v/v) in PBS pH 7.4 at 37 °C for 24 h, then centrifuged at 100 *g* for 5 min to facilitate antibody-antigen interactions.

Moreover, the presence of preserved bacterial antigens on microparticles LBG:BL 10:0.2 (*w*/w) were qualitatively assessed using a commercial immunochromatographic strip test, the CerTest Strep A (Certest Biotec, Spain), for detection of *S. pyogenes* capsule antigens. A dispersion of 1.4 mg/mL microparticles in the supplied assay buffer (2 M sodium nitrate and 0.15 M acetic acid) was applied to the test strip to examine antigen recognition by the specific mouse monoclonal antibodies.

2.11. Moisture content determination

LBG:BL microparticles (10:0.2 w/w) samples were precisely weighed (±0.1 mg) in glass test tubes after collection from the spray-dryer, then dried at 105 °C for 27 h. Samples were immediately transferred to a desiccator and cooled to room temperature to prevent moisture reabsorption before reweighing with an analytical balance. Moisture content (%) was calculated in triplicate using the following equation:

$$\text{Humidity (\%)} = \frac{\text{Weight before drying} - \text{Weight after drying}}{\text{Weight after drying}} \times 100 \quad (3)$$

2.12. Evaluation of mucoadhesive properties

The mucoadhesive properties of microparticles were evaluated using a texture analyser (TA-XTplus, Stable Micro Systems, Surrey, UK) equipped with a temperature-controlled chamber. Mucin discs were prepared from porcine stomach mucin. Tested microparticles comprised either unloaded (LBG MP) or BL-loaded microparticles (LBG:BL = 10:0.2, *w*/w). Lactose and chitosan were used as negative and positive controls, respectively. All tablets were produced using a hydraulic press, compressing 200 mg of each material at 10 tons for 30 s (mucin) or 120 s (microparticles, lactose, and chitosan). The microparticle or control tablets were affixed to the analyser probe, and mucin discs were secured beneath using double-sided adhesive tape. Experiments were conducted at 25 °C and 37 °C, and each sample was tested 3 times. Before testing, mucin discs were hydrated with phosphate-buffered saline (PBS, pH 7.4) for 30 s, with excess liquid gently blotted away. The probe was lowered until the sample tablets contacted the mucin disc, applying a 0.1 N downward force for 30 s. Subsequently, the probe was retracted at 0.5 mm/s, and the detachment force was measured. Force-distance plots were generated and analyzed using Exponent software (Version 6.2.2.0, Stable Micro Systems) to determine the maximum detachment force as a measure of mucoadhesive strength.

2.13. In vitro release profile

To study the release profile of BL, the microparticles (LBG:BL = 10:1.2, w/w) were aerosolized onto a glass microfiber membrane using an insufflation device developed by the research group [18,19]. The amount of powder deposited was adjusted to around 30 mg. The membrane was then pre-wetted with PBS (pH 7.4) and placed in a modified Transwell insert support, which was placed in a 6-well plate containing 1.5 mL of PBS (pH 7.4) in the basolateral chamber, allowing for contact with liquid at the bottom of the membrane instead of complete submersion. The plate was kept under horizontal shaking (OS-20 Orbital Shaker, Biosan, Latvia) at 60 rpm and 37 °C. At pre-determined time points up to 24 h, aliquots of the medium were collected and replaced with fresh PBS. The released BL was quantified using the Bradford protein assay, and the cumulative drug release was calculated as the percentage of the total BL amount in the powder formulation ($n = 6$).

2.14. Storage stability study

LBG:BL microparticles (10:0.2 w/w) and commercial OM-85 (Broncho-Vaxom®) stability were assessed following international regulatory guidelines for pharmaceutical products [20,21]. Microparticle samples (~100 mg) were sealed in dark glass vials within platinum bags containing silica desiccant and stored at 25 ± 2 °C with 60 ± 5 % relative humidity (RH) in a calibrated climatic chamber (TK 252; Nüve, Turkey) for 12 months. Protein content in LBG microparticles was measured periodically using the Bradford assay as described in Section 2.8. OM-85 samples underwent identical storage conditions and were used as control. All measurements were performed in triplicate.

2.15. Metabolic activity assay

The MTT assay was used to assess metabolic activity as an indicator

of cell viability following exposure to microparticles. A549 cells were cultured in DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) penicillin/streptomycin, 1 % (v/v) L-glutamine, and 1 % (v/v) non-essential amino acids. Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO2/95 % air. A549 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated overnight before testing. Subsequently, cells were exposed to different concentrations of BL-loaded (10:0.2–10:1.2, *w*/w) and unloaded microparticles up to 2000 μg/mL. After 24 h exposure, the medium was removed and 30 μL of MTT solution added to each well, followed by 2 h incubation at 37 °C. The resulting formazan crystals were solubilized with 50 μL of DMSO. The absorbance was measured by spectrophotometry (Synergy H4 Hybrid, BioTek, Vermont, USA) at 540 nm, with background correction at 640 nm. CCM alone and 2 % (*w*/*v*) SDS solution were used as positive and negative controls of cell viability, respectively. Cell viability was calculated as described below:

$$\text{Cell viability } (\%) = \frac{A - S}{CM - S} \times 100 \quad (4)$$

where A represents the absorbance measured upon exposure to each sample, CM represents the absorbance measured for cells incubated with CCM and S is the absorbance measured for the cells exposed to 2 % SDS.

The data enabled the determination of half maximal inhibitory concentration (IC50) values, representing the sample concentrations that reduced cell viability to 50 % under the tested conditions. IC50 values were determined through sigmoidal curve fitting using GraphPad Prism® software (GraphPad Software, version 9.31, USA). Measurements were performed at least in triplicate and the assay repeated at least 3 times.

2.16. Statistical analysis

The results of microparticle characterization were analyzed using *t*-test for comparisons between two groups and one-way analysis of variance (ANOVA) followed by the Holm-Sidak method for pairwise multiple comparisons among three or more groups. As for the mucoadhesion, the data were analyzed by means of a one-way ANOVA using the Dunnett's multiple comparisons test. All analyses were run using the GraphPad Prism® statistical program (Version 9.31); $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation of BL-loaded LBG microparticles

The commercial LBG was purified *via* ethanol precipitation to remove protein impurities, which are of particular concern in therapeutic applications. This process resulted in an average yield of approximately 70 %, effectively eliminating protein contaminants [22]. All subsequent references to LBG in this study pertain to the purified form. The characterization of the specific LBG used in this study was already reported [22] and includes molecular weight determination through high performance size exclusion chromatography, Fourier-transform infrared spectroscopy analysis and thermophysical analysis. The spray-drying conditions established to develop the BL-loaded LBG microparticles were selected from previous studies on LBG-based microparticles, which showed that lower temperatures did not provide adequate drying of the final microparticles, owing to the large size of LBG molecule. Moreover, these conditions resulted in suitable powder properties and process yield for this application [19,22].

Unloaded LBG microparticles and BL-loaded LBG microparticles were then produced by spray-drying, from dispersions containing a constant LBG concentration (1 %, *w*/*v*). The BL content in microparticles was variable, the LBG:BL ratios ranging from 10:0.2 to 10:1.2 (*w*/w), as described in Table 1. A high LBG proportion was intentionally maintained to enhance recognition by APC for the intended application.

Table 1
Characteristics of different formulations of unloaded and bacterial lysates (BL)-loaded locust bean gum (LBG) microparticles (mean ± SD; $n \geq 3$). Different letters represent significant differences in each parameter ($p < 0.05$).

LBG:BL (w:w)	Spray drying yield (%)	Feret's diameter (μm)	Association efficiency (%)	Loading capacity (%)
10:0.0	61 ± 8^{a}	$4.3 \pm 4.4^{b*}$	n.a.	n.a.
10:0.2	60 ± 4^{a}	3.6 ± 4.9^{b}	81.0 ± 6.7^{d}	1.9 ± 0.2^{f}
10:0.7	60 ± 6^{a}	7.2 ± 5.3^{c}	71.9 ± 3.4^{d}	2.8 ± 0.1^{g}
10:1.2	56 ± 2^{a}	7.1 ± 5.4^{c}	56.0 ± 2.0^{e}	2.7 ± 0.1^{g}

n.a.: not applicable; *data published in [22], reprinted with permission from Elsevier.

The spray-drying process exhibited consistency, yielding approximately 60 % across all tested formulations. This demonstrates the robustness of the production method and its independency of the incorporation of different BL amounts.

3.2. Physical and morphological characterization of microparticles

The morphology of microparticles was evaluated using FESEM, which revealed that all microparticles displayed a convoluted structure and some deformities on their surface (Fig. 1). This characteristic morphology is attributed to a well-known phenomenon in spray-drying, where surface enrichment can lead to the formation of a solid shell during drying while the interior retains moisture. The resulting pressure imbalance causes the shell to collapse, creating the distinctive deflated appearance. This behavior is commonly associated with amorphous polymers such as LBG, accounting for the convoluted microparticle shape [23]. Theoretically, the presented morphology suggests appropriate flowing properties and reduced airflow resistance, which could enhance the potential to reach the lung [23]. Furthermore, an increase in surface roughness was observed as the BL content raised. A closer observation of the microparticle surface for the formulation containing the higher amount of BL shows the presence of needle-shaped crystals (Fig. 1.f), which clearly contrasts with the smooth surface of unloaded microparticles (Fig. 1.e). This phenomenon is likely due to the presence of excipients from the OM-85 formulation, such as mannitol, which can form small crystals on the particle surface during spray-drying [23].

Size estimation based on Feret's diameter (Table 1) indicated a heterogeneous size distribution, observed through high standard deviations, with the lowest BL ratio (LBG:BL = 10:0.2, w/w) producing microparticles with a mean diameter of approximately 3.6 μm, which was similar to that of unloaded LBG microparticles. In contrast, formulations with higher BL content (10:0.7 and 10:1.2 w/w) produced larger microparticles ($p < 0.05$), averaging around 7 μm in Feret's diameter. These size differences are consistent with theoretical predictions, as an increase in the overall material content within the formulation directly influences the final microparticle size.

The physical attributes of microparticles, including shape parameters, were further examined using Morphologi 4® and the results are displayed in Table 2. Unloaded LBG and LBG:BL = 10:0.2 (*w*/w) microparticles were characterized. The average circular equivalent diameter of BL-loaded LBG microparticles was approximately 6.3 ± 0.2 μm, which was notably larger compared to the unloaded LBG microparticles (4.5 ± 0.1 μm, $p < 0.05$). Although a value comparable to that obtained as Feret diameter (Table 1) was observed for unloaded particles, the same cannot be said for BL-loaded microparticles, where a much larger diameter was registered. This discrepancy could potentially be attributed to size overestimation caused by aggregation effects when the Morphologi 4® is used, where the analysis software identifies aggregates as single particles.

Shape factor analysis indicated that, regardless of BL loading, the microparticles exhibited a circular, non-elongated morphology, as evidenced by high circularity (0.87–0.89) and low elongation values

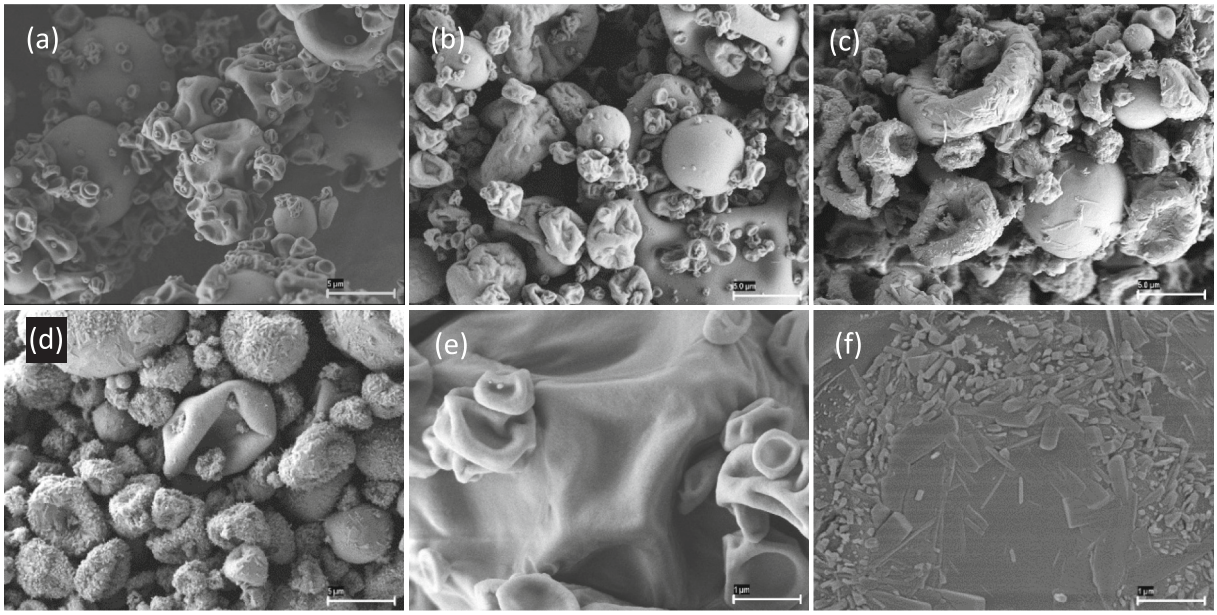

Fig. 1. Field emission scanning electron microscopy images of LBG microparticles at different LBG:BL mass ratios: (a) 10:0.0, (b) 10:0.2, (c) 10:0.7, (d) 10:1.2 (w/w); and surface detail of (e) 10:0.0 and (f) 10:1.2 (w/w) microparticles. Scale bars: 5 μm (a–d), 1 μm (e, f).

Table 2
Size and shape parameters of unloaded and bacterial lysates (BL)-loaded locust bean gum (LBG) microparticles (mean ± SD, $n = 3$), as obtained from Morphologi 4®. Different letters represent significant differences in each parameter ($p < 0.05$).

Microparticles LBG:BL (*w*/w)	Circle equivalent diameter (μm)	Circularity	Elongation	Convexity
10:0.0	4.5 ± 0.1[a]	0.894 ± 0.006[c]	0.210 ± 0.006[e]	0.960 ± 0.004[g]
10:0.2	6.3 ± 0.2[b]	0.874 ± 0.004[d]	0.234 ± 0.003[f]	0.949 ± 0.004[h]

(0.21–0.23) [24]. Spherical particle geometries have been shown to be more efficiently internalized by phagocytic cells compared to elongated or non-spherical particles [25], which favours the application proposed herein. Convexity values around 0.95 also reflect the presence of slightly convoluted/collapsed particles observed in SEM microphotographs (Fig. 1). This indicates that particles have a slight deviation from spherical particles (convexity =1), which is associated with an increased surface area, potentially favouring the deposition pattern within the lungs and enhancing drug delivery efficiency [26].

The spatial distribution of BL within the microparticles could also provide insights into potential delivery behavior. To visually determine the spatial distribution of BL and LBG within the microparticles, fluorescent labelling of the components combined with CSLM was applied. In this approach, the BL components were labelled with a red fluorescent probe (BL-RITC), and LBG was conjugated to a green fluorescent dye (LBG-FL). As shown in Fig. 2, the CSLM observations indicated that both the BL antigens and the LBG carrier material had a ubiquitous localization throughout the microparticles. This distribution, with the presence of BL both on the surface and distributed through the microparticle matrix, can potentially enhance the presentation and uptake of the antigens by immune cells, facilitated by the LBG-mediated interaction with APC. The co-localization of BL and LBG could allow for a more controlled and dynamic presentation of the antigens to the APC, thereby stimulating a more robust and specific immune response.

3.3. Determination of association efficiency (AE) and loading capacity (LC)

The AE of BL and LC of microparticles were determined using the Bradford protein assay. BL are a mixture of components and their quantification as a whole would be complex. As a main component of BL comprises proteins, the Bradford protein assay was used to measure the total protein content, providing a proxy for quantifying the encapsulation of the total BL components within the microparticles, by extrapolation. The results are displayed in Table 1, showing higher AE (81 %, $p < 0.05$) for the formulation with the lower BL content (LBG:BL = 10:0.2, *w*/w), which progressively decreased to reach 56 % in the microparticles containing the higher amount of BL (10:1.2, w/w). Interestingly, the LC remained relatively constant at around 2–3 % across all formulations, a

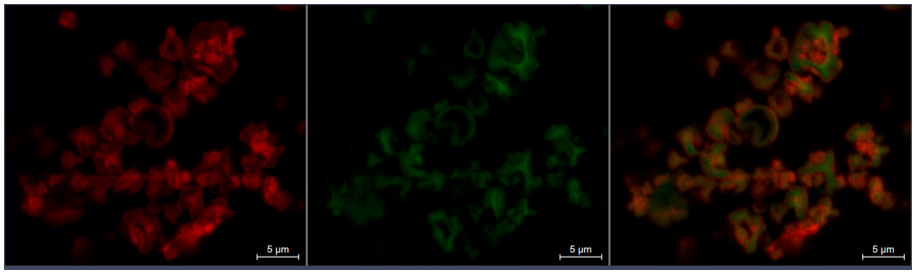

Fig. 2. Confocal laser scanning microscopy images of fluorescence-labelled locust bean gum (green) microparticles loaded with bacterial lysates (red). Scale bar = 5 μm).

level considered acceptable for the intended application and aligned with reported studies of respiratory immunization. In the context of inhaled antigens, the immune system is highly sensitive, and even microgram or lower quantities presented in a pathogen-mimicking manner can elicit robust responses [27].

3.4. Aerodynamic particle size distribution

The aerosolization properties of dry powders intended for inhalation stand as the most relevant characteristics. In the application proposed herein it is intended to reach the mid-lung region and smaller airways, thus an aerodynamic diameter below 5 μm is desired. Fig. 3 shows the stage-by-stage deposition profile of BL (extrapolated from protein content) associated to the tested microparticles. For all formulations, it is possible to observe high deposition in the induction port (IP, 30–35 %), which represents the oropharyngeal area. While the microparticles composed of LBG:BL = 10:0.2 (*w*/w) (Fig. 3.A) showed a relatively uniform deposition (3–7 %) across the intermediate stages, dry powders with higher BL content demonstrated a progressive tendency toward increasing deposition in the higher stages. This behavior reflects the less favourable aerodynamic properties of the microparticles with higher BL content, as seen in Table 3.

The aerodynamic properties were determined from the registered deposition (Table 3). The aerodynamic particle size analysis revealed that the MMAD increased with increasing BL content. Specifically, the microparticles with the lowest BL amount (LBG:BL = 10:0.2, w/w) exhibited the most suitable MMAD (<5 μm) for pulmonary delivery applications. This MMAD is comparable with that of the unloaded microparticles, while the other formulations containing BL (higher amounts) registered values over 5 μm that do not favour lung deposition. MMAD determines aerosol deposition within the lungs, with particles in the 1–5 μm range capable of reaching the lower regions of the respiratory tract [28].

The MMAD of 4.6 μm achieved by the LBG:BL = 10:0.2 (w/w) microparticles is particularly suitable for immunomodulation applications, as this intermediate size targets mid-lung regions where APCs are most active, while it further enables interaction with multiple compartments of the respiratory tract [9,10]. This aerodynamic characteristic positions the formulation to deliver antigens from the diverse pathogenic species present in OM-85 (*S. aureus*, *S. pyogenes*, *K. pneumoniae*, and others) to anatomical sites matching their natural colonization patterns, potentially enhancing the induced immune response [4,8].

Table 3
Aerodynamic parameters of unloaded and bacterial lysates (BL)-loaded locust bean gum (LBG) microparticles (mean ± SD; $n \geq 2$). Different letters represent significant differences in each parameter ($p < 0.05$).

LBG:BL (w:w)	Emitted dose (%)	MMAD (μm)	GSD	FPD (μg)	FPF (%)
10:0.0	70.2 ± 19.8^{a}	4.8 ± 0.3^{c}	2.2 ± 0.1^{e}	4.2 ± 0.6^{h}	25.9 ± 1.3^{j}
10:0.2	96.8 ± 1.6^{b}	4.6 ± 0.4^{c}	1.9 ± 0.1^{f}	23.1 ± 4.5^{i}	29.0 ± 3.8^{j}
10:0.7	98.3 ± 0.1^{b}	7.4 ± 0.3^{d}	1.8 ± 0.2^{f}	23.1 ± 1.3^{i}	13.6 ± 0.5^{k}
10:1.2	99.5 ± 0.7^{b}	8.2 ± 3.7^{d}	1.5 ± 0.1^{g}	16.9 ± 6.6^{i}	11.3 ± 3.1^{k}

FPD: fine particle dose; FPF: fine particle fraction; GSD: geometric standard deviation; MMAD: mass median aerodynamic diameter.

In line with the MMAD results, the microparticles composed of LBG:BL = 10:0.2 (*w*/w) demonstrated the highest FPF, around 30 %. This was deemed appropriate, given that many marketed inhalable products have similar pulmonary deposition values, with FPF typically around 20–30 % [29]. All the microparticles exhibited a polydisperse size profile, as evidenced by GSD values ranging between 1.5 and 2.2. This supported the observations made through SEM microphotographs and the high standard deviations obtained from the Feret's diameter measurements.

The deposition of unloaded microparticles (LBG:BL = 10:0.0, w/w) could not be quantified by protein content, thus a gravimetric-based procedure was tentatively applied for the determination of aerodynamic parameters. A different dose was used, in comparison with the testing of BL-loaded microparticles, which accounts for the observed differences in FPD.

The gravimetric approach was also tested for BL-loaded microparticles alongside the protein quantification methodology, to compare methods and assess gravimetric accuracy. Gravimetric determination involves quantifying powder mass deposited in all parts but has

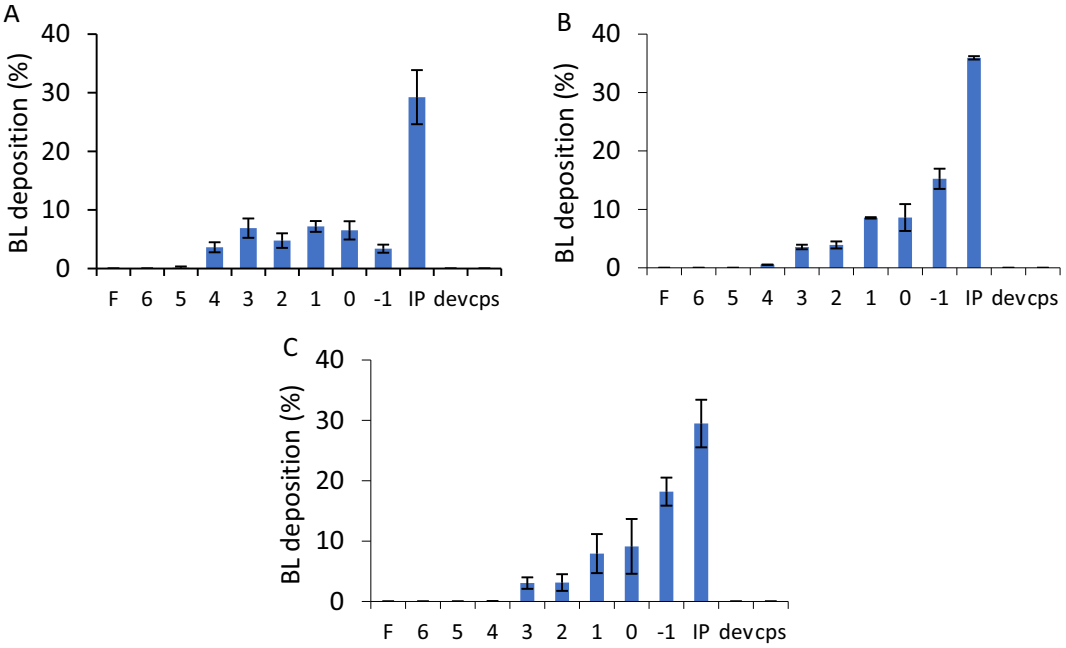

Fig. 3. *In vitro* deposition of bacterial lysates (BL) in Anderson cascade impactor stages, according to the protein quantification after aerosolization of locust bean gum (LBG) microparticles: (A) LBG:BL = 10:0.2 (w/w); (B) LBG:BL = 10:0.7 (w/w); (C) LBG:BL = 10:1.2 (w/w). Values are mean ± SD, $n \geq 2$. F: filter; IP: induction port; dev: inhaler device; cps: capsules.

limitations for weighing powder on the induction port due to its heavy weight. Relevant differences between methods were observed for IP stage deposition, with gravimetric measurements consistently overestimating BL amounts compared to protein quantification. Given its ability to directly measure protein concentration and enable powder recovery from intermediate stages, protein detection was deemed the more valid quantification approach.

3.5. Antigenicity preservation

The process of microencapsulation through spray-drying exposes the materials to high temperatures and shear stress, which could potentially degrade antigens within the BL, compromising the capacity to elicit an immune response. Therefore, evaluating the antigenicity of BL after the microencapsulation process appeared as an essential endeavor. To investigate these effects, *Streptococcus pyogenes*, a relevant bacterial strain present in the OM-85 formulation, was used as the reference. To obtain antibodies against this strain, rabbits were immunized with inactivated bacteria to produce specific serum.

A microplate agglutination test (MAT) was performed to assess the retention of BL antigenicity within spray-dried microparticles. Aqueous microparticle dispersions were incubated with serum containing antibodies against *S. pyogenes*. While no agglutination was observed for unloaded LBG microparticles, those containing BL showed agglutination when incubated with serum, whereas no agglutination was observed in the absence of serum (Fig. 4A). These results demonstrate that the agglutination effect is specifically due to antigen-antibody interactions between BL and the serum. Interestingly, microparticles with lower BL content and smaller diameters exhibited stronger agglutination. These enhanced antibody-antigen interactions are attributed to the increased epitope availability resulting from the higher surface area of smaller particles per unit concentration.

To further validate these findings, a commercially available rapid test for *S. pyogenes* capsule detection was utilized. After optimization, a concentration of 1.4 mg/mL of the microparticle formulation produced a positive result (Fig. 4B). This assay confirmed the presence of capsular antigens on the microparticles and demonstrated that these key bacterial antigens remained preserved despite the processing conditions, as evidenced by their recognition by monoclonal antibodies.

3.6. Analysis of mucoadhesion

Mucoadhesion can prolong the lung retention time of inhaled particles, making it a desirable property for certain pulmonary drug delivery applications [30,31]. To further evaluate the suitability of LBG microparticles for this strategy, their mucoadhesive properties were assessed using a texture analyser and results are displayed in Fig. 5. Lactose served as a negative control, representing minimal adhesion, while chitosan was used as a positive control due to the well-established mucoadhesive characteristics of the polymer [32,33].

Tablets of the test samples were compressed and brought into contact with mucin discs, mimicking the lung mucosal layer. The detachment force required to separate the microparticle tablets from mucin was measured at both 25 °C and 37 °C (Fig. 5). The LBG microparticles, regardless of BL loading, exhibited consistent mucoadhesive properties, with a work of adhesion ranging from 1.8 to 2.3 N·mm. BL-loaded or unloaded LBG microparticles displayed significantly higher work of adhesion values ($p < 0.05$) compared to the negative control (lactose). Notably, they showed a tendency toward higher values than chitosan, which is recognized for its mucoadhesive properties, despite no statistical difference being observed. Furthermore, a variation in temperature (25 °C and 37 °C) was not found to significantly impact the mucoadhesion of the tested materials. The reported findings suggest that LBG microparticles may prolong the lung residence time of the incorporated BL, thereby enabling favourable interaction with the APC and sustained release of the bacterial antigens at the target site.

3.7. In vitro release profile

To better simulate powder delivery to the lungs, as occurs during inhalation, an in-house developed insufflation device (Fig. 6A, patent pending) was used to disperse the dry powder formulation onto a membrane surface [18]. This device intends to better mimic the effect of powder dispersion occurring within the respiratory tract. To further simulate conditions within the lung, the release experiments were conducted providing the contact of the basal side of the membrane with a thin layer of PBS (pH 7.4), while no liquid was added on the apical side, thus preventing microparticle submersion in liquid.

The release profile was examined using a representative microparticle formulation (LBG:BL = 10:1.2, w/w), which had a relatively higher BL loading, thus permitting the quantification even when low amounts of protein are released. The results showed that approximately 50 % of BL released within 2 h, reaching 80 % after 6 h and stabilising around 85 % at 24 h (Fig. 6B). The observed sustained release suggests the promotion of a more robust immune response by enabling an extended antigen-APC interaction and mimicking natural infection through controlled release. A continuous release of BL may also lead to a smaller dose of the antigen being required to achieve the same immunomodulation effect and could help maintain antigen levels within a safe and effective range, minimizing potential side effects. These results are consistent with the even distribution of BL within the microparticles, as described in Section 3.2. The dual-phase release profile may enhance the overall immune response by combining immediate activation with sustained antigen presentation.

3.8. Stability study

European regulations require pharmaceutical stability testing under

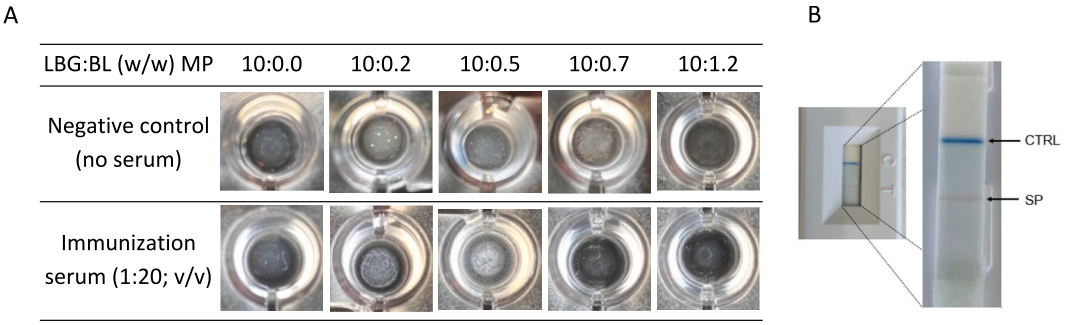

Fig. 4. A. Microplate agglutination test with various LBG:BL ratios in microparticles using a specific immune serum against *Streptococcus pyogenes* [in PBS (1:20; v/v) at 37 °C for 24 h]. B. Specific immunochromatography assay against *S. pyogenes* capsule using LBG:BL = 10:0.2 (w/w) microparticles (MP) at 1.4 mg/mL. SP: immobilized specific monoclonal antibodies; CTRL: control line.

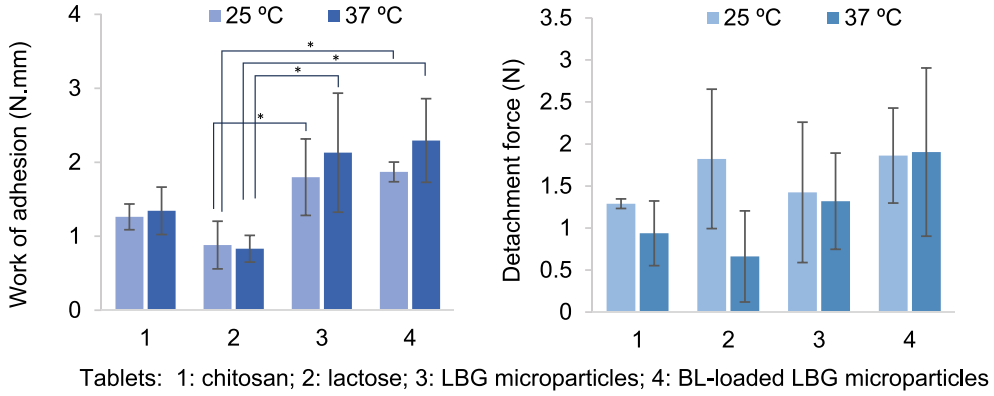

Fig. 5. Work of mucoadhesion between mucin discs and tested tablets; Results expressed as mean ± SD ($n = 3$). Statistically significant differences are indicated with an asterisk ($p < 0.05$).

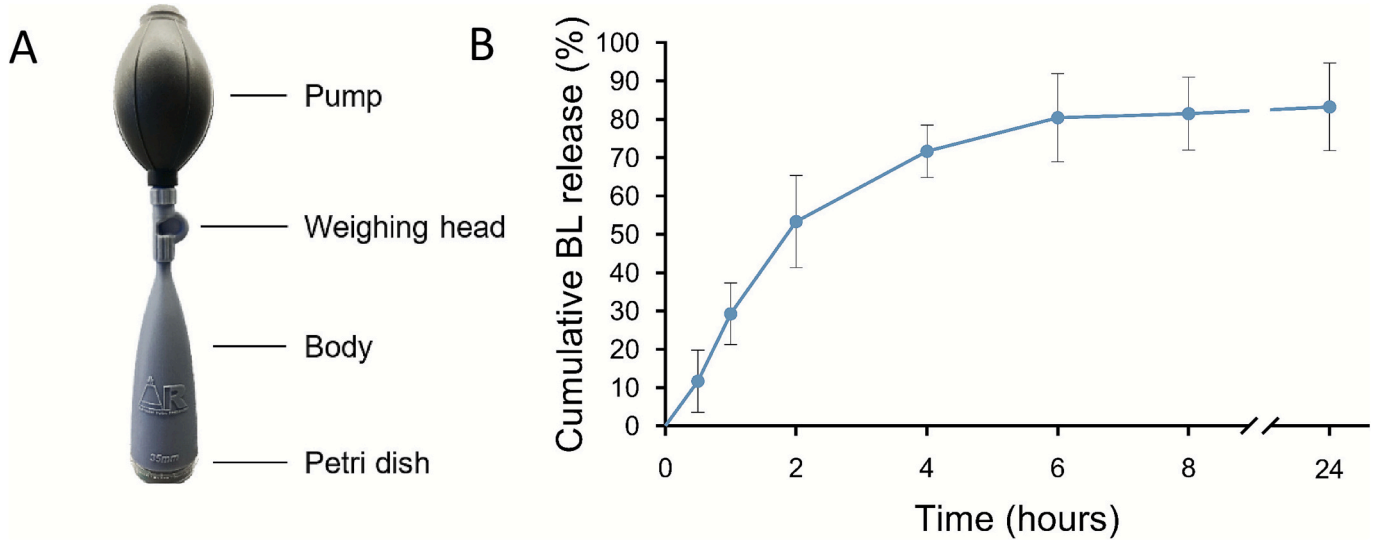

Fig. 6. A - Aerosolization device developed in-house. B - *In vitro* release profile of BL from microparticles (LBG:BL = 10:1.2, w/w) in PBS (pH 7.4). Data represents mean ± SD (n = 6).

specific environmental conditions, Portugal being assigned Zone II (subtropical/Mediterranean) [34,35]. In this study, microparticles (LBG:BL = 10:0.2, *w*/w) were tested regarding their storage stability and compared with the commercial formulation of OM-85, used as control. Following ICH guidelines, a long-term (12 months) stability study was performed at a temperature of 25 ± 2 °C and relative humidity of 60 ± 5 % RH. The results are presented in Fig. 7.

The protein content analysis revealed distinct behavior for LBG:BL microparticles and the non-encapsulated commercial OM-85 formulation (BL) over the 12-month storage period. The protein content in commercial OM-85 demonstrated preservation of stability throughout the study period, maintaining levels around 100 % after an initial minor decrease to approximately 95 % ($p < 0.05$). On the other hand, the LBG:BL microparticles were stable up to 2 months and the determination of protein content at 12 months also indicated stability (*ca.* 100 %). However, an intermediate determination at 6 months indicated a decrease to 65 % of the initial content ($p < 0.05$). Assuming that the occurrence of a real protein decrease would be irreversible, the observed variation suggests potential challenges in protein dispersion efficiency specific to the microparticle formulation during the process of protein quantification. The applied methodology (Bradford assay) requires complete protein dissolution. The fact that LBG forms viscous solutions may hamper the reproducibility of pipetting and the homogenization of the dispersion with the Bradford reagent during the process, all

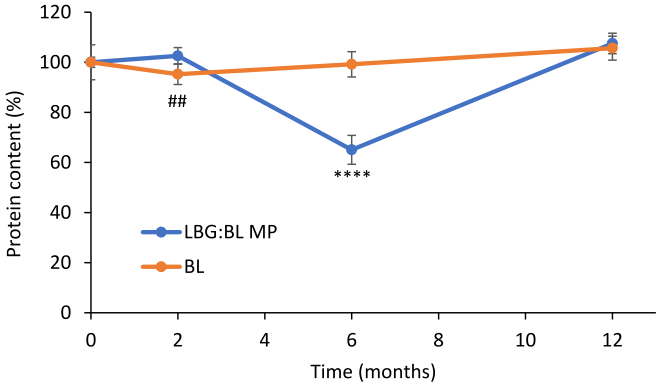

Fig. 7. Protein content changes in commercial BL (OM-85) and LBG:BL microparticles (10:0.2 w/w) during 12-month storage at 25 ± 2 °C and 60 ± 5 % RH. Mean ± SD ($n = 3$). Statistical significance levels denoted as ****$p < 0.0001$ for LBG:BL MP and as ##$p < 0.01$ for OM-85, compared with day 0.

contributing to method variability. The moisture content of the microparticle formulation was found to be 2.7 ± 0.6 % (*w*/w), which is within the recommended 3 % limit for dried biological formulations [36] and, thus, is not expected to interfere in the reported observations. The use of

a more accurate analytical technique, such as HPLC, for the determination of protein would probably help solving the observed limitations, but the complex mixture of substances comprising the lysates hampers this option.

3.9. Metabolic activity screening of microparticles

As an indicator of cell viability, the metabolic activity of A549 cells, used herein as model of respiratory epithelium, was determined following the exposure to BL-loaded microparticles, using the MTT assay. The study design included controls of non-encapsulated OM-85 and of the unloaded LBG microparticles, comprised only of the matrix material, to distinguish between effects caused by the active compound and those potentially induced by the microparticle matrix or its particulate nature. The assessment was performed after a 24 h exposure period, a duration chosen based on *in vivo* research demonstrating that the majority of particle clearance by phagocytosis occurs within this timeframe [37]. According to ISO 10993-5:2009 guidelines, cytotoxicity was defined as cell viability below 70 % [38].

The results of the MTT assay revealed a concentration-dependent effect of BL-loaded microparticles on A549 cell viability (Fig. 8). As a whole, as the concentration increased, a gradual decline in cell viability was noticed in the tested range of concentrations. Importantly, the results evidenced absence of significant toxicity from microparticles at physiologically-relevant concentrations, up to 125 μg/mL. Indeed, at such conditions cell viability generally remained above the 70 % threshold, only reaching 68 % in the formulation containing the higher amount of BL (LBG:BL = 10:1.2, w/w).

The cell viability subsequent to microparticle exposure gradually decreased to values around 55–60 % at concentrations up to 1 mg/mL, but only for the formulations LBG:BL = 10:0.7 (w/w) and LBG:BL = 10:1.2 (w/w). Microparticles of LBG:BL = 10:0.2 (w/w) behaved similarly to unloaded LBG microparticles, generally showing absence of toxicity. This trend was mirrored in experiments testing non-encapsulated BL, suggesting that the observed effect is likely attributable to the BL itself rather than the microparticle formulation. Notably, the microparticle formulation with a LBG:BL ratio of 10:0.2 (w/w) demonstrated exceptional biocompatibility. Even at concentrations as high as 1000 μg/mL, which clearly exceeds a possible concentration, no cytotoxicity was observed (cell viability of 69 %). Interestingly, these microparticles predominantly induced higher cell viability values compared to unloaded microparticles, which was more evident at higher concentrations. When comparing BL-loaded microparticles to the corresponding non-encapsulated BL content, the cytotoxicity profiles were similar.

Concentrations ranging from 0.1 to 1 mg/mL are typically tested in the context of lung delivery applications, but this range includes overestimated concentrations, considering that the lung has a surface area of up to 80–100 m^2 [39]. Biorelevant concentrations are therefore up to around 125 μg/mL and the results of this study indicated absence of significant toxicity in those conditions. This supports the suitability of the developed delivery platform and particularly LBG:BL = 10:0.2 (*w*/w) microparticles for an application in lung inhalation, which exhibit promising cytocompatibility profile. Furthermore, the tested concentrations reached 2 mg/mL in order to allow the calculation of the maximal inhibitory concentration (IC_{50}). The parameter was determined whenever applicable to microparticles and the corresponding BL content (before microencapsulation) (Table 4). IC_{50} values of microparticles ranged from 1.199 mg/mL (unloaded microparticles) to 1.762 mg/mL for the BL-loaded microparticles with the highest BL content (LBG:BL = 10:1.2, w/w). These results indicate that increasing drug loading correlates with enhanced cytotoxicity. Nevertheless, for the 10:0.2 w/w formulation and corresponding BL amount, since the maximum concentration exposure did not generate viability below 50 %, an accurate determination IC_{50} was not possible. Therefore, these results suggest that this low drug loading may represent an optimal balance between therapeutic efficacy and cytotoxicity.

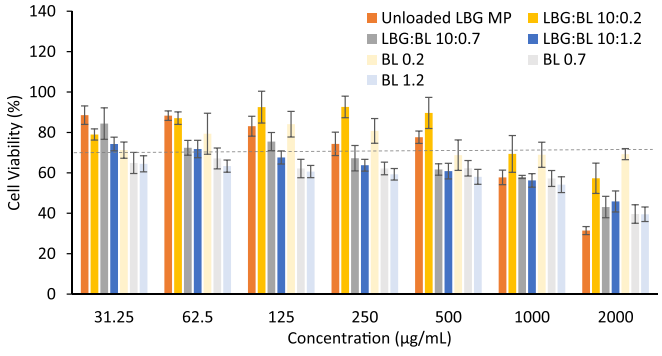

Fig. 8. A549 cell viability measured by the MTT assay after 24 h exposure to BL-loaded and unloaded LBG MP, as well as non-encapsulated BL. Results are expressed as mean ± SEM ($n \geq 4$, three replicates per experiment at each concentration). Dashed line represents 70 % viability.

4. Conclusion

This study presents the development and characterization of novel inhalable microparticles composed of LBG and BL for potential use in pulmonary immunization against respiratory infections. First, the spray-drying process successfully produced microparticles with consistent yield across various LBG:BL ratios, demonstrating a robust and reproducible production method. The optimal formulation achieved high association efficiency and suitable loading capacity, indicating efficient encapsulation of BL within the LBG matrix. Physicochemical characterization revealed favourable microparticle properties for pulmonary delivery, including appropriate size distribution and aerodynamic characteristics. Microparticles composed of LBG:BL = 10:0.2 (w/w), the lowest BL content, demonstrated the most promising features for lung deposition, essential for effective pulmonary immunization. Importantly, LBG-based microparticles exhibited superior mucoadhesive properties compared to control substances. This characteristic could potentially prolong the residence time of the formulation in the lung, allowing for extended interaction with the pulmonary immune system. Furthermore, *in vitro* release studies showed a sustained release profile of BL up to 6 h, suggesting the potential for both rapid initiation and more prolonged stimulation of the immune response. The combination of targeted pulmonary delivery, mucoadhesive properties, and sustained antigen release comprises a promising approach for enhancing immune responses against respiratory pathogens. Preliminary cytotoxicity studies showed absence of toxicity of the microparticles tested at physiologically relevant concentrations. While these results are encouraging, several aspects require further investigation, including the study of immunomodulatory effects, *in vivo* immunogenicity and comparative studies with traditional immunomodulation methods. Additionally, exploring the efficacy of an inhalable formulation of isolated lysates could be more informative of potential results. In conclusion, this study developed an inhalable immunomodulation delivery system that shows promise for an application in lung immunization

Table 4
IC_{50} values (mg/mL) determined in A549 cells following 24 h exposure to locust bean gum (LBG) microparticles with varying bacterial lysate (BL) loading and the respective non-encapsulated BL content.

LBG:BL (w/w)	IC_{50} (mg/mL)			
	10:0.0	10:0.2	10:0.7	10:1.2
Microparticles	1.199	n.a.	1.472	1.762
Non-encapsulated BL	n.a.	n.a.	1.576	1.030

n.a.: not applicable (IC_{50} not reached under tested conditions or no BL present).

against respiratory infections.

CRediT authorship contribution statement

Joana Pinto da Silva: Writing – original draft, Methodology, Investigation. **Melibea Berzosa:** Investigation. **Lucas Chiarentin:** Investigation. **Alberto Delgado-López:** Investigation. **Maria Patrícia Almeida:** Investigation. **Carla Vitorino:** Validation, Supervision, Methodology. **Ana Figueiras:** Validation, Supervision, Methodology. **Ana M. Rosa da Costa:** Writing – review & editing, Validation, Supervision, Methodology. **Carlos Gamazo:** Validation, Supervision, Methodology, Conceptualization. **Ana Grenha:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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