Carbohydrate Polymers 363 (2025) 123729

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Locust bean gum (LBG) – A potential excipient for inhalation purposes: Excipient characterisation and *in vitro* and *in vivo* toxicological evaluation

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ARTICLE INFO

Keywords:
Dry powder
Inhalation
Locust bean gum
Lung drug delivery
Polysaccharide
Toxicological profile

ABSTRACT

Dry powders proposed for lung drug delivery typically involve excipients not approved for inhalation. The physicochemical characterisation of excipients is informative regarding their interaction with different body structures. Locust Bean Gum (LBG) has been proposed for diverse applications, including inhalation, benefiting from targeting ability towards phagocytic cells owing to the presence of mannose moieties. The aim of the present study was to characterise the physicochemical parameters of LBG and, for the first time, draw a toxicological profile for this excipient. LBG from three different suppliers was evaluated and eventual changes on polymer characteristics induced by purification and microparticle production were assessed, which were observed to not occur. The commercial samples showed identical FTIR spectra, as well as TGA and DSC profiles, M_w around 3.60×10^6 Da, and T_g near −39 °C. All microparticles presented similar morphology and Feret diameters around 4 μm. *In vitro* assays performed on respiratory (A549) cells evidenced no impact of LBG microparticles on cell viability (> 80 %) when biorelevant concentrations (≈ 0.1 mg/mL) were used. Additionally, inhalation of LBG microparticles by mice provided indications of a safe profile, without induction of allergic reactions. The basis is laid for further exploration of this material in inhalation.

1. Introduction

Lung drug delivery strategies have been studied significantly in recent years, certainly benefiting from increased awareness of their potential after Covid-19. Most of the reported approaches involve drug repurposing (Kumbhar et al., 2022), the use of medicinal chemistry (Pasqua et al., 2022) and/or new excipients (Zillen et al., 2021), which foster new therapeutic approaches. New excipients are usually proposed due to inherent properties that may improve the behaviour of the drug formulations. Nevertheless, a very reduced number of excipients is currently approved for inhalation purposes, which is a real limitation to the development of new medicines. Among the new excipients being described in the literature, polysaccharides are a class receiving high attention (Valente et al., 2022). Our lab has been working in the development of inhalable dry powders for a long time, where polysaccharides that are not much explored and, in any case, not approved for inhalation, are very frequently included in the formulations. Polysaccharides present structural flexibility, are usually available at low cost and frequently present other functional abilities that further enhance their attractiveness. Locust bean gum (LBG), a galactomannan, is one of the materials being explored (Grenha et al., 2020; Yadav & Maiti, 2020). Apart from the beneficial features of polysaccharides, the great interest of this material relies on its reported ability to favourably interact with phagocytic cells, such as macrophages (Rodrigues et al., 2017), thus assuming a potential role in cell targeting that could be relevant in the management of intracellular diseases such as tuberculosis. LBG is further reported as a mucoadhesive material (Sudhakar et al., 2006), which can be beneficial in applications involving mucosal

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https://doi.org/10.1016/j.carbpol.2025.123729
Received 30 December 2024; Received in revised form 10 April 2025; Accepted 8 May 2025
Available online 11 May 2025
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surfaces. Current Food and Drug Administration (FDA) approval status of LBG refers only to oral dosage forms (Meunier et al., 2014). The approval of a material is specifically linked to a pharmaceutical dosage form and route of administration, requiring detailed and extensive characterisation of the excipient for the specific conditions (Elsayed, 2019; Nyamweya, 2021). In fact, similar to drugs, excipients also interact with the human body, and this interaction often remains to be fully understood and characterised (Martinez, Sinko, et al., 2022; Martinez, Wu, et al., 2022). As a novel excipient, LBG requires a thorough physicochemical characterisation, which is critical for subsequent evaluation of the toxicological profile, information of utmost importance for the approval of drug products. Comprehensive characterisation of LBG is not available in the literature and certainly not in the context of an application in lung delivery. The scarcity of information regarding physicochemical and biological aspects of the excipients is a limitation for industry, hampering their use in formulation development and, ultimately, hindering progress in the development of new therapeutic products (Bejarano et al., 2019; Elder et al., 2016).

In this sense, the aim of this work was to perform a comprehensive characterisation of LBG as excipient and ascertain if it reveals a suitable toxicological profile to enable its application in the context of lung inhalation. Because geographic origin and climatic factors, as well as seed processing, may impact LBG molecular structure and physicochemical properties, raw material from three different suppliers was tested in this study. Due to the high level of purity demanded for all components in any pharmaceutical formulation, and being well established that commercial samples of LBG contain 3–7 % of protein as impurity, which decreases to 0.1–0.7 % in clarified gum (Kawamura & Joint FAO/WHO Expert Committee on Food Additives, 2016), a previous purification step was performed. Moreover, testing the different forms (commercial polymer, purified polymer and microparticles) was also deemed relevant to evaluate the effect of heating (both in purification and spray-drying) on the polymer. The work is, thus, expected to fulfil an existing gap on the available information on LBG, fostering its widespread use in drug delivery applications, particularly in inhalation.

2. Materials and methods

2.1. Materials

Three LBG raw materials were obtained from different sources: one was acquired from Sigma-Aldrich® (polymer origin: Italy, batch: 098 K0123) and the other two were kind gifts from Industrial Farense® (Faro, Portugal; polymer origin: Portugal, batch: 02.2020/01) and C.E. Roeper® (Germany; polymer origin: Mediterranean region, unspecified, batch: 8855019). Sodium azide (NaN_3), L-glutamine solution (200 mM), non-essential amino acids solution, penicillin/streptomycin (10,000 units/mL, 10,000 g/mL), trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), trypan blue solution (0.4 %), thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), Triton-X 100, phosphate buffered saline (PBS) tablets pH 7.4 and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich®. Lactate dehydrogenase (LDH) kit was obtained from Takara Bio (Tokyo, Japan) and fetal bovine serum (FBS) from Gibco (Life Technologies, USA). Ham's F-12 and RPMI 1640 media were from Lonza Group AG (Switzerland). Ultrapure water (Milli-Q, Millipore, Watford, UK) was used throughout. All other chemicals were reagent grade.

2.2. Cell culture

Human lung epithelial adenocarcinoma cells (A549) were provided by the American Type Culture Collection (ATCC, USA) and used in passages 20–38. Cells were cultured in flasks (75 cm^2), at 37 °C, in an incubator with 5 % CO_2/95 % humidified atmospheric air. Cell culture medium (CCM) was Ham's F-12, supplemented with L-glutamine 200 mM (1 %, *v*/v), non-essential amino acids (1 %, v/v), penicillin/streptomycin at 1 % (*v*/v) and FBS at 10 % (v/v). CCM was exchanged two to three times per week.

2.3. Raw material purification and identification

2.3.1. Polymer purification

LBG raw materials are known to contain proteins (≈7 %) and other impurities. Therefore, a purification step was required and applied to the three LBG samples, which was based on a protocol reported elsewhere (Braz et al., 2018). In brief, an amount of LBG was dispersed in ultrapure water heated at 85 °C, for 1 h. After cooling to room temperature, the dispersion was centrifuged (22,000 xg, 20 °C, 20 min), and the supernatant precipitated in an equal volume of ethanol. The precipitate was collected using a stainless-steel sieve, separated into small threads, and dried in a vacuum oven at 30 °C, for 72 h.

2.3.2. Fourier-transform infrared spectroscopy (FTIR) analysis of LBG

LBG samples were blended with KBr in a mortar and compressed into a disc. Spectra were collected by means of a 25-scan interferogram, in transmittance mode, in the 4000 to 400 cm^{-1} region, with a 4 cm^{-1} resolution (Bruker Tensor 27, Bruker OPTIK GmbH, Germany). OPUS software (version 6.5) was used for data acquisition and analysis.

2.4. Spray-drying to obtain LBG microparticles

Purified LBG (three different sources) was spray-dried (Buchi B-290 laboratory mini spray-dryer, Buchi Labortechnik AG, Switzerland) to produce microparticles. To do so, an amount of purified LBG was weighed and dispersed in ultrapure water (1 %, *w*/*v*) at 85 °C for 1 h for solubility improvement (García-Ochoa & Casas, 1992). After cooling to room temperature, the dispersion was left under mild stirring overnight. The next day, the LBG dispersion was heated again (85 °C) before spray-drying, to decrease its viscosity. The spray-dryer was equipped with a high-performance cyclone and operated in open mode using compressed air as fluid (spray flow rate of 473 L/h). Other conditions were as follows: inlet temperature of 130 °C, aspirator at 100 % and feed flow rate set at 1.0 mL/min. After each spray-drying process, the yield was calculated using eq. 1:

$$\textit{Process yield}\ (\%) = \frac{\textit{Weight of microparticles}\ (g)}{\textit{Total amount of solids}\ (g)} \quad (1)$$

2.5. Characterisation of LBG microparticles

The morphology of the microparticles was determined by field emission scanning electron microscopy (FESEM; FESEM Ultra Plus, Zeiss, Oberkochen, Germany), using a protocol detailed in (Guerreiro et al., 2021). The size of the microparticles was determined as the Feret's diameter, which was estimated as the mean of 300 particles measurement ($n = 300$), registered from the photographs obtained by FESEM.

Particle size distribution was evaluated, with D10, D50 and D90 being determined from the cumulative particle size distribution, representing the size below which 10 %, 50 % or 90 %, of all particles are found, respectively.

2.6. Molecular weight distribution

High Performance Size Exclusion Chromatography (HPSEC) with a refractive index detector (Knauer K-2300, Berlin, Germany) was used to analyse commercial and purified LBG from three different suppliers, as well as the corresponding LBG-based microparticles. Two columns OHpak SB-806 M HQ of 300 × 8.0 mm were used in series with a 50 × 6.0 mm OHpak SB-G 6B guard column (Shodex, Tokyo, Japan). Ultrapure water with 0.02 % NaN_3 was used as mobile phase at a flow rate of 1 mL/min. Samples were dissolved in the solvent used as mobile phase at a concentration of 1 mg/mL and filtered through a 0.45 μm size-pore

filter (VWR, Portugal). A set of 12 pullulan standards (ReadyCal-Kit, Lot No: pulkitr1h-03, PSS Polymer Standards, Germany) was injected in the same conditions of the samples and used to draw a conventional calibration curve, as used elsewhere (Gómez-Ordóñez et al., 2012; Guerreiro et al., 2021; Zhang et al., 2003). Results were analysed using a commercially available chromatography software (Clarity, version 8.8.0, DataApex). The equation for the analysis model (Eq. 2) was as follows:

$$y = 0,00022x^3 - 0,02579x^2 + 0,06427x + 10,18816 \quad (2)$$

The model presented above was employed in the chromatogram of the materials analysed for the calculation of molecular weight distribution results (M_n = number-average molecular weight, M_w = weight-average molecular weight). Furthermore, the broadness of the peaks obtained in the chromatogram that translates into sample dispersity was calculated using the former parameters (Zhou et al., 2000) in Eq. 3:

$$Đ = \frac{M_w}{M_n} \quad (3)$$

2.7. Thermophysical features of LBG and LBG-based microparticles

2.7.1. Thermogravimetric analysis

The thermal stability of all LBG-based samples (commercial LBG, purified LBG and microparticles) was evaluated *via* Thermogravimetric Analysis (TGA 209-F1 Iris®, Netzsch, Germany). The samples (up to 15 mg) were heated from 20 °C up to 1000 °C, at a heating rate of 10 °C/min, under a nitrogen atmosphere.

2.7.2. Differential scanning calorimetry

The thermal behaviour of the various LBG samples (commercial, purified and microparticles) was evaluated using a differential scanning calorimeter (DSC Q200, TA Instruments, Germany). Each sample was weighed (5–15 mg) into aluminium pans, which were sealed, and their lids pierced. Measurements were performed between −80 °C and 250 °C with a heating rate of 10 °C/min, under a nitrogen atmosphere, and for three cycles per sample.

2.8. Cytotoxicological profiles of LBG and LBG-based microparticles

2.8.1. Preparation of test samples for in vitro assays

A stock solution of 2 mg/mL of commercial and purified LBG, as well as of LBG-based microparticles was prepared for the three polymers obtained from different sources. In brief, each LBG sample (commercial, purified and LBG-based microparticles) was dispersed in ultrapure water (Milli-Q, Millipore, Watford, UK), previously heated at 85 °C, for 1 h under constant magnetic stirring. After that, four dilutions were prepared with CCM, in the laminar flow hood and using a vortex for homogenisation, to reach concentrations of 0.1 mg/mL, 0.25 mg/mL, 0.50 mg/mL and 1 mg/mL. The dispersions were used immediately after preparation, being mixed in the vortex before loading into 96-well plates for performance of the assays.

2.8.2. Determination of metabolic activity

The metabolic activity of A549 cells was determined after exposure to the various LBG samples (commercial, purified and microparticles) from different suppliers at various concentrations between 0.1 and 1 mg/mL. The cells were seeded in 96-well plates at 1×10^4 cells/well, incubated overnight, and exposed to the samples after that. CCM and SDS at 2 % (*w/v*) were used as positive and negative controls, respectively. After 3, 24 and 48 h of exposure, the samples were removed and replaced by 30 μL of MTT (5 mg/mL in PBS pH 7.4), which remained under incubation for 2 h, at 37 °C. After that, 50 μL of DMSO were added to each well to solubilise the formazan crystals. The absorbance was read by spectrophotometry (Infinite M200; Tecan, Austria) at 540 nm, with background correction at 640 nm. The cell viability was calculated using Eq. 4:

$$\textit{Cell viability}\ (\%) = \left[\frac{A - S}{CM - S}\right] \times 100 \quad (4)$$

where A is the absorbance obtained upon exposure to each example, S represents the absorbance measured for SDS 2 % and CM is the absorbance read for the cells incubated with CCM. The assay was performed at least 3 times, with 3 replicates for each tested concentration.

The obtained data allowed the determination of the half maximal inhibitory concentration (IC_{50}), which was calculated for samples reaching 50 % of cell viability in the conditions of the study. IC_{50} values were determined by sigmoidal fitting of the data in the GraphPad Prism statistical program (GraphPad Software, version 9.4.0.673, USA).

2.8.3. Determination of cell membrane integrity

Cell membrane integrity was assessed by the quantification of the cytoplasmic enzyme LDH on the supernatant of A549 cells after 24 h and 48 h exposure to various LBG samples at concentrations of 0.50 mg/mL and 1 mg/mL. CCM was used as negative control and Triton X-100 (1:100 dilution) as positive control of LDH release, the latter being assumed as the 100 % of released LDH. Supernatants were centrifuged (16,000 xg, 5 min) and then, 75 μL of sample were inserted in the wells of a 96-well plate, to which 75 μL of the LDH reagent were added, after preparation following the indications of the supplier. Absorbances were measured in a spectrophotometer (Infinite M200, Tecan, Grödig, Austria) at 490 nm and the background was corrected at 690 nm. The LDH released (%) upon exposure to each sample was determined by comparison with the positive control using Eq. 5:

$$\textit{LDH release}\ (\%) = \frac{A - CCM}{L} \times 100 \quad (5)$$

where A is the absorbance of the samples corrected with the background, CCM is the absorbance obtained for cell culture medium (negative control) and L is the absorbance obtained for Triton X-100 (positive control). The assay was carried out at least three times with three replicates for each tested concentration.

2.9. In vivo safety evaluation

2.9.1. Preparation of test samples for in vivo assays

LBG microparticles prepared with the polymer from Sigma-Aldrich® were used in the *in vivo* assays described in the following sections. Their preparation is detailed in section 2.4.

2.9.2. Animals

Male BALB/c mice (21–27 days) were obtained from Charles River (France). The animals were maintained in standard hygienic conditions with access to commercial chow and acidified drinking water *ad libitum*. All the experiments performed with the animals were approved by the ethical committees of the Faculty of Pharmacy of the University of Lisbon in accordance with the European Union Directive (2010/63/EU) and Portuguese laws (DL 113/2013, 2880/2015, 260/2016 and 1/2019) for the use and care of animals in research.

2.9.3. Lung administration

Animals (6–8 weeks; 22–28 g) were divided in three groups, naïve (control), LBG 1× and LBG 10×, of six animals each. The groups LBG 1×

and 10× were exposed, respectively, to a single dose (acute exposure) and to ten doses (subacute exposure) of LBG microparticles over a period of 2 weeks. The naïve group corresponded to untreated animals which received no LBG microparticles. The administration of the powder was performed using a homemade apparatus. Mice were restrained in a 50 mL tube and their nose positioned in a small hole in the far end (bottom) of the tube to promote the inhalation of the powder. In parallel, LBG powder was weighed in a 15 mL tube, the powder being then administered using a small pump connected to the upper part of the tube, creating an air stream. The air stream facilitated the insufflation of the powder and subsequent inhalation by the mice, through inspiration. Previous experiments revealed that *ca.* 10 % of the powder loaded in the apparatus and insufflated is inhaled by the animals (Grenha et al., 2020), thus the administered dose was approximately 240 mg/kg. To perform the inhalation, each dose loaded in the apparatus corresponded to 60 mg of LBG microparticles, to which the mice were exposed for 2 min (inhaling effectively 10 % of that = 6 mg). The weight of the animals was registered before each administration.

One day after the last administration (or the single administration when applicable), animals from all groups were anesthetised with isoflurane (IsoVet® 1000 mg/g, Piramal Healthcare, UK). Then, blood was extracted from the retinal blood vessels (retro-orbital bleeding technique) and stored in vials containing ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation. The animals were sacrificed by cervical displacement under anaesthesia and organs such as liver, spleen, lung, and kidneys were collected, weighed, and stored for further analysis.

2.9.4. Biochemical and histological analysis

2.9.4.1. Leucocyte count and differentiation. Blood samples were collected from all mice groups and divided into two fractions: one was sent to DNAtech (Lisbon, Portugal) to determine the leukocyte count and differentiation and the other was used in the analyses described below.

2.9.4.2. Immunoglobulin E (IgE) evaluation. The determination of immunoglobulin E (IgE) levels was performed in plasma samples. The blood volume fraction collected in a previous step was centrifuged in a refrigerated centrifuge (Sigma-202 MK, 10 min, 2000 x g) to separate the cellular part from the plasma. The latter (supernatant) was collected and IgE levels determined using a mouse IgE kit (RayBiotech, Norcross, GA, USA), following the indications provided by the supplier and using a dilution of 1:100.

2.9.4.3. Tissue index. Liver, spleen, lung, and kidneys were collected and weighed. The respective tissue index (%) was determined using Eq. 6:

$$Tissue\ index = \sqrt{\frac{Organ\ weight}{Animal\ weight}} \times 100 \quad (6)$$

The results obtained were further compared with the naïve group, used as control.

2.9.4.4. Histology. Lungs were weighed and stored in tubes containing a formalin solution (10 %, *v*/v, Sigma-Aldrich, Germany). Histological analysis of the lungs was performed at Instituto Nacional de Investigação Agrária e Veterinária, I.P. (INIAV, Lisbon, Portugal).

2.10. Statistical analysis

For *in vitro* assays, the results were analysed considering three different groups, pertaining to the three different suppliers of LBG. Within the same group, data were analysed considering two different approaches: a time- and concentration-dependent analysis. For MTT, the data were analysed using a one-way ANOVA using the Tukey's multiple comparisons test. As for the LDH, the data were analysed by means of a one-way ANOVA using the Dunnett's multiple comparisons test. For *in vivo* assays, three ANOVA tests were performed on the data presented: one-way ANOVA (Tukey and Dunnett's tests), two-way ANOVA (Dunnett's test) and the nested ANOVA (Dunnett's test) with the pairwise multiple comparison procedure being used to compare multiple groups with the naïve set. All analyses were run using GraphPad Prism® statistical program (GraphPad Software, version 9.4.0.673), and differences were considered significant at a level of $p < 0.05$.

3. Results and discussion

For an easier understanding of this section, the following nomenclature was considered:

- In-text mentions of LBG 0 correspond to the commercial form of LBG. As an example, LBG 0 Sigma, refers to the LBG raw material obtained from Sigma-Aldrich®.
- In-text mentions of LBG 1 correspond to the purified form of LBG. As an example, LBG 1 Sigma, refers to the purified LBG prepared from the raw material of Sigma-Aldrich®.
- In-text mentions of LBG 2 correspond to the microparticles prepared with the purified form of LBG. As an example, LBG 2 Sigma, refers to the microparticles prepared with the purified LBG prepared from the raw material of Sigma-Aldrich®.

3.1. Characterisation of LBG microparticles

Polymer purification is an important step for pharmaceutical formulation development, especially in the case of LBG, which is known to have protein content. Its purification rendered a yield between 50 and 67 % on average, and the purified LBG was used to further prepare microparticles by spray-drying, a process that registered an average yield between 55 and 61 %. As mentioned previously, the performed studies tested LBG from three different sources. The obtained results were generally similar among the three and, therefore, LBG from Sigma-Aldrich® was the one used in some occasions to show in figures the most complete results and was selected to perform the *in vivo* studies. The remaining data is discussed in the text and available in Supplementary Materials. The choice of the polymer from Sigma-Aldrich® is justified by the worldwide access to the provider when compared with the other two sources.

Fig. 1 depicts LBG microparticles as viewed by FESEM, showing irregular particles with many convexities, a feature that theoretically benefits the flowing properties (Yaqoubi et al., 2021), a relevant issue when inhalation applications are envisaged.

The resulting appearance can be explained through the concepts of particle engineering, an area of pharmaceutical technology that has been gaining attention for some years (Ordoubadi et al., 2021; Vehring, 2008). When a droplet is formed at the spray-dryer injector and considering that a single droplet results in one microparticle, uneven distribution of the polymer on the droplet usually occurs. During drying, the polymer migrates to the surface of the droplet in a process termed surface enrichment. Initially, it is suggested that this might function as a protective shield, but when water dries out, this protective cover collapses, resulting in the appearance shown in the photograph (Vehring, 2008). In lung delivery applications that require good flowing ability,

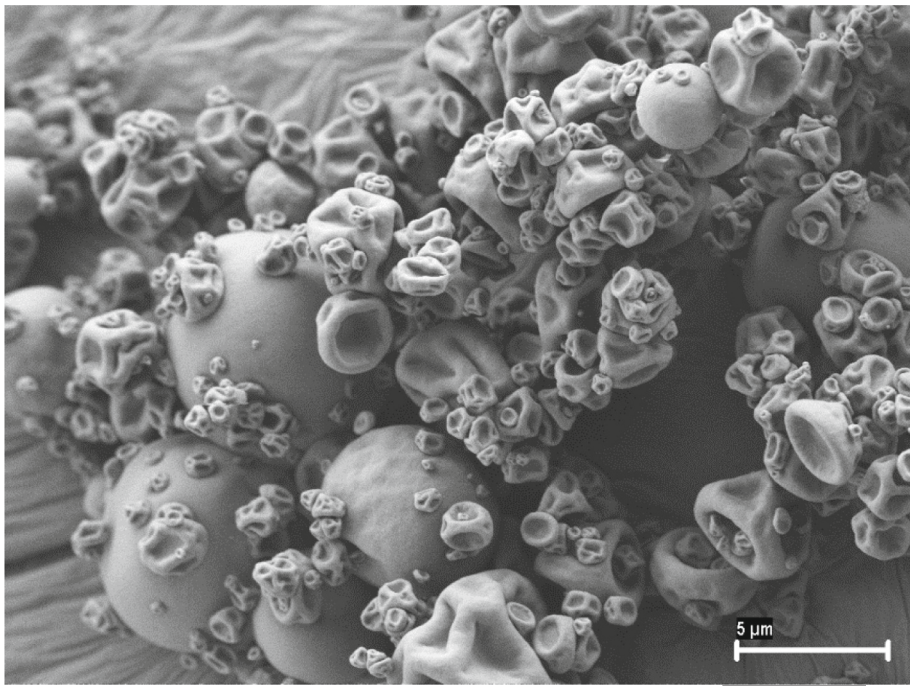

Fig. 1. Representative microphotograph of LBG-based (Sigma-Aldrich®) microparticles obtained by field emission scanning electron microscopy (FESEM).

these microparticles usually perform better than spherical ones, as their higher surface area leads to lower air resistance upon flying, thus enabling reaching deeper regions of the lung (Yaqoubi et al., 2021).

The Feret diameter of microparticles was determined to be 3.95 ± 4.25 µm. Regarding the distribution of size, D10 was registered as 1.02 µm, D50 was 2.06 µm and D90 was 7.51 µm. The remaining formulations showed similar morphology (Fig. S1), and Feret diameters of 4.31 ± 4.39 µm and 3.76 ± 4.54 µm. Values of D50 ranged between 2.2 and 2.6 µm.

3.2. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of commercial and purified LBGs are shown in Fig. 2A and B, respectively.

All spectra correspond to the spectrum of a polysaccharide, showing three characteristic bands (marked with black arrows in Fig. 2B). The first one is around 3425 cm^{-1}, which is related to the stretching of hydroxyl (O—H) groups. The other two bands, 2925 and 1026 cm^{-1}, can be associated with the C—H stretching of -CH and -CH_2 groups and the stretching of the C-O-H bond, respectively.

The only difference between the spectra of commercial LBG and those of the purified samples is the presence of a small band at 1524 cm^{-1} in the former (marked with black arrows in Fig. 2A), attributable to the amide II band of protein. The small intensity of the band is consistent with the low amount of protein in the samples, and its disappearance in the spectra of the purified gums corroborates this attribution. The amide I and amide III bands, expected around 1650 and 1300 cm^{-1} respectively, are not visible. The former is masked by the strong band of the O—H bending of adsorbed water at 1645 cm^{-1} and the latter is a weak band, easily masked by the polysaccharide bands in that region.

These findings are in accordance with others reporting LBG analysis (Jana & Sen, 2017; Kaity, Isaac, & Ghosh, 2013; Kaity, Isaac, Kumar, et al., 2013). The fact that the three spectra of the commercial LBG are very similar to each other evidences the resemblance of the samples.

3.3. High performance size exclusion chromatography (HPSEC)

The molecular weight of the various forms of different LBGs was characterised with HPSEC analysis. Fig. 3 displays the chromatograms obtained for the purified forms of the LBGs from three different sources. The remaining chromatograms are available as Supplementary Materials (Figs. S2 and S3, Tables S1 and S2).

These peaks allow the mathematical calculation of molecular weight (M_n and M_w) using the calibration curve shown in Eq. 2.

The calculations for the weight-average molecular weights of the purified samples of LBG resulted in the following data: 3.04×10^6 Da for the one from Sigma-Aldrich®, 3.40×10^6 Da for the one from Industrial Farense® and 3.57×10^6 Da for the one from C. E. Roeper® (Table 1). These results are within the same range as those obtained for the commercial samples (Table S1). Information concerning the molecular weight of this polysaccharide is scarce, and the data that exists elsewhere varies greatly depending on the sample tested: 1.8×10^6 Da (Fidan et al., 2020) and between 3.0×10^5 and 3.6×10^5 Da (Maier et al., 1993). An important aspect to take into consideration is the fact that the results obtained in this study are relative to pullulan, selected as standard for its similarity to LBG (a neutral polysaccharide) and commercial availability (LBG standards are not available), which makes comparison to reported values difficult. Nevertheless, the purpose of this analysis is to ascertain the effect of heating on polymer chains, in which the type of calibration does not impact. A very similar chromatogram was reported elsewhere (Pollard et al., 2007), also showing two peaks, one of which due to the presence of oligomers, upon elution of LBG samples. A slight decrease in M_w to 2.46×10^6, 2.38×10^6, and 2.57×10^6 Da was observed in the microparticles prepared with LBG from Sigma-Aldrich®, Industrial Farense® and C. E. Roeper®, respectively (Table S2). A similar observation was made by our group in microparticles prepared from konjac glucomannan (Guerreiro et al., 2021). Therefore, heating the polymer to 85 °C for enhanced solubilisation in the purification step and to 130 °C for spray-drying does not seem to have a significant impact in polymeric chains. This is not surprising

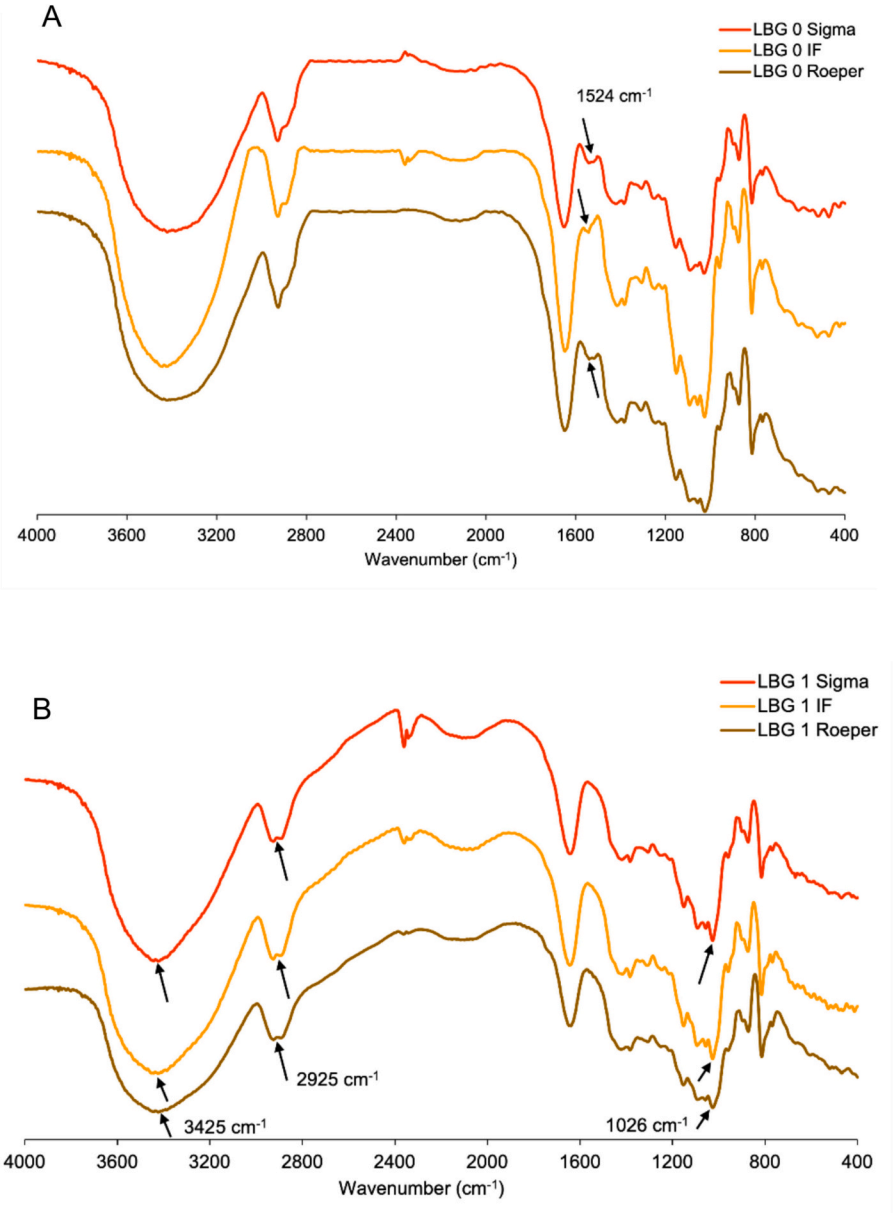

Fig. 2. FTIR spectra of A) commercial locust bean gum (LBG 0) and B) purified locust bean gum (LBG 1) obtained from three different suppliers, Sigma-Aldrich®, Industrial Farense® and C. E. Roeper®. Arrows indicate bands characteristic of groups present in the LBG molecules.

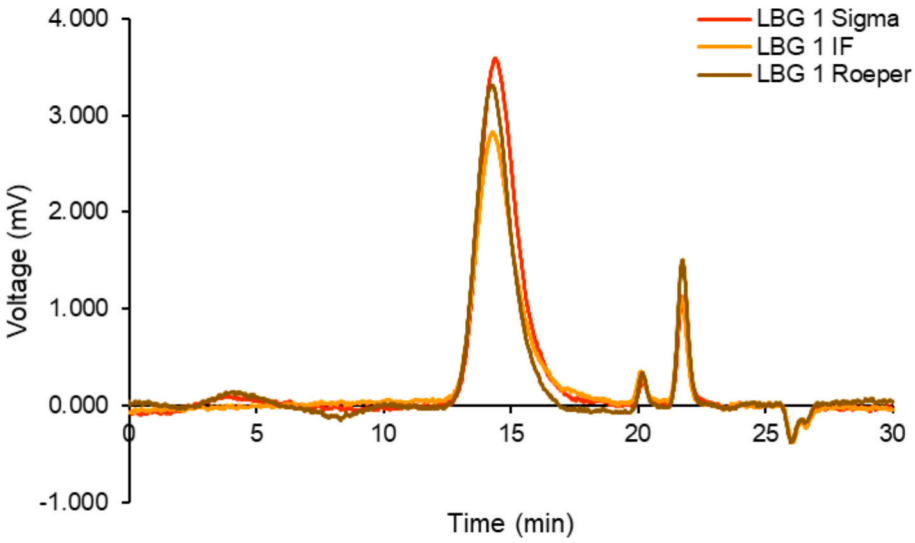

Fig. 3. HPSEC chromatograms of purified locust bean gum (LBG 1) from three different suppliers.

Table 1
HPSEC analysis of purified locust bean gum (LBG 1) from three different suppliers. The different parameters are as follows: M_n = number-average molecular weight, M_w = weight-average molecular weight, $Đ$ = dispersity.

Polymer		Purified: LBG 1 Sigma	Purified: LBG 1 IF	Purified: LBG 1 Roeper
M_n (Da)*	Peak 1	1,173,729	1,400,104	1,590,226
	Peak 2	628	705	652
	Peak 3	44	48	46
M_w (Da)*	Peak 1	3,038,912	3,398,325	3,574,440
	Peak 2	662	748	697
	Peak 3	49	53	50
$Đ$	Peak 1	2.59	2.43	2.25
	Peak 2	1.05	1.06	1.07
	Peak 3	1.11	1.11	1.11

* Apparent molecular weight, relative to pullulan.

considering TGA data discussed below, that points to a stability of LBG until near 300 °C. To complement TGA data, the DSC thermograms, show no visible alteration of LBG's solid state until 250 °C.

3.4. Thermophysical features of LBG

3.4.1. Thermogravimetric analysis (TGA)

One of the possibilities to verify the effect of temperature on LBG behaviour is to use TGA. This technique allows not only the determination of a temperature threshold for the initiation of a degradation process but can also be an alternative to the Karl-Fischer technique for the determination of the water content of a sample. The curve obtained for LBG from Sigma-Aldrich® is shown in Fig. 4.

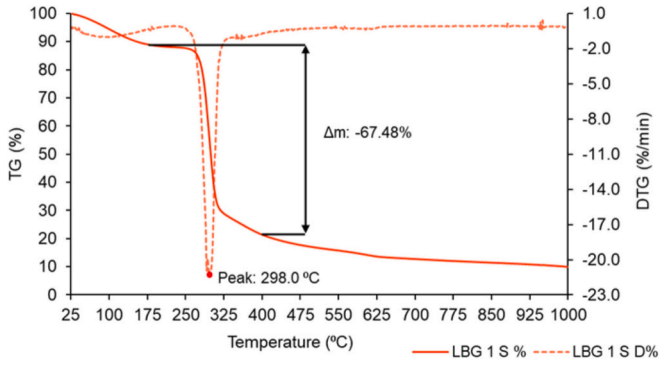

Fig. 4. Thermogravimetric analysis (TGA) curve for purified locust bean gum (LBG) from Sigma-Aldrich®.

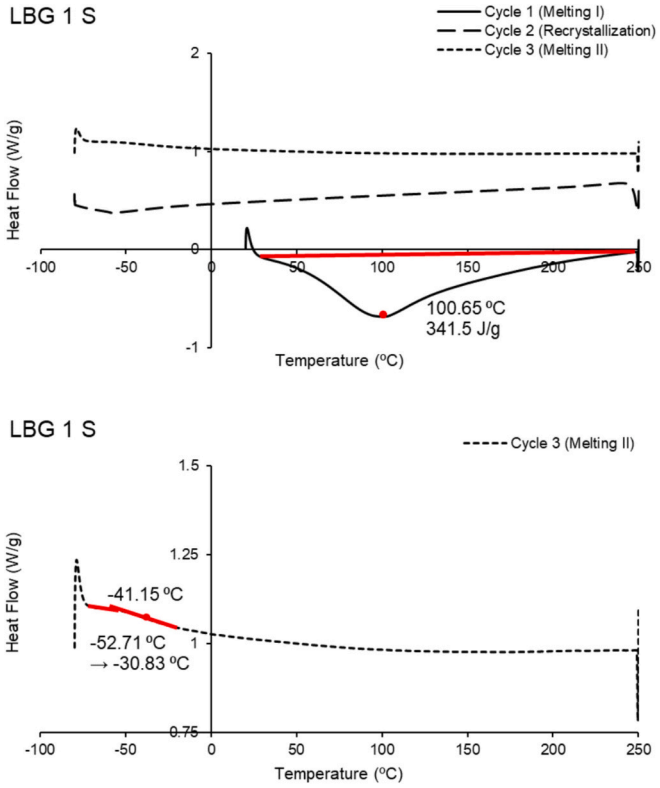

Fig. 5. Differential Scanning Calorimetry (DSC) curve for purified locust bean gum (LBG 1) from Sigma-Aldrich®. The panel on the top shows the thermal behaviour of the sample, and the panel on the bottom details the glass transition temperature (T_g) found for the respective sample.

The TGA curve presented herein evidences two different peaks: one at an earlier temperature (*ca.* 100 °C) and another very intense event at a higher temperature. The first is attributed to the loss of water associated with the sample. In the case of the purified sample (LBG 1 S), this loss of mass occurs around 100 °C, whilst lower temperatures were registered for the other cases (Figs. S4-S6). The more intense event can be associated with the degradation of LBG, occurring around 300 °C, as observed elsewhere (Li et al., 2021). Despite some differences found in the

thermal profiles, all analysed samples show similar behaviour (Figs. S4-S6). The TGA can be even more useful when defining a DSC protocol, especially for samples that are still relatively unknown. In this specific case, the DSC method, detailed in section 2.7.2., could not be extended beyond 300 °C.

3.4.2. Differential scanning calorimetry (DSC)

The previous TGA section evidenced that the thermal analysis of this polymer could not occur at temperatures higher than 300 °C, at which degradation takes place. In this regard, a DSC protocol was set, and samples analysed. Fig. 5 shows the curve for purified locust bean gum (LBG 1) sample from Sigma-Aldrich®.

The curve showed herein evidenced a first heating cycle by an endothermic peak around 100 °C. This peak is associated with the loss of water that is bound to LBG, which does not appear in the subsequent cycles (Li et al., 2021). The second cycle corresponds to a cooling phase, thus it enables the search for a temperature that induces recrystallisation, if the sample is indeed crystalline. In the case of LBG, a recrystallisation temperature was not found, suggesting this polymer is amorphous. In fact, a previous work performing XRD analysis indicated the amorphous nature of LBG (Alves et al., 2016). The third cycle, which is a second heating, intends to look for a melting temperature, if the sample is crystalline, and for a glass transition temperature (T_g). In the case of the LBG from Sigma-Aldrich®, the T_g seems to be at −41.15 °C with an onset starting at −52.71 °C, an observation that was also discussed elsewhere (Rogers et al., 2006). This behaviour was similar to those found for the other two forms of LBG (commercial and microparticles; Supplementary Materials, Figs. S7-S9). In all, the results shown above, and in the Supplementary Materials, evidence the amorphous state of LBG, which aligns with previous reports by our group (Alves et al., 2016). Relevant for the application envisaged herein is the fact that amorphous excipients improve the aerosolization properties in dry powders intended for lung administration, as discussed elsewhere (Adhikari et al., 2022). Nevertheless, as the amorphous state further attributes physical instability (*e.g.* facilitated water uptake), a great compromise in the powder properties must be attained and the stability of drug formulations adequately tested.

3.5. In vitro toxicological profile

The use of excipients in drug delivery applications requires demonstration of biocompatibility and absence of toxicity. The performance of *in vitro* studies is very useful and informative for subsequent *in vivo* assessment. The evaluation of the adequacy of an excipient is closely connected with its application, including the route of administration. *In vitro* testing conditions must align with the application of the drug formulation as much as possible to enable adequate assessment. LBG is not approved for lung delivery applications, although its potential for inhalation-related applications has been suggested several times (Alves et al., 2016; Grenha et al., 2020; Rodrigues et al., 2017). Providing data on its toxicological profile is a need to support proposals for applications in the formulation of inhalable medicines.

In this regard, an *in vitro* assessment considered the evaluation of two different parameters to provide data on cell toxicity. Both cell metabolic activity and the release of a cytoplasmic enzyme (LDH) were determined upon exposure to the LBG samples, testing not only the different LBG suppliers but also the different forms of the polymer explored in this paper (commercial polymer, purified polymer and spray-dried microparticles). Alveolar epithelial cells (A549) were used, as they are frequently applied as model of respiratory epithelium.

3.5.1. Cell metabolic activity

Cell metabolic activity data were interpreted as cell viability and ISO 10993-5-2009 (International Organization for Standardization (ISO), 2009) was used as reference, assuming toxicity to occur when cell viability goes beyond the threshold of 70 %.

Considering the overall similarity of the obtained results, which are independent of different origins/suppliers of LBG, the polysaccharide obtained from Sigma-Aldrich® was deemed representative and its specific results shown in Fig. 6. This displays the results of cell viability obtained upon exposure to LBG concentrations ranging from 0.1 to 1 mg/mL in periods up to 48 h. Results of the LBG from the other suppliers are available in Supplementary Materials (Fig. S10 and S11).

The analysis of the data generated two essential conclusions. There is a time-dependent effect on cell viability, especially if short (3 h) and long exposure times (24 and 48 h) are compared. The effect is significant particularly for purified LBG and microparticles produced with polymers from Sigma-Aldrich® and Industrial Farense®, tested at the higher concentrations (0.5 and 1.0 mg/mL, $p < 0.05$). Interestingly, some

Table 2
IC_{50} (mg/mL) calculated for different samples of locust bean gum (LBG) upon 24 h and 48 h contact with A549 cells.

IC_{50} (mg/mL)				
Exposure time	LBG 1 Sigma	LBG 2 Sigma	LBG 1 I. Farense	LBG 2 I. Farense
24 h	0.395	0.495	0.329	0.759
48 h	0.393	0.505	0.439	0.746

LBG1: purified LBG; LBG2: LBG microparticles.

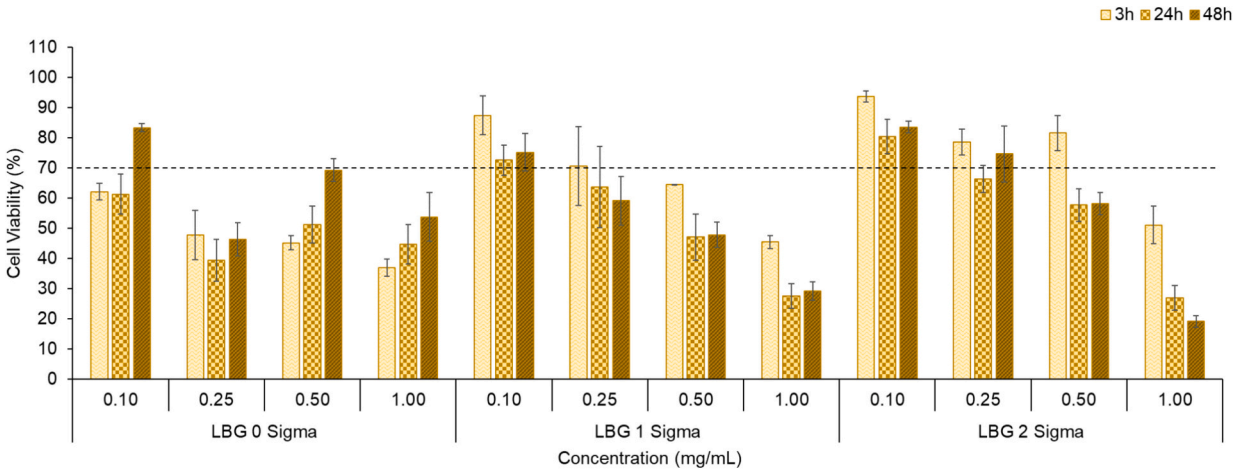

Fig. 6. A549 cell viability upon 3 h, 24 h and 48 h exposure to (a) commercial locust bean gum (LBG 0), (b) purified locust bean gum (LBG 1) and (c) locust bean gum microparticles (LBG 2) (LBG from Sigma-Aldrich®; textured columns in yellow and brown: ●●●). Data represent mean ± SEM ($n = 3$).

commercial samples registered an increase of cell viability with time, which is possibly due to the presence of other compounds in the raw material samples that were afterwards eliminated during purification, and which may have a role as nutrients. LBG from C. E. Roeper® was the one with milder toxicological profile (Fig. S11). In the context of time-dependent effect it is relevant to mention that lung mechanisms of defence ensure that the surface is generally free from inhaled material after 24 h, thus the testing at 48 h being mainly useful to register observations on extreme and unusual conditions.

The second relevant conclusion pertains to a concentration-dependent effect. In fact, for all forms of LBG and the different suppliers, higher concentrations (0.5 and 1.0 mg/mL) generally resulted in lower cell viability ($p < 0.05$). The dissolution of LBG produces viscous solutions. At lower concentrations, the viscosity is reduced, having little impact on the access of cells to nutrients and gases available in the culture medium. However, higher LBG concentrations show a marked viscosity (only registered visually), which could possibly create a physical and viscous barrier, complicating the biological processes of the cells and resulting in reduced cell viability. This justification is supported by literature reports indicating that medium viscosity affects cell growth and functions (Khorshid, 2004) and suggesting that polymeric networks reduce the diffusion of cell nutrients, leading to decreased cell viability (Abdelmoneim et al., 2023). Spray-dried microparticles produced with LBG from Sigma-Aldrich® were previously reported to not have significant toxicity in A549 cells up to 1.0 mg/mL (Alves et al., 2016) but the conditions of microparticle preparation differed significantly, limiting the relevance of comparisons. In that case, HCl was included in the microparticle production process to reduce LBG viscosity.

The half maximal inhibitory concentration (IC_{50}) was calculated for purified LBG and LBG microparticles from Sigma-Aldrich® and Industrial Farense®, as these samples displayed cell viability below 50 %. The results are compiled in Table 2.

The exposure time was found to generally not have a significant impact on the IC_{50}. The most important observation resulting from IC_{50} determination was related with the type of LBG sample being tested (purified - 1 or microparticle - 2), which had an impact on cell interaction. The processing of LBG to produce microparticles results in IC_{50} increase, demonstrating a lower toxicological effect. This observation can be justified with LBG characteristics. Purified LBG was found to produce a more viscous dispersion compared with the microparticles (visual observation only), which may hinder the access of cells to oxygen and nutrients of CCM, as previously discussed and reported elsewhere (Abdelmoneim et al., 2023; Alves et al., 2016; Khorshid, 2004). In turn, due to further processing in spray-drying, LBG microparticles appeared to result in a dispersion of lower viscosity, which benefits access of cells to nutrients. Determination of viscosity would be needed, however, to better support these observations. It is also relevant to indicate that, considering the area of the lung (80–100 m^2), real concentrations of excipients or formulations are not expected to overpass 0.1–0.2 mg/mL. IC_{50} values are highly above that, and the toxicological effect observed at the referred concentrations is rather mild, thus making the obtained results very satisfactory. Despite the rational supporting these observations, the indications on the acute toxicity classification of the United Nations Health Hazards (United Nations, 2007) should also be considered. This classification indicates that a concentration of 0.1 % (1 mg/mL) for the IC_{50} tested in cellular systems is a critical concentration, below which toxicological effects may occur. Under the light of this standard, the IC_{50} values obtained herein, ranging between 0.50 and 0.76 mg/mL (equivalent to 0.05 % and 0.076 %), cannot be directly taken as safe.

3.5.2. *Cell membrane integrity*

The assessment of the impact of LBG samples on cells was further complemented with the quantification of the cytoplasmatic enzyme LDH released to the culture medium. This occurrence implies the disruption of the cell membrane and is, therefore, a sign of cell damage. The higher concentrations tested in the MTT assay (0.5 mg/mL and 1.0 mg/mL) were those assessed, and results are depicted in Fig. 7.

A closer observation of the results reveals that, comparing the different samples at the same timepoint, only LBG microparticles produced with polymer from Sigma-Aldrich® at 1.0 mg/mL evidenced a statistically significant increase in the released LDH as compared with the control (cell culture medium; $p < 0.05$). In any case, a maximum release of 6.5 % was observed. Overall, the obtained results are very mild, with very low release of LDH, which indicates that the exposure to LBG in any of its forms does not compromise the integrity of cell membrane (Moura et al., 2014).

As a whole, the performed *in vitro* toxicological studies revealed mild effect of LBG microparticles in respiratory cells, although the robustness of results would be improved by testing in cells of normal epithelium instead of cancer-derived cells and expanding the evaluated parameters. In any case, the results encouraged the performance of *in vivo* studies to assess the potential toxicity of LBG microparticles. The registered observations are discussed in the following section aiming at contributing to the clarification of the potential of LBG as an excipient in lung delivery applications.

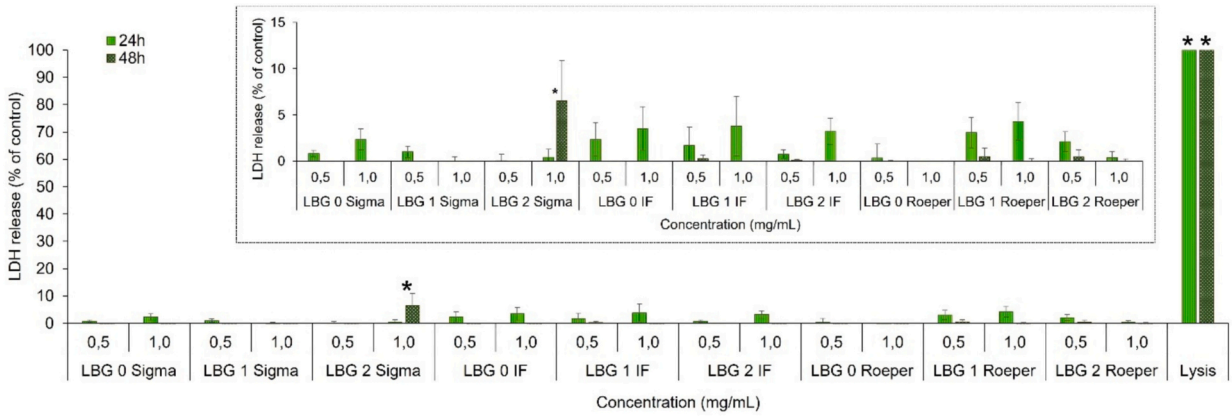

Fig. 7. Lactate dehydrogenase (LDH) released from A549 cells (textured columns in green: ●●) upon 24 h and 48 h (LBG) concentrations corresponding to commercial (LBG 0), purified (LBG 1) and LBG microparticles (LBG 2) from different suppliers (mean ± SEM, $n = 3$). The same figure showing expanded detail for % LDH release up to 15 % was added for clarification. Statistically significant differences are indicated with an asterisk ($p < 0.05$) compared with respective cell culture medium (negative control).

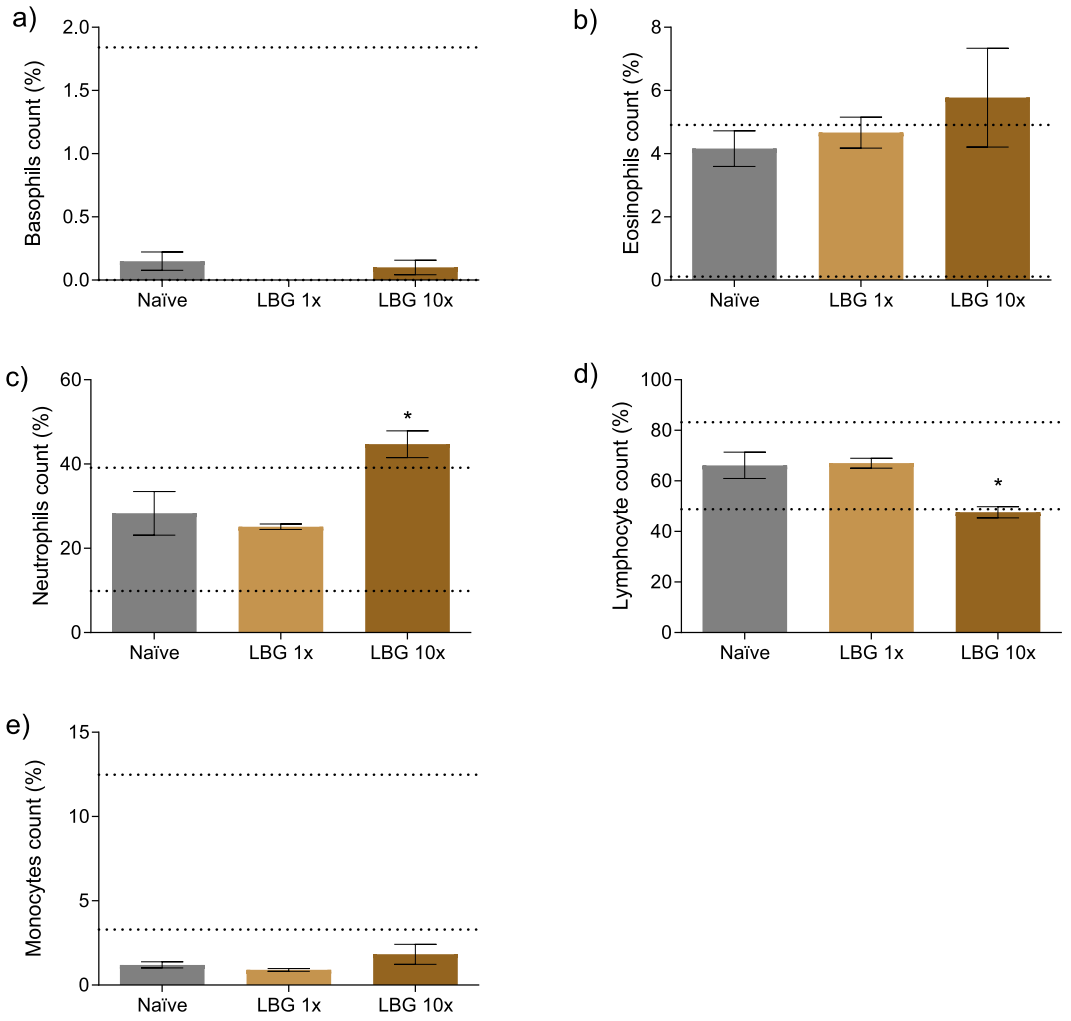

Fig. 8. Leucocyte count in mice blood (mean ± SEM, $n = 6$): (a) Basophils, (b) Eosinophils, (c) Neutrophils, (d) Lymphocytes and (e) Monocytes. Dashed lines represent reference intervals for each type of white blood cell. Statistical significance comparing with control (naïve group) is indicated with an asterisk (*) for $p < 0.05$.

3.6. *In vivo safety evaluation*

Many cellular events occur, in the human body, upon contact with foreign substances. The characterisation of these events when inhalation is considered is very important, as inflammation or structural changes in lung epithelia may be observed, which may compromise normal lung functions. Biosafety parameters are, thus, relevant in formulation development. Although the regulations mention requirements for data on the complete dosage form, excipients to be used in formulation also demand characterisation of their biological impact and, ultimately, toxicological profile. In this regard and considering that LBG is proposed herein as an excipient for lung delivery applications, safety parameters were studied *in vivo*, using mice as an animal model. In brief, mice were exposed to LBG microparticles (composed of excipient only and produced with polymer from Sigma-Aldrich®) by inhalation, following an acute (single dose) or subacute (ten doses during two weeks) exposure. The animals were monitored for clinical signs of morbidity and, after sacrifice, organs and plasma were analysed to evaluate toxicity biomarkers and allergic response.

3.6.1. *Monitorisation of body weight*

The body weight of mice exposed to ten doses of LBG microparticles was monitored every day, since the day of first dose administration until moments before their sacrifice and compared with naïve mice (Fig. S12).

The results revealed an absence of major alterations in the weight of mice within the same group, for both groups, despite the inhalation of microparticles in one group. Some concerns on the evolution of mice weight were raised at the beginning. Due to the swelling properties of LBG (Dionisio & Grenha, 2012; Kawamura & Joint FAO/WHO Expert Committee on Food Additives, 2016), and the amount of powder that was to be inhaled, a concern arose pertaining to some of the powder entering the pharynx into the gastrointestinal tract of the mice, leading to an alteration of their overall weight. However, despite this concern, and even if the swallowing of LBG-based microparticles did occur, it did not have a measurable influence throughout the assay. Mice body weight was also assessed in the group receiving a single dose of LBG-based microparticles, before and after administration (data not shown), without significant alterations being observed.

3.6.2. *Counting of differentiated leucocytes*

White blood cells play a pivotal role in maintaining body homeostasis. The immune system is comprised of several types of cells, involved in both innate and adaptative immunity. Modifications of their normal numbers frequently indicate the occurrence of molecular events, such as inflammation or infection (Guo et al., 2020), which is helpful in the monitorisation of health conditions. A deeper study of the white blood cell population could, thus, provide some relevant data on the reactions to lung drug delivery approaches. As such, blood leucocytes from animals receiving LBG microparticles were characterised and compared with naïve mice, and results are shown in Fig. 8.

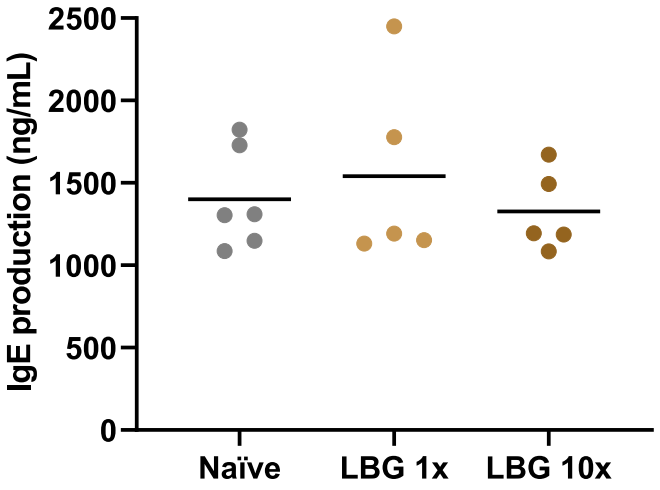

Fig. 9. IgE levels determined in plasma of the mice from naïve and locust bean gum (LBG) groups of acute (LBG 1×) and subacute (LBG 10×) administration of LBG microparticles by inhalation. Lines represent the mean IgE secretion expressed in ng/mL ($n = 6$).

Basophils are the only type of white blood cell that is within the reference levels in all the tested groups (Charles River Laboratories International, 2012) and no statistical differences were found between the three groups. The case changes for eosinophils, which, much like neutrophiles, are associated with inflammation and allergy reactions (Lee et al., 2021). The eosinophil count was within the reference levels for the naïve group and the mice receiving a single dose of LBG microparticles. However, when subacute exposure (ten doses administered over two weeks), eosinophil count overpassed the reference. The increase was, however, not statistically significant due to a large standard deviation. Neutrophils can also be associated with an allergy or an inflammatory episode in mice (Ding et al., 2021). Herein, both naïve and acute exposure groups had results within the reference interval (Charles River Laboratories International, 2012). However, the subacute exposure generated a significant increase ($p < 0.05$) comparing with the naïve group. The observed neutrophilia could be associated with an inflammatory reaction, but more studies would be required to confirm that and establish an eventual causative effect with the exposure to inhaled LBG microparticles.

With regards to lymphocytes, these cells surprisingly decreased below the reference levels for the group of the subacute exposure. Lymphocytes may also be indicator of inflammation (Gilmore et al., 2021), being typically recruited to inflamed tissues in a process termed 'homing' (Sakai & Kobayashi, 2015). The detected lymphocytopenia might be explained by this 'homing', as lymphocytes may have possibly abandoned the blood circulation to concentrate in the inflamed tissue. As for the monocytes, their count was below the reference levels for every group analysed. No explanation was found for the effect so far but, importantly, monocyte levels did not change significantly after exposure to LBG microparticles (Auffray et al., 2009).

Information regarding a possible pro-inflammatory effect of LBG, either as a polymeric dispersion or in a drug delivery platform, is limited. Literature reports the works of Chen et al., in which they tested the production of pro-inflammatory factors by both native LBG and its hydrolysed form. While the latter did not induce inflammation, native LBG did trigger the production of tumour necrosis factor (TNF)-α in RAW264.7 cells at concentrations ranging within 0.05 % and 0.2 % (Chen et al., 2018).

Upon the analysis of leucocyte counts provided herein, a possible inflammation is suggested to occur that is possibly not associated with an infection.

Table 3
Tissue index for liver, spleen, lungs, and kidneys for naïve and treatment groups (LBG 1×: acute exposure; LBG 10×: subacute exposure) receiving locust bean gum (LBG) microparticles (mean ± SD, $n = 6$). Different letters mean different levels of significance for each parameter ($p < 0.05$).

	Liver (%)	Spleen (%)	Lungs (%)	Kidneys (%)
Naïve	22.6 ± 0.8^{a}	5.7 ± 0.2^{b}	8.6 ± 1.1^{c}	12.7 ± 0.3^{d}
LBG 1×	22.7 ± 1.1^{a}	5.9 ± 0.3^{b}	9.0 ± 0.9^{c}	12.8 ± 0.3^{d}
LBG 10×	22.5 ± 0.9^{a}	5.6 ± 0.1^{b}	8.4 ± 1.0^{c}	13.0 ± 0.4^{d}

3.6.3. *Immunoglobulin E (IgE) quantification*

The inhalation of materials is generally suggested to be a possible trigger of allergic reactions (Dubey et al., 2015; Metwali et al., 2024). Besides inflammation, the potential of induction of an allergic reaction upon inhalation of LBG dry powder was considered relevant and was objectively assessed. IgE antibodies were therefore quantified in plasma, as they are associated with the release of inflammatory mediators, among other roles (Haniuda & Kitamura, 2021; Kelly & Grayson, 2016). Basal values of IgE in humans and mice are the lowest of all antibodies, and highly regulated (Hnasko, 2015; Wu & Zarrin, 2014). The results obtained in the study are shown in Fig. 9.

Mean values of IgE production of 1400 ng/mL, 1541 ng/mL, 1326 ng/mL for the naïve, acute and subacute exposure groups, respectively, were determined in plasma. No statistically significant differences were found between the groups, suggesting that no allergic reaction was initiated in the sequence of the inhalation of LBG microparticles.

3.6.4. *Tissue index*

The determination of the tissue index can also be an indicator of toxicity upon exposure to materials and, in this case, an excipient (Michael et al., 2007; Sellers et al., 2007). After the inhalation period, mice were sacrificed and the organs of interest, such as liver, spleen, lungs, and kidneys, collected and weighed. Afterwards, tissue indexes were calculated, and compared with those obtained for the naïve group, which received no treatment. Results are shown in Table 3.

Considering the results obtained for the treatment groups, by comparison with the naïve set, no significant differences were detected. The organs of interest maintained their tissue index despite the administration of a dry powder formulation.

3.6.5. *Histology*

Both cellular molecular events and morphology of organs are assumed elements of relevance in *in vivo* experiments. Through all the previous sections, data have been suggesting absence of overt toxicity upon the inhalation of LBG microparticles. Nevertheless, an eventual inflammatory event cannot be discarded considering the results of leucocyte counts, but further studies would be required to confirm that possibility. This section describes the observations of the histological analysis performed on lung tissue obtained from animals of each test group of this study, to complement the observations of previous sections. Results are depicted in Fig. 10.

Lung and bronchi were, in general, normal, with no cellular modifications worthy of note. However, in the images B and D of Fig. 10, although variable between mice, there seems to occur an infiltration of mononucleated cells in both groups, naïve and the group that received the microparticles. These observations might suggest an infiltration of neutrophils, which is common in lung injury scenarios (Johansson & Kirsebom, 2021; Lax et al., 2014; Sunil et al., 2020) and lung inflammation (Cho et al., 2013). However, these cannot be attributed to the inhalation of LBG microparticles because it was also observed in naïve mice.

In conclusion, the toxicological assessment of LBG herein presented and discussed in the context of an application as excipient for inhalation purposes generally provides indications towards a safe profile. *In vitro* assays provide good indications if lung biorelevant concentrations are considered. The *in vivo* assessment mostly delivered positive indications,

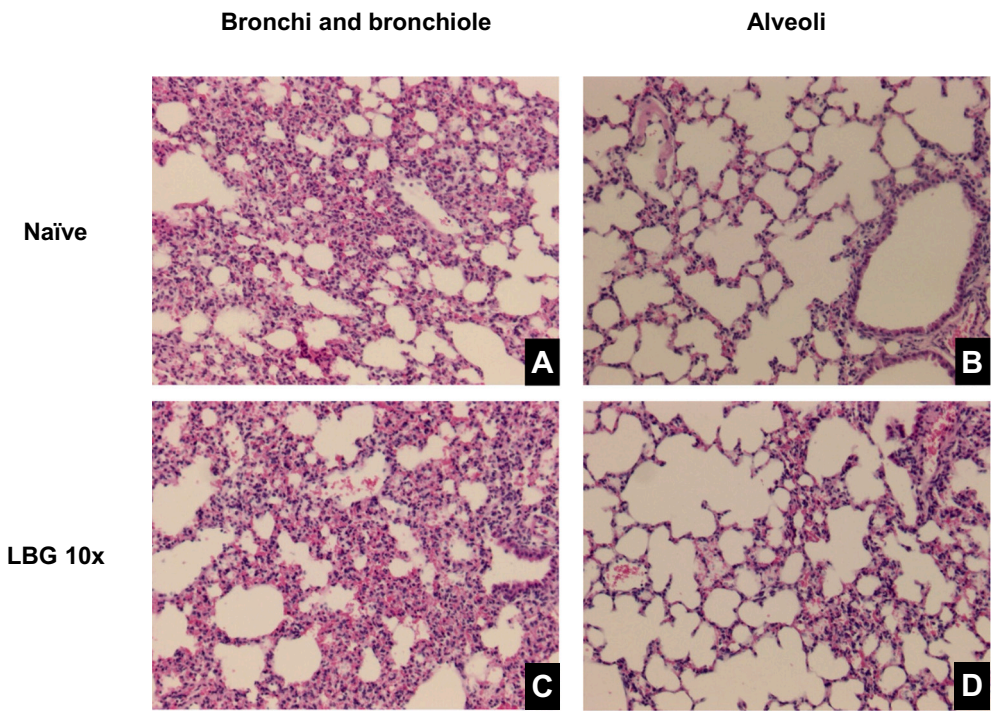

Fig. 10. Representative haematoxylin and eosin (H&E) stain lung histology sections (100×) of the bronchi and bronchioles (a, c) and alveoli (b, d) for the naïve group and the group of subacute exposure (LBG 10×).

although the occurrence of an eventual inflammatory event cannot be discarded, which requires future clarification. The data herein presented comprise, as a whole, a valuable contribution on the characterisation and safety profile of LBG. The experiments outlined and discussed, although preliminary, show a toxicological profile that is favourable for an application as excipient in lung delivery. In any case, gathering safety data for excipient approval is necessarily a joint effort between academia and industry, mainly because of limitations regarding funding. This work provides data to kick-off the discussion and a more profound evaluation towards having more excipients available for use in inhalable formulation development.

4. Conclusions

LBG is one of many polymers being investigated regarding new applications in drug delivery strategies. This study aimed at characterising the physicochemical properties of LBG and performing a biological safety assessment to, ultimately, draw a toxicological profile for this excipient. In this regard and considering the amount of impurities present in its commercial forms, LBG was purified and the production of dry powders corresponding to LBG microparticles was successful when spray-drying was used as production methodology. Selected microparticle formulations were thoroughly characterised using FTIR, DSC, TGA and HPSEC, and the results suggested that LBG characteristics were not impacted by the temperatures used in both the process of purification and in the preparation of microparticles. The biological safety assessment performed *in vitro* in cells representing the respiratory epithelium evidenced a time- and concentration-dependent effect of LBG: longer exposure times paired with higher concentrations are associated with lower cell viability. Nevertheless, IC_{50} values benefit from further processing of LBG (from purification to microparticle production), increasing when microparticles were the tested sample. Moreover, the real concentration expected to be observed upon lung delivery of microparticles is closer to 0.1 mg/mL, and the obtained results are very promising at that concentration. As for the *in vivo* assessment, it generally provided positive indications. No clinical signs of morbidity were observed, and an allergic response was not detected, although an eventual inflammatory event could not be ruled out. The fact that microparticles prepared with LBG from different sources presented a similar behaviour is positive when considering a wide use of this polysaccharide as excipient for inhalation formulations.

CRediT authorship contribution statement

Jorge F. Pontes: Writing – original draft, Methodology, Investigation. **Filipa Guerreiro:** Methodology. **Joana P. Silva:** Methodology, Investigation. **Maria P. Almeida:** Methodology, Investigation. **Annalisa Rosso:** Methodology. **Ana M. Rosa da Costa:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition. **Géraldine Agusti:** Investigation. **Giovanna Lollo:** Writing – review & editing, Funding acquisition. **Maria Manuela Gaspar:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Ana Grenha:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

Jorge F. Pontes PhD scholarships (PD/BD/137064/2018 and COVID/BD/152626/2022) from the Portuguese Foundation for Science and Technology (FCT) are acknowledged.

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.

Acknowledgements

This research was funded by the Portuguese Foundation for Science and Technology (FCT) grant numbers UIDB/04326/2020 (DOI:10.544

99/UIDB/04326/2020), UIDP/04326/2020 (DOI:10.54499/UIDP/04326/2020) and LA/P/0101/2020 (DOI:10.54499/LA/P/0101/2020).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2025.123729.

Data availability

Data will be made available on request.

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