International Journal of Antimicrobial Agents 63 (2024) 107001

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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

Development of inhalation powders containing lactic acid bacteria with antimicrobial activity against *Pseudomonas aeruginosa*

Stefania Glieca[a,1], Eride Quarta[a,1], Benedetta Bottari[a,1], Elena Bancalari[a], Saverio Monica[a], Erika Scaltriti[b], Martina Tambassi[b], Lisa Flammini[a], Simona Bertoni[a], Annalisa Bianchera[a], Valentina Fainardi[c], Susanna Esposito[c], Giovanna Pisi[d], Ruggero Bettini[a,e], Fabio Sonvico[a,e], Francesca Buttini[a,e,*]

[a] *Food and Drug Department, University of Parma, Parma, Italy*
[b] *Risk Analysis and Genomic Epidemiology Unit, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Parma, Italy*
[c] *Paediatric Clinic, Department of Medicine and Surgery, University of Parma, Parma, Italy*
[d] *Cystic Fibrosis Unit, Paediatric Clinic, Az. Ospedaliera, Universitaria di Parma, Parma, Italy*
[e] *Interdepartmental Centre for Innovation in Health Products, Biopharmanet_TEC, University of Parma, Parma, Italy*

ARTICLE INFO

Article history:
Received 12 May 2023
Accepted 1 October 2023

Editor: Dr Y-W Lin

Keywords:
Lactiplantibacillus plantarum
Lung probiotics
Inhalation powders
P. aeruginosa infection
Cystic fibrosis
Lung microbiota

ABSTRACT

Objectives: The aim of the project was to develop and characterise powders containing a probiotic (*Lactiplantibacillus plantarum* [*Lpb. plantarum*], *Lacticaseibacillus rhamnosus, or Lactobacillus acidophilus*) to be administered to the lung for the containment of pathogen growth in patients with lung infections.
Methods: The optimised spray drying process for the powder manufacturing was able to preserve viability of the bacteria, which decreased of only one log unit and was maintained up to 30 days.
Results: Probiotic powders showed a high respirability (42%–50% of particles had a size < 5 μm) suitable for lung deposition and were proven safe on A549 and Calu-3 cells up to a concentration of 10^7 colony-forming units/mL. The *Lpb. plantarum* adhesion to both cell lines tested was at least 10%. Surprisingly, *Lpb. plantarum* powder was bactericidal at a concentration of 10^6 colony-forming units/mL on *P. aeruginosa*, whereas the other two strains were bacteriostatic.
Conclusion: This work represents a promising starting point to consider a probiotic inhalation powder a value in keeping the growth of pathogenic microflora in check during the antibiotic inhalation therapy suspension in cystic fibrosis treatment regimen. This approach could also be advantageous for interfering competitively with pathogenic bacteria and promoting the restoration of the healthy microbiota.

1. Introduction

Although for many years the lung had been considered sterile, in the last decade, the presence of a microbial population was demonstrated [1]. The lung microbiota is preserved by a balance between the translocation of microorganisms from the upper respiratory tract and their elimination by defence [2]. In chronic respiratory diseases, this balance is lost as the propagation of pathogenic microorganisms exceeds the defence capacity of the lung [1]. In cystic fibrosis (CF) patients, the presence of a highly viscous mucus on lung epithelium promotes colonisation and chronic infections by opportunistic pathogens, which are primary responsible for patients' mortality. The widespread use of systemic and inhaled antibiotics to treat infections led to the development over time of microorganisms (like *Pseudomonas*) that are resistant to all commonly used antibiotics [3].

An innovative therapeutic approach for chronic respiratory diseases may be to act on the microbial community by administering probiotics: live microorganisms which confer a health benefit to the host [4]. The most widely used oral probiotics belong to the genus *Lactobacillus,* which demonstrated excellent immunomodulatory effects and potentially the role of modulating respiratory microbial community [5]. Moreover, it was recently demonstrated that both a direct intratracheal and intranasal prophylactic administration of *Lactobacilli* decreased the *Pseudonomas aeruginosa* (*P. aeruginosa*) lung burden in the murine model of pneumonia, avoiding the fatal complications of infection [6,7].

* Corresponding author. Mailing address: Food and Drug Department, University of Parma, Parco Area delle Scienze, 27/A, 43124 Parma, Italy.
E-mail address: francesca.buttini@unipr.it (F. Buttini).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijantimicag.2023.107001

Table 1
Matrix of the central composite design of experiments performed to produce *Lactiplantibacillus plantarum* dry powders investigating three factors (k) at two levels.

Powder #	Factor 1 excipient concentration (%w/v)	Factor 2 inlet temperature (°C)	Factor 3 feed rate (mL/min)
1	1.00	145	4.0
2	1.00	125	3.5
3	1.25	145	3.5
4	1.00	135	4.0
5	1.00	135	4.5
6	1.25	145	4.0
7	0.75	135	4.5
8	0.75	125	4.5
9	1.00	135	3.5
10	1.25	135	3.5
11	0.75	125	4.0

Note: The number of runs were calculated as $2^k + k$.

Despite these encouraging efficacy data, the development of lactic acid bacteria (LAB) formulations and their delivery to the lung is still unexplored. This approach could allow the LAB adhesion to the respiratory epithelium, where they could both competitively interfere with pathogenic bacteria and promote the restoration of the healthy microbiota.

The aim of this work was to develop different inhalable powders containing *Lactiplantibacillus plantarum* (*Lpb. plantarum*), *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*), or *Lactobacillus acidophilus* (*L. acidophilus*)—all previously proven to be effective in vitro in the containment of *P. aeruginosa* growth [8,9]. Spray drying was the process chosen to produce the powders, as it allows to obtain micronised particles suitable for inhalation and at the same time to partially preserve the viability of the probiotics [10]. More aggressive processes such as jet-milling or high shear mixer are not suitable owing to the high mechanical stress to which the product undergoes.

The LAB powders produced were then tested for their tolerability and adhesion capacity on lung cell lines. Subsequently, the study of the antimicrobial activity of the powders against three *P. aeruginosa* strains, representative of the intraspecies virulence variability, was evaluated.

2. Materials and methods

2.1. Cultivation of the bacterial strains

Frozen stocks cultures of *L. acidophilus* LA14 (ATCC SD5212), *Lcb. rhamnosus* GG (ATCC 53103), and *Lpb. plantarum* 4265 belonging to University of Parma Culture Collection were recovered in Man, Rogosa, and Sharpe broth (MRS; Oxoid, Ltd., UK) by two overnight subculturing, as detailed in the Supplementary Material (section 1). The cultures were then washed with phosphate buffer saline (PBS; Oxoid, Ltd., UK) to remove the cultural medium.

2.2. Preliminary investigation and optimization of probiotic powders

Spray-dried powders were produced using a B-290 Mini Spray Dryer (Buchi, Switzerland) starting from 50 mL of *Lpb. plantarum* suspension in PBS or Ringer's solution (VWR) at three different cell concentrations: 10^9, 5×10^9, and 10^{10} colony-forming units (CFU)/mL. The parameters set were: inlet temperature of 135°C, air flow rate at 600 L/h, aspiration at 35 m^3/h, feed rate at 4 mL/min, and nozzle of 0.7 mm.

Then, the manufacturing process was optimised by applying a central composite Design of Experiments (DoE) with three factors and two levels (Table 1), and the significance of the model was calculated using the Design-Expert v13 software (Stat-Ease Inc., USA). The excipient consisted of lactose (Respitose ML001; DFE pharma, Germany) and L-leucine (ACEF Spa, Italy) in a ratio of 70:30 (w/w) and added in such quantities as to reach the concentration indicated by the DoE for each run.

By adopting the best conditions emerging from the DoE, two other powders containing *Lcb. rhamnosus* and *L. acidophilus* were produced in addition to a new one of *Lpb. plantarum*. These powders were stored in sealed amber glass vials at 25°C with 60% relative humidity, and the viability, cultivability, and vitality were measured up to 30 days.

2.3. Cultivability, viability, and vitality of LAB in the spray-dried powders

LAB strain cultivability (CFU/mL) after spray drying was assessed by suspending 150–220 mg of inhalation powder in 5 mL of sterile distilled water, ten-fold serially diluting in 0.9% Ringer's solution, and spreading onto MRS agar plate for 48 h.

The viable cells in the powders were evaluated with the LIVE/DEAD Baclight Bacterial Viability kit (Molecular Probes, USA) and fluorescence microscopy. One mililiter of each diluted powder sample was stained with LIVE/DEAD Baclight, filtered, and visualised under fluorescence microscope ECLIPSE 80*i* (Nikon, Japan) to count live and dead cells [11]. Results were expressed as viable cells on total cells: the sum of viable and nonviable cells (cells/mL).

The vitality was analysed with the BacTrac 4300 Microbiological Analyser system (Sylab, Austria). Powders resuspended in distilled water were inoculated (2% v/v) into MRS broth and incubated. The E%, the relative change in the capacitance at the electrode surface, was recorded every 10 min for 20 h [12]. The data were analysed using the Excel add-in 'DMfit version 2.1' to obtain the growth parameters of lag time, rate, and yEnd (maximum value of % change in conductivity in the medium) [12]. At the end of the analysis, the pH value was measured (3000 series; Beckman Instruments, USA).

2.4. In vitro respirability

The in vitro respirability was evaluated using a fast screening impactor (Copley Scientific Limited, UK) using a RS01 (Plastiape, Italy) inhaler loaded with 40 mg of *Lpb. plantarum* powder. Details of the aerodynamic performance test are illustrated in the Supplementary Material (section 4). The emitted fraction was calculated as the percentage ratio between emitted powder mass and the one loaded in the inhaler. The respirable fraction (RF) was calculated as the percentage ratio between the powder with an aerodynamic diameter (d_{ae}) < 5 µm and the emitted powder.

The aerodynamic distribution of the powders was assessed using the next generation impactor (Copley Scientific Limited, UK). The 40 mg of aerosolized powder contained LAB in the range of 10^6–10^8 CFU/mg, according to the formulation.

The emitted dose (ED) and powder mass deposited in the impactor led the calculation of the mass median aerodynamic diameter (MMAD), the mass of powder with d_{ae} < 5 µm, and d_{ae} < 2 µm (i.e., fine particle dose (FPD) and extra-fine particle dose [EFPD]). The fine particle fraction (FPF) and extra-fine particle fraction (EFPF) were calculated as the percentage ratio of fine particle dose and EFPD with respect to ED.

The sample quantification in the different portions of the next generation impactor was done both gravimetrically by determining the mass of powder deposited and by counting the LAB CFU after the powder resuspension in 10 mL of Ringer's solution and spreading on MRS agar plate for 48 h.

2.5. Thermogravimetric analysis and scanning electron microscopy

The moisture content in the spray-dried powders was measured using thermogravimetric analysis (Mettler Toledo, USA) under a flux of dried nitrogen from 25°C to 160°C at a rate of 10°C/min by integrating the weight loss between 25°C and 130°C. Morphological analysis of powders was performed by scanning electron microscopy (Supra 4000; Carl Zeiss, Germany) on uncoated samples with an electron high tension at 1.00 kV.

2.6. Cell viability assay and adhesion experiment on Calu-3 and A549 cells

A549 and Calu-3 (ATCC) cells seeded in 96-well tissue culture–treated plates were stimulated with probiotic powders suspended in medium. The LAB concentrations tested were chosen considering that the 40 mg containing 10^6–10^8 CFU would be dispersed in 20 mL of pulmonary fluid. Hence, a concentration of 10^6 CFU/mL for *Lpb. plantarum* and *Lcb. rhamnosus* and 10^5 CFU/mL for *L. acidophilus* were selected, and two additional concentrations of 2 log higher were tested for the LAB in the hypothesis of an increase in the inhaled dosage. Excipients alone as spray-dried powder were tested as well (Supplementary Material, section 5). After 2 h of incubation, 100 µL of medium containing penicillin/streptomycin 2% was added to eliminate the interference of LAB with the MTS (3-[tributylammonium]-propyl methanethiosulfonate bromide solution) signal. After 1 h, 20 µL of MTS solution was added to each well and absorbance read at 490 nm after 2 h by a Tecan Spark plate reader (Tecan, Italy). The viability of cells was expressed as a percentage of the absorbance to control (PBS). Viability above 80% was considered acceptable (ISO 10993–5).

For adhesion experiments, 100 µL of bacterial powder suspension (10^6 CFU/mL of LAB) was added to the well seeded with A549 and Calu-3 cells. After 2 h, the cells were washed with PBS, and LAB adhering to the cells were collected by adding 50 µL of trypsin/EDTA solution. The enzymatic treatment was left in contact for 10 min at 37°C, followed by the addition of PBS 200 µL. Lactobacilli were collected and counted by plating them on solid MRS. The LAB adhesion was calculated as the percentage ratio between the amount of bacteria adhering to those added to the well.

2.7. Antimicrobial effect of the powders on P. aeruginosa growth

P. aeruginosa strains ATCC 9027, ATCC 15692, and ATCC BAA-47 and LAB powders, resuspended in saline, were diluted to 1×10^6 CFU/mL, and 100 µL of both suspensions were used to inoculate the 2 mL of MRS/Trypticase Soy Broth (Oxoid, UK) medium. The LAB concentrations tested were chosen considering that the 40 mg containing 10^6–10^8 CFU would be dispersed in 20 mL of pulmonary fluid. They were then incubated at 37°C for 24 h. Serial ten-fold dilution of co-cultures were plated on Gassner medium (Merck Life Science, Germany) and incubated for 24 h for the *P. aeruginosa* cell count. Control with excipients only (lactose:leucine 70:30 spray-dried) was tested at a concentration of 0.02 mg/mL, equivalent to the concentration present when LAB composite powders were tested. The pH of the three *P. aeruginosa* strains without LABs and after culturing with the LABs was measured and compared.

2.8. Statistical analysis

Statistical analysis was conducted using the analysis of variance (ANOVA test) with a post hoc test using Prism 9 (GraphPad Software, v.9.4.0). Dunnett's multiple comparison post hoc test was used to compare the means of all experimental groups against a control group. Statistical significance between groups was considered with $P \leq 0.05$ ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

3. Results

The viability of *Lpb. plantarum* in all six powders produced decreased of only 1 log after spray-drying production (Fig. 1). The in vitro respirability was very low in the case of Ringer's solution medium, whereas using PBS it reached values of 70% at the starting concentration of 5×10^9 cell/mL. Considering these results, it was therefore decided to produce powders by a DoE using this composition and adding the excipients. Table 2 lists the results of the parameters identified of critical effect on the product quality i.e., the moisture content affecting the physical stability of the powder [13], the viability of the LAB, and respirability. All the powders were efficiently produced, as the yield of the spray-drying process was higher than 80%. The moisture content in the powders was the lowest when the excipient (lactose-leucine) content was up to 1.25% w/v. The ANOVA analysis showed that none of the factors investigated had a significant effect on these two critical quality attributes (CQAs).

The bacteria viability (Fig. 2) varied widely from 2.9%–82%, depending on the powder batch produced. The ANOVA analysis indicated that both the feed solution concentration ($P = 0.0004$) and the inlet temperature ($P = 0.0321$) had a significant effect on this CQA.

As shown in Fig. 3A, the viability of *Lpb. plantarum* was very high with the highest values of both inlet temperature and feed solution concentration (i.e., 145°C and 1.25% w/v). Regarding the respirability, all the powders had excellent emitted dose values (>93%), and none of the investigated factors had a statistically significant effect on this CQA. In contrast, the inlet temperature ($P = 0.0241$) and the feed rate ($P = 0.0442$) had a significant effect on the RF, which was greater than 50% for all the formulations with some values reaching 66%–68%. The highest RF was obtained when the feed rate of the process was at the lowest value (3.5 mL/min) and the inlet temperature was the highest (i.e., 145°C) (Fig. 3B).

Lpb. plantarum cells analysed before spray drying showed a rod-like structure with a length of ca. 2–3 µm (Supplementary Fig. S1). When the LABs were spray-dried starting from a suspension in a buffer (PBS), the particles obtained showed a spherical shape whose walls were composed of many small structures that could be identified as microorganisms (Fig. 4A). In the microparticles obtained by adding the excipients, bacteria were no longer individually identifiable, as they were embedded into a matrix of material organized in the form of collapsed particles of ca. 3–4 µm (Fig. 4B-D).

Considering the DoE results, powder #3 was identified as the best, and its process parameters were adopted to produce formulations containing *Lcb. rhamnosus* and *L. acidophilus* (Fig. 4C,D). The yield of the processes was higher than 80% for both the powders, and their morphology did not differ from the one containing *Lpb. plantarum*, although the original bacteria length size was ca. 5 and >10 µm for *Lcb. rhamnosus* and *L. acidophilus*, respectively (Supplementary Fig. S2). After production, the residual moisture was about 2.8%–3.0% for both powders, and the viability of *Lcb. rhamnosus* was of the same order of magnitude as *Lpb. plantarum* (10^{10})—unlike that of *L. acidophilus*, which was lower (4.86×10^8 cells/mL) (Fig. 5). The bacteria viability in the powders tended to decrease compared with time zero during a 15- and 30-day storage to a different extent, depending on the strain. *Lpb. plantarum* was the strain that better preserved the viability, as no great differences were observed after 15-day storage, and only 1 log reduction was observed at day 30. For this strain, the lag time values remained unaffected, as the value increased only slightly from 5.07 to 5.61

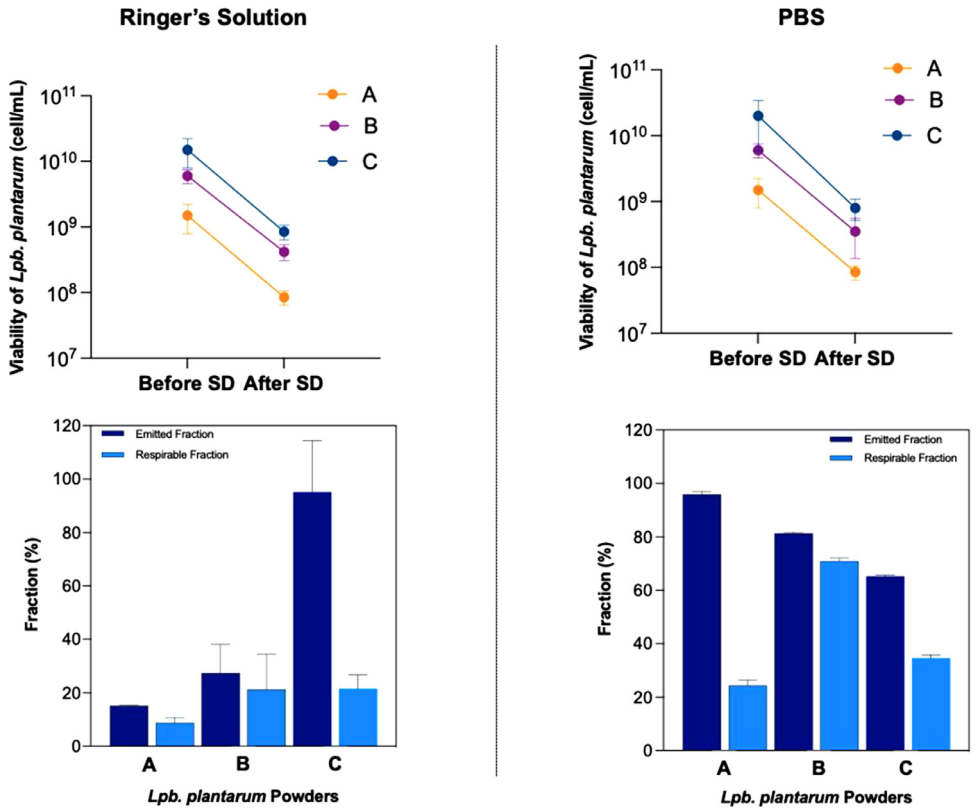

Fig. 1. Viability before and after the spray-drying process (SD) and respirability parameters obtained via fast screening impactor (expressed as emitted fraction % and respirable fraction %) of the *Lactiplantibacillus plantarum* (*Lpb. plantarum*) powders when obtained starting from a Ringer's solution (left panel) and phosphate buffered saline (PBS; right panel). The cell concentrations in the starting suspension were 10^9 cells/mL (A), 5×10^9 cells/mL (B), and 10^{10} cells/mL (C), respectively (n = 3, mean ± standard deviation).

Table 2
Results of central composite Design of Experiments (n = 3, mean ± standard deviation) on the *Lactiplantibacillus plantarum* powders.

Powder #	Moisture content (%w/w)	Viability of *Lactiplantibacillus plantarum* (%)	Emitted fraction (%)	Respirable fraction (%)
1	3.0 ± 0.2	21.6 ±1.3	93.6 ± 1.9	58.9 ± 6.0
2	3.5 ± 0.1	29.2 ± 2.3	95.8 ± 1.5	58.8 ± 6.7
3	2.5 ± 0.2	82.0 ± 1.9	97.8 ± 1.2	69.5 ± 1.2
4	3.1 ± 0.1	14.0 ± 0.4	98.6 ± 0.9	57.0 ± 3.4
5	3.7 ± 0.2	7.5 ± 0.4	92.9 ± 0.6	55.3 ± 0.5
6	2.5 ± 0.2	74.0 ± 1.9	96.7 ± 1.7	65.6 ± 1.2
7	3.6 ± 0.2	5.0 ± 0.1	93.6 ± 2.9	54.3 ± 0.6
8	4.4 ± 0.3	3.0 ± 0.3	94.6 ± 1.8	51.5 ± 1.0
9	2.4 ± 0.1	9.5 ± 0.2	96.3 ± 1.1	56.7 ± 3.3
10	2.4 ± 0.2	77.0 ± 2.3	98.3 ± 2.2	61.1 ± 4.5
11	3.9 ± 0.3	2.9 ± 0.2	98.2 ± 1.5	54.6 ± 7.4

h. A similar trend was observed for *Lcb. rhamnosus,* which had a longer lag time at day 30 and therefore a longer time for adapting and to start growing, meaning that the vitality of the cells was less preserved. This was not the case of *L. acidophilus,* which showed a reduction in viability of more than 1 log already at 15 days, and no vitality detected after 30 days of storage.

The aerodynamic distribution determined gravimetrically was similar for all three LAB powders (Fig. 6A): the ED was greater than 37 mg, and a high deposition on the stages collecting particles <5 µm was observed. *Lpb. plantarum* and *Lcb. rhamnosus* had a FPD of ca. 19 mg, leading to a FPF of 50% and a MMAD around 3.1 µm (Table 3). Even more respirable was the *L. acidophilus* powder which, because of an even higher amount of powder on the micro-orifice collector, showed a FPD of 21.4 mg and a FPF of 56%.

Figure 6B illustrates the deposition of the LAB CFU in the impactor. *Lactobacillus acidophilus* had a low amount of total collected CFU due to its already low initial cultivability in spray-dried powder. In all the cases, the sum of the quantified CFU represented at least 80% of the CFU embedded in the 40 mg of aerosolized powder. Interestingly, the CFU quantification analysis revealed that the powder deposited in the last stages was mostly composed of excipients, whereas the LABs were primarily deposited in stages 1–4, collecting particles >1.6 µm. This resulted in a MMAD between 4.5 and 5.1 µm and a FPF between 42% and 50%. Because of this different deposition behaviour between LAB powders or LAB CFU, different values of EFPD and EFPF were obtained between the two set of measurements (Table 3).

The effect of LAB on A549 and Calu-3 cell viability was generally very low (Fig. 7). In particular, *Lcb. rhamnosus* treatment was well tolerated by cells up to 10^8 CFU/mL; similarly, *Lpb. plantarum* did not impair the viability of the two cell cultures until 10^7 CFU/mL, showing a slightly negative effect only at 10^8 CFU/mL.

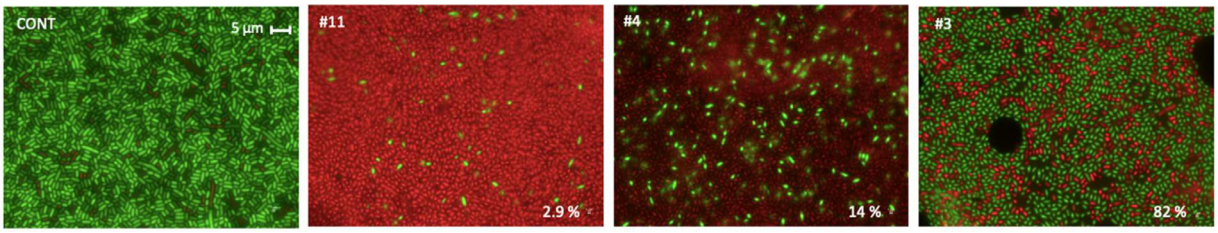

Fig. 2. Viability measurement by the live-dead fluorescence count of *Lactiplantibacillus plantarum* control (fresh culture-CONT) and spray-dried powders #11, #4, and #3 of the Design of Experiments. Live cells appear as green, and dead cells appear as red. Magnification × 100. Each pixel corresponds to 0.1 µm.

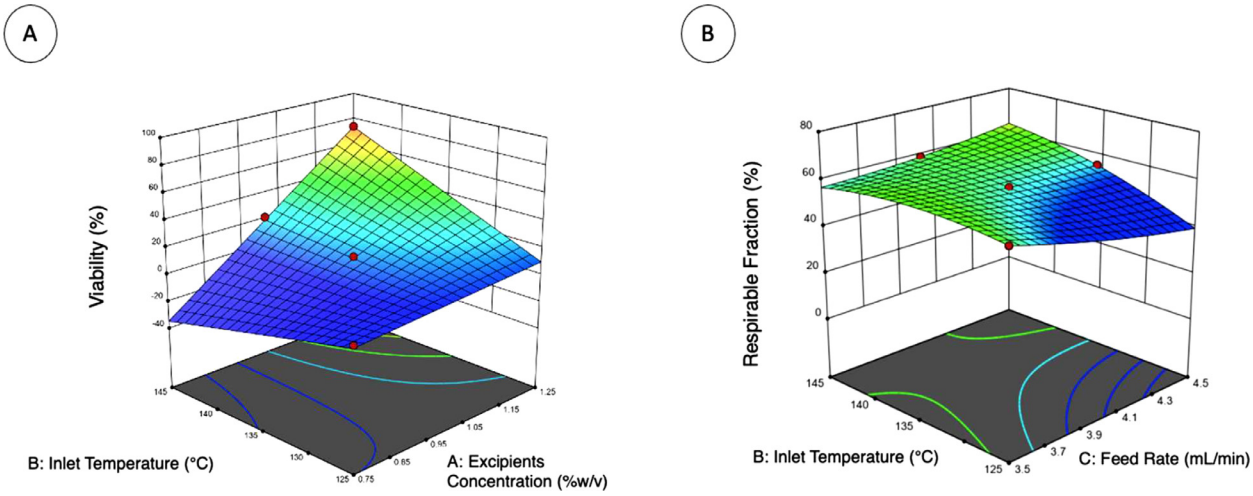

Fig. 3. Three-dimensional surface plots illustrating the effect of the inlet temperature and the excipient concentration used on the *Lactiplantibacillus plantarum* viability (A) and the effect of the inlet temperature and feed rate of process on the respirable fraction (B).

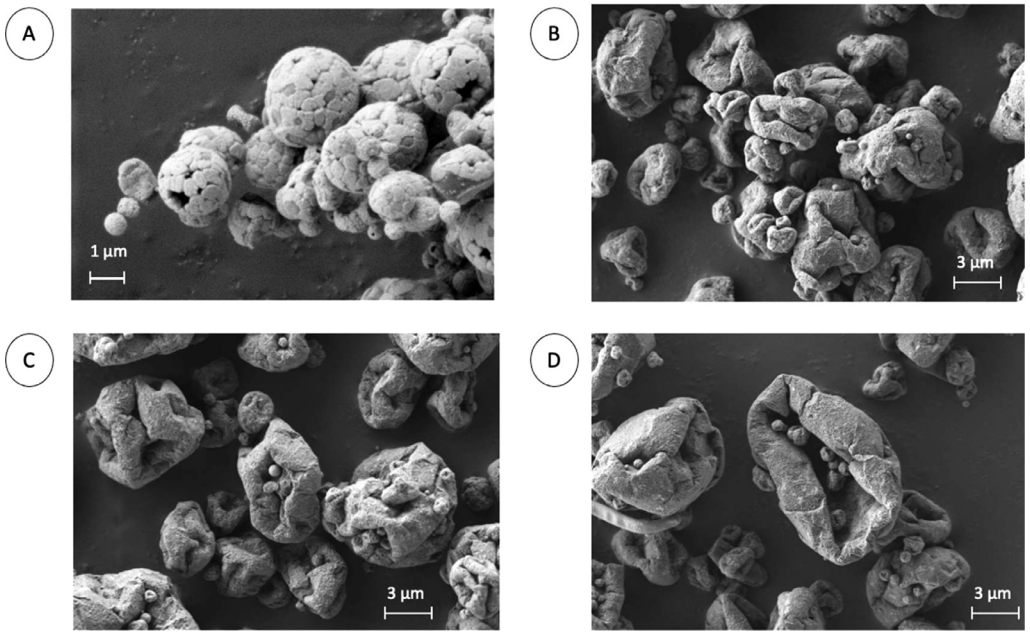

Fig. 4. Scanning electron microscope images of *Lactiplantibacillus plantarum* (*Lpb. plantarum*) powder without excipients (× 20 000) (A), *Lpb. plantarum* lead powder (B), *Lacticaseibacillus rhamnosus* spray-dried powder (C), and *Lactobacillus acidophilus* spray-dried powder (× 10 000) (D).

Lactobacillus acidophilus was the less-tolerated bacteria and caused a strong reduction of cell culture viability at 10^7 CFU/mL.

The adhesion capacity of *L. acidophilus* was lower than the one of *Lpb. plantarum* after 2 h, for both cell lines. On the other hand, the adhesion capacity of *Lcb. rhamnosus* was statistically lower than that of *Lpb. plantarum* only on Calu-3 cells. In addition, the capacity of adhering of the latter was statistically higher on Calu-3 cells than on A549 cells, with values of 24.4% and 12.8%, respectively (Fig. 8).

Three *P. aeruginosa* strains with different virulence and different antibiotic resistance (see Supplementary Table S1) were included in the studies to cover the bacterium intraspecies genetic variability. *Lpb. plantarum* powder showed bactericidal activity on all three strains, as none of them grew when combined with the treatment

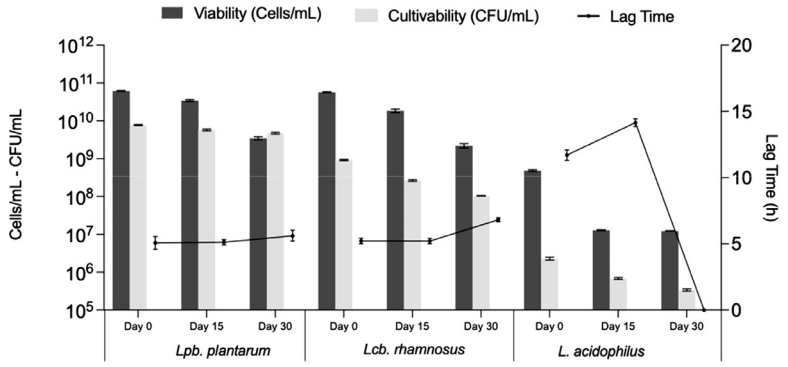

Fig. 5. Viability (cells/mL) represented as dark grey bars, cultivability (colony-forming units [CFU]/mL) represented as light grey bars, and vitality (lag time) represented as black lines of *Lactiplantibacillus plantarum* (*Lpb. plantarum*), *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*), and *Lactobacillus acidophilus* (*L. acidophilus*) at time 0, 15, and 30 days after storage at 25°C of the spray-dried powders (n = 3, mean ± standard deviation).

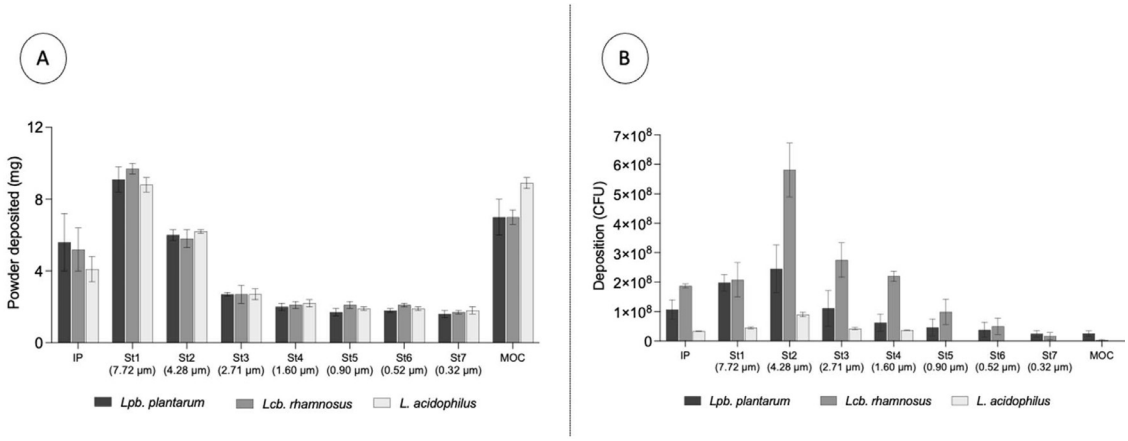

Fig. 6. Aerodynamic profile of the three probiotic spray-dried powders expressed as powder mass deposited in induction port (IP), stages (St) 1–7, and micro-orifice collector (MOC) determined by gravimetric analysis (A) and by quantifying the bacterial cells (colony-forming units [CFU]) in the powder deposited in the next generation impactor portions (B) (n = 3 mean ± standard deviation). In brackets are the effective cut-off diameter of stages. *L. acidophilus, Lactobacillus acidophilus; Lcb. rhamnosus, Lacticaseibacillus rhamnosus; Lpb. plantarum, Lactiplantibacillus plantarum.*

Table 3
Aerodynamic performance of the three probiotic inhalation powders.

Gravimetrical quantification of powder						
	ED (mg)	FPD <5 µm (mg)	FPF <5 µm (%)	EFPD <2 µm (mg)	EFPF <2 µm (%)	MMAD (µm)
Lpb. plantarum	38.2 ± 0.3	18.8 ± 1.3	49.6 ± 3.2	13.7 ± 1.3	35.8 ± 3.1	3.10 ± 0.15
Lcb. rhamnosus	38.3 ± 0.4	19.3 ± 1.0	50.3 ± 3.4	13.9 ± 0.3	36.3 ± 1.3	3.08 ± 0.12
L. acidophilus	37.8 ± 0.1	21.4 ± 0.2	56.0 ± 0.4	20.4 ± 6.2	42.1 ± 1.1	2.38 ± 0.07
CFU quantification by plate count						
	ED (CFU)	FPD <5 µm (CFU)	FPF <5 µm (%)	EFPD <2 µm (CFU)	EFPF <2 µm (%)	MMAD (µm)
Lpb. plantarum	$8.9 \pm 1.4 \times 10^8$	$3.8 \pm 1.1 \times 10^8$	43.9 ± 4.6	$1.9 \pm 0.5 \times 10^8$	22.2 ± 5.7	5.04 ± 0.57
Lcb. rhamnosus	$1.6 \pm 2.3 \times 10^9$	$8.5 \pm 3.6 \times 10^8$	49.7 ± 1.6	$3.2 \pm 0.7 \times 10^8$	19.4 ± 4.3	4.46 ± 0.16
L. acidophilus	$2.5 \pm 0.8 \times 10^8$	$1.1 \pm 0.8 \times 10^8$	42.0 ± 2.3	$2.9 \pm 0.8 \times 10^7$	12.0 ± 2.5	5.12 ± 0.21

Note: Parameters were determined by gravimetric evaluation and quantification of the colony-forming units (CFU) in the different portions of the next generation impactor. The mass of powder loaded into the device was 40 mg containing *Lactiplantibacillus plantarum* 1.10×10^9 CFU, *Lactobacillus rhamnosus* 1.95×10^9 CFU, or *Lactobacillus acidophilus* 3.36×10^8 CFU (n = 3, mean ± SD)
ED, emitted dose; EFPD, extra-fine particle dose; EFPF, extra-fine particle dose; FPD, fine particle dose; FPF, fine particle fraction; *L. acidophilus, Lactobacillus acidophilus; Lcb. rhamnosus, Lacticaseibacillus rhamnosus; Lpb. plantarum, Lactiplantibacillus plantarum*; MMAD, mass median aerodynamic diameter.

(Fig. 9). *Lcb. rhamnosus* decreased the growth of the three *P. aeruginosa* strains, and a bacteriostatic effect was observed against ATCC 9027. Moreover, no *P. aeruginosa* growth was observed when the co-culture was plated without prior dilution (Supplementary Fig. S5). *Lactobacillus acidophilus* limited the growth of *P. aeruginosa* ATCC 9027 and ATCC 15692, even if at a low extent, whereas it had no effect on strain ATCC BAA-47. No bactericidal activity against *P. aeruginosa* was observed with spray-dried excipients only, suggesting that lactose and leucine did not contribute to the reduction of *P. aeruginosa* growth when the LAB formulations were tested. Co-culture with LABs resulted in a decrease of pH compared with *P. aeruginosa* without treatment (ca. 8.1): the pH of *Lpb. plantarum* with all three *P. aeruginosa* strains was the lowest (ca. 4.17) as compared with the other two LABs (Supplementary Fig. S6).

Finally, considering all the impedometric parameters (Table 4), *L. acidophilus* showed the worst growing and adaptability char-

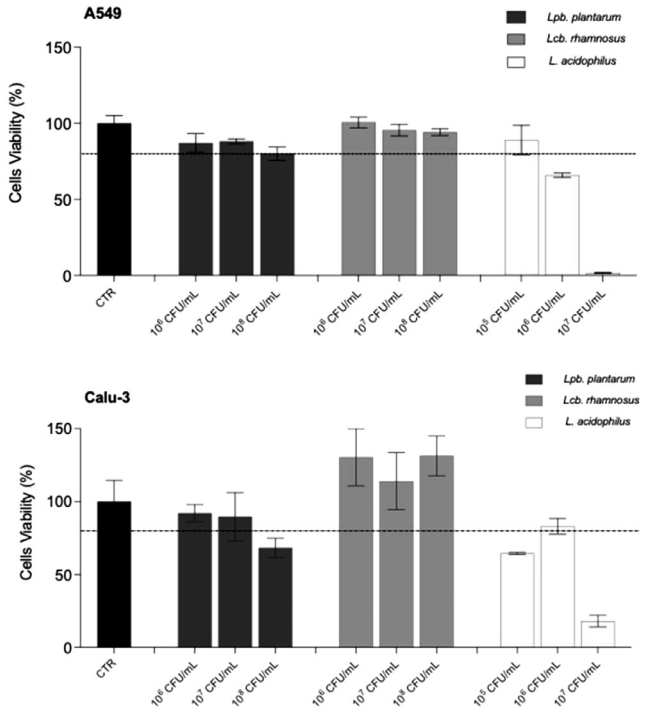

Fig. 7. Viability of A549 and Calu-3 cell cultures exposed to phosphate-buffered saline (PBS) (CTR), *Lactiplantibacillus plantarum* (*Lpb. plantarum*) and *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*) powders at concentrations of 10^6, 10^7, and 10^8 colony-forming units (CFU)/mL and *Lactobacillus acidophilus* (*L. acidophilus*) powder at concentrations of 10^5, 10^6, and 10^7 CFU/mL. The dashed lines represent the 80% viability limit above which cell viability is considered acceptable. Data are expressed as a percentage with respect to CTR (n = 6, mean ± standard deviation).

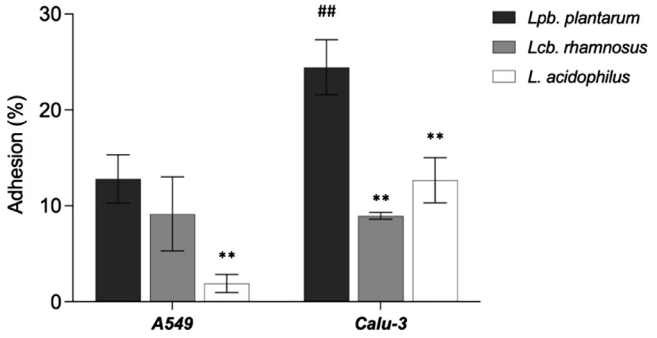

Fig. 8. Adhesion capacity of the *Lactiplantibacillus plantarum* (*Lpb. plantarum*), *Lacticaseibacillus rhamnosus* (*Lcb. rhamnosus*), and *Lactobacillus acidophilus* (*L. acidophilus*) spray-dried powders on A549 and Calu-3 cells (n = 3, mean ± standard deviation). $^{**}P \leq 0.01$ vs. *Lpb. plantarum*; $^{\#\#}P \leq 0.01$, vs. *Lpb. plantarum* on A549.

acteristics, with long lag time, low rate and yEnd, and highest pH, proving to be the most affected by the production process. *Lpb. plantarum* showed the best growth performance, whereas *Lcb. rhamnosus* showed the fastest rate, and therefore the shortest time to reach the final pH.

4. Discussion

Despite the need to consider safety issues, the idea of administering living bacterial cells to promote human health is quite intriguing. Thanks to the long history of safe use and the beneficial effects of *Lactobacillus* species against various infectious diseases, their use for potentially preventing pulmonary infection has been recently proposed [14,15]. By delivering the probiotics directly to the lungs, the primary site of infection, it may be possible to achieve beneficial direct outcomes that are not reachable by oral administration, and whose effects, to be exerted, require the activation of the gut-lung axis [16].

In this study, probiotics were included by spray drying in an inhalable powder to avoid the instability of LAB-free cells in a liquid medium [17] and to offer an easy and convenient way of their delivery to the lung [18].

Preliminary studies were addressed to investigate the effect of the diluting solution used for the suspension of the LABs and their concentration on viability and respirability of the powder. These tests were performed on *Lpb. plantarum*, which is a wild strain with less available information on stress tolerance, unlike the other two commercial strains [19,20]. The viability of the *Lpb. plantarum* decreased 1 log, indicating that neither the suspension diluting solution nor the cells concentration in the stock suspension had a significant effect on this feature. The decrease of 1 log is negligible, considering the very high content of viable cells, and literature reports that ca. 1 log is lost using both spray drying and other manufacturing processes [10]. On the other hand, the respirability of the powders produced was strongly affected by the use of Ringer's solution, and the powders were characterised by visible particles aggregates. In this regard, the calcium chloride present in this solution, being a strong desiccant, could have absorbed moisture, making the particles more cohesive and therefore with poor aerosolization properties.

The product optimization was carried out adding to the LAB suspension two excipients, lactose and L-leucine, to maximize the

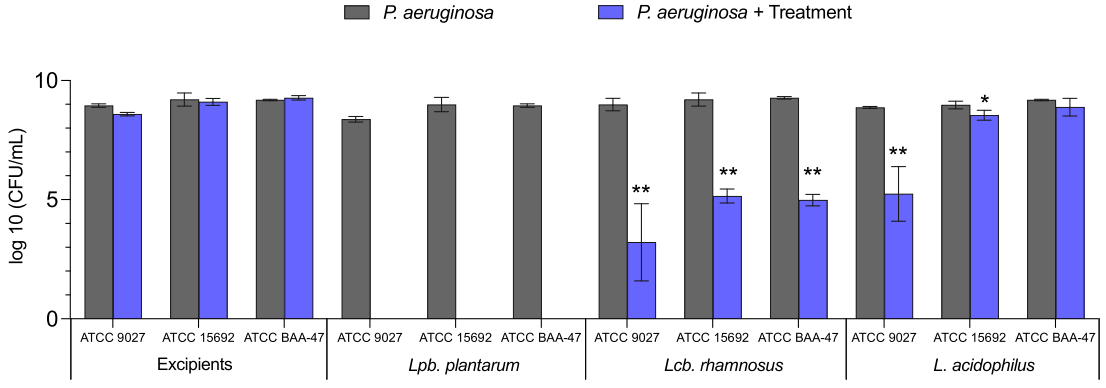

Fig. 9. Antimicrobial activity of lactic acid bacteria (LAB) spray-dried powders on *Pseudonomas aeruginosa* (*P. aeruginosa*) growth and, as control, of the spray-dried excipients (n = 6, mean ± standard deviation). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. *P. aeruginosa* in the absence of treatment (LAB or excipients). CFU, colony-forming units; *L. acidophilus, Lactobacillus acidophilus; Lcb. rhamnosus, Lacticaseibacillus rhamnosus; Lpb. plantarum, Lactiplantibacillus plantarum*.

Table 4
Microbial evolution with time after re-suspension of spray-dried inhalation powders in water: lag time, rate, yEnd, and pH values for *Lpb. plantarum, Lcb. rhamnosus,* and *L. acidophilus* (n = 3, mean ± SD).

Strains	Lag time (h)	Rate	yEnd (%)	pH
Lpb. plantarum	5.07 ± 0.16	9.11 ± 0.35	50.74 ± 0.15	3.70 ± 0.01
Lcb. rhamnosus	5.22 ± 0.45	12.66 ± 0.64	52.55 ± 1.63	3.75 ± 0.03
L. acidophilus	11.70 ± 1.19	6.31 ± 0.07	14.22 ± 1.71	4.27 ± 0.04

L. acidophilus, Lactobacillus acidophilus; Lcb. rhamnosus, Lacticaseibacillus rhamnosus; Lpb. plantarum, Lactiplantibacillus plantarum; yEnd, maximum value of change in conductivity in the medium.

LAB viability and to improve the in vitro respirability. Lactose is usually used for the formulation of spray-dried biologic powders because it forms a glassy matrix that provides protection during technological processes and storage [21]. Leucine precipitates on the surface of the dried particle, thereby protecting the powder from water adsorption and improving the flowability and stability. In the powder composition, leucine content of 30% was selected, as it allows to obtain the surface coating sufficient to improve aerosolisation of dried particles [22].

In addition to the contribution of the excipients, the effect of spray-drying inlet temperature was investigated as a critical factor. Temperature must be high enough to ensure water evaporation but not excessive to damage the cells [23]. Our data suggest that an increase of the inlet temperature together with the maximization of excipients content leads to improved cell viability. This result can be justified considering that a large part of the heat is used for the evaporation of the solvent, and that the range of temperatures investigated was not particularly high. Rather, the effect of residual water in the powder, not efficiently removed when operating at 125°C, could be responsible for this correlation, as a low level of moisture content and water activity has been shown to be more effective in maintaining probiotic cells in a partially inactivated state, thus preserving their viability [24].

Overall, the viability of *Lpb. plantarum* dropped off 1 log compared with the starting stock suspensions, and this result was also observed with the other two strains. These data are in accordance with those reported by Su et al. regarding the production of spray-dried powders containing *Lcb. rhamnosus GG* for food implementation, which showed a drop in cell viability of 1 log [25].

The respirable fraction is the characteristic that most affects the efficacy of an inhaled product, as it represents the fraction of the inhaled particles capable of reaching the lower airways. For this purpose, the RS01 device was chosen as being capable to deliver high-powder doses (up to 60 mg) [26], and thanks to its peculiar capsule spinning movement, it is highly efficient in the powder aerosolisation [27].

Although the bacteria themselves had dimensions in length ranging from 2–10 µm depending on the strain, all were incorporated in similar microparticles with maximum dimensions around 3–5 µm. Therefore, the strain with the longest structure (*L. acidophilus*) also evidently underwent a folding inside the particle.

The extra-fine fraction of powders (<0.9 µm) was largely composed of excipients, as few CFUs were found in this size range. This subpopulation could already be present in the powders as such (see spherical submicron structure in scanning electron microscopy) or generated by large particle collision during aerosolisation. Indeed, probiotics were found only in the population of larger particles deposited in the first stages of the impactor (size range 1.6–8 µm) after aerosolisation. The aerodynamic LAB parameters indicate that our formulation would allow their deposition in the endobronchial space of airways, where in CF patients' *P. aeruginosa* colonise the highly viscous mucus [28] and where probiotics could have a fruitful interaction with the lung microbiota.

Even if *Lactobacilli* are recognized as safe considering their oral administration, by modifying the route of administration, preclinical tests are needed. Their toxicity was evaluated in vitro on the immortalized cell of both the bronchial and alveolar epithelium. The safety of *Lpb. plantarum* and *Lcb. rhamnosus* powders up to a concentration of 10^7 cells/mL are consistent with previously published data on the toxicity of LAB suspension [29]. The higher toxicity of *L. acidophilus* than the other strains was due to the large amount of powder applied to the cells to reach the selected concentrations of CFU to be tested. The same concentration of excipients (40 mg/mL) applied alone damaged the cells (Supplementary Fig. S3), probably because of the hypertonic environment created following their dissolution.

The LAB's capacity to adhere to lung cells and the powder's antimicrobial effect are the main mechanisms that the host can benefit from, representing the discriminants for the choice of probiotic strains. The adhesion of the probiotics to the host epithelium represents a benefit, as this not only increases the residence time of the substrate and therefore its colonization, but it also involves the release of molecules with immunomodulatory and antimicrobial effect [30]. The adhesion normally involves interactions of external strain-specific portions of the bacterium (adhesins, polysaccharides, and proteins) with collagen, fibronectin, or mucus of the host [30]. Despite the presence of key adhesive pili on the *L. rhamnosus GG* surface, in our study its capacity of adhesion was even lower than that of the *Lpb. plantarum.* Because it has been demonstrated that pili are shear-sensitive structures that can easily be sheared off [31], they could be damaged during the drying. As pili are also known to provide an essential mechanism for colony formation in bacteria [32], this hypothesis would be also supported by the low cultivability observed in *Lcb. rhamnosus* after spray drying, despite the high viability count. On the other hand, *Lpb. plantarum* showed the greatest ability to adhere to host cell surface on both cell lines, indicating its higher resistance to the spray-drying process, allowing the retention of extracellular protein involved in the adherence. This hypothesis appears be supported by the survival-rate results after the spray drying, where *Lpb. plantarum* formulation showed higher viability, cultivability, and vitality compared with the other two strain powders. Moreover, glycoproteins with a high proportion of sulphates are known to be the cell membrane structures involved in the adhesion of bacteria to model lung cells [33]. The higher adhesion of *Lpb. plantarum* to Calu-3 can be explained considering that these cells express higher levels of MUC5 mucins than A549. In addition, Calu-3 MUC5 mucins were shown to be sulphated, in contrast to those secreted by A549 cells [34].

After the *Lactobacilli* have adhered to the mucus layer, they can consume the nutrients necessary for the survival of the pathogenic bacteria, and they produce molecules and peptides with antimicrobial properties, like bacteriocins [8]. Although it is reported in literature that all LABs can potentially produce those substances [35], their ability to interact with the growth of pathogenic bacteria could be impaired by the powder manufacturing thermal process.

In our study, *Lpb. plantarum* had a bactericidal effect on *P. aeruginosa; Lcb. rhamnosus* showed a bacteriostatic effect by significantly limiting the bacterial growth; and *L. acidophilus* was bacteriostatic as well, although in a lower manner. These findings agree with the study by Dubourg et al., which analysed the antimicrobial activity of eight lactobacilli strains, tested as such without undergoing any formulation, and demonstrated that *Lpb. plantarum* and *Lcb. rhamnosus* were active towards *P. aeruginosa,* whereas *L. acidophilus* was not effective [36]. Interestingly, the favourable antimicrobial results, demonstrated in our study, were provided by *Lpb. plantarum* formulated as dry powder, indicating that the process did not compromise its metabolic activity. Comparing the powder antimicrobial efficacy with that of the native LAB suspensions, it is possible once again to observe that *Lpb. plantarum* maintained its full activity, whereas for the other two strains the bactericidal activity was lost (see Supplementary Fig. S4). The fact that *Lcb. rhamnosus* in our study was found to have a marked bacteriostatic effect against *P. aeruginosa* ATCC 9027 when undiluted could indicate that the synthesized antimicrobials were effective only at high concentrations.

Another major factor that affects the *P. aeruginosa* growth is the environmental pH, as the growth of the pathogen was reported to be hindered at pH below 3.9 [9]. This aspect can further strengthen our results, supporting the high efficacy of *Lpb. plantarum* against *P. aeruginosa* being the strain that allowed us to obtain the lower co-culture pH (4.1) compared with *Lcb. rhamnosus* (4.2) or *L. acidophilus* (4.5) as well as the lowest values when tested alone (pH: 3.7).

5. Conclusions

This work highlights for the first time how, by applying a multidisciplinary approach, it was possible to produce inhalation powders including beneficial lactic acid bacteria. Thanks to the optimisation of the drying process, powders were characterised by a high viability and cultivability of Lactobacilli. Probiotics, providing a FPF in the range of 42%–50% and a MMAD of ca. 4.5–5.0 µm, demonstrated to be suitable for their deposition on the conductive airways.

Among the tested strains, the inhalation powder including *Lpb. plantarum* showed the best performance in terms of bacterial viability, tolerability, adhesion, and bactericidal properties against *P. aeruginosa.*

The work represents a promising starting point to drive the scientific community to explore the modulation of the pulmonary microbiota and management of lung infection following the administration of a probiotic directly in contact with microflora. In the specific field of CF, the proposed inhalation product containing *Lpb. plantarum* might be useful in keeping the growth of pathogenic microflora in check during the antibiotic inhalation therapy suspension in treatment regimen.

Funding: This work was supported by a grant by the University of Parma (FIRB2021).

Competing interests: None declared.

Ethical approval: Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.107001.

References

[1] Wypych TP, Wickramasinghe LC, Marsland BJ. The influence of the microbiome on respiratory health. Nat Immunol 2019;20:1279–90. doi:10.1038/s41590-019-0451-9.
[2] Natalini JG, Singh S, Segal LN. The dynamic lung microbiome in health and disease. Nat Rev Microbiol 2023;21:222–35. doi:10.1038/s41579-022-00821-x.
[3] Fainardi V, Neglia C, Muscarà M, Spaggiari C, Tornesello M, Grandinetti R, et al. Multidrug-resistant bacteria in children and adolescents with cystic fibrosis. Children 2022;9:1–7. doi:10.3390/children9091330.
[4] . Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Food and Agriculture Organization and World Health Organization; 2001. p. 1–34.
[5] Yuksel N, Gelmez B, Yildiz-Pekoz A. Lung microbiota: its relationship to respiratory system diseases and approaches for lung-targeted probiotic bacteria delivery. Mol Pharm 2023;20:3320–37. doi:10.1021/acs.molpharmaceut.3c00323.
[6] Fangous MS, Alexandre Y, Hymery N, Gouriou S, Arzur D, Blay G Le, et al. Lactobacilli intra-tracheal administration protects from *Pseudomonas aeruginosa* pulmonary infection in mice—a proof of concept. Benef Microbes 2019;10:893–900. doi:10.3920/BM2019.0069.
[7] Fangous MS, Gosset P, Galakhoff N, Gouriou S, Guilloux CA, Payan C, et al. Priming with intranasal lactobacilli prevents *Pseudomonas aeruginosa* acute pneumonia in mice. BMC Microbiol 2021;21:1–11. doi:10.1186/s12866-021-02254-7.
[8] Al-malkey MK, Ismeeal C, Abo J, Mohammed SW, Nayyef HJ. Antimicrobial effect of probiotic *Lactobacillus spp.* on *Pseudomonas aeruginosa.* J Contemp Med Sci 2017;3:218–23. doi:10.22317/jcms.v3i10.169.
[9] Chappell TC, Nair NU. Engineered lactobacilli display anti-biofilm and growth suppressing activities against *Pseudomonas aeruginosa.* NPJ Biofilms Microbiomes 2020;6:48. doi:10.1038/s41522-020-00156-6.
[10] Killeit U. Current trends in the production of probiotic formulations. Foods 2022;11:2330. doi:10.3390/foods11152330.
[11] Bottari B, Santarelli M, Neviani E, Gatti M. Natural whey starter for Parmigiano Reggiano: culture-independent approach. J Appl Microbiol 2010;108:1676–84. doi:10.1111/j.1365-2672.2009.04564.x.
[12] Bancalari E, Bernini V, Bottari B, Neviani E, Gatti M. Application of impedance microbiology for evaluating potential acidifying performances of starter lactic acid bacteria to employ in milk transformation. Front Microbiol 2016;7:1–11. doi:10.3389/fmicb.2016.01628.
[13] Buttini F, Rozou S, Rossi A, Zoumpliou V, Rekkas DM. The application of Quality by Design framework in the pharmaceutical development of dry powder inhalers. Eur J Pharm Sci 2018;113:64–76. doi:10.1016/j.ejps.2017.10.042.
[14] Alexandre Y, Le Berre R, Barbier G, Le Blay G. Screening of *Lactobacillus spp.* for the prevention of *Pseudomonas aeruginosa* pulmonary infections. BMC Microbiol 2014;14:107. doi:10.1186/1471-2180-14-107.
[15] Bottari B, Castellone V, Neviani E. Probiotics and Covid-19. Int J Food Sci Nutr 2021;72:293–9. doi:10.1080/09637486.2020.1807475.
[16] Batoni G, Maisetta G, Kaya E, Esin S. Lung-directed bacteriotherapy in cystic fibrosis: could it be an option? Antibiotics 2022;11:326. doi:10.3390/antibiotics11030326.
[17] Huang S, Vignolles ML, Chen XD, Le Loir Y, Jan G, Schuck P, et al. Spray drying of probiotics and other food-grade bacteria: a review. Trends Food Sci Technol 2017;63:1–17. doi:10.1016/j.tifs.2017.02.007.
[18] Lavorini F, Buttini F, Usmani OS. 100 years of drug delivery to the lungs. Handb Exp Pharmacol 2019;260:143–59. doi:10.1007/164_2019_335.
[19] Broeckx G, Vandenheuvel D, Henkens T, Kiekens S, van den Broek MFL, Lebeer S, et al. Enhancing the viability of *Lactobacillus rhamnosus GG* after spray drying and during storage. Int J Pharm 2017;534:35–41. doi:10.1016/j.ijpharm.2017.09.075.
[20] Albertini B, Vitali B, Passerini N, Cruciani F, Di Sabatino M, Rodriguez L, et al. Development of microparticulate systems for intestinal delivery of *Lactobacillus acidophilus* and *Bifidobacterium lactis*. Eur J Pharm Sci 2010;40:359–66. doi:10.1016/j.ejps.2010.04.011.
[21] Pilcer G, Wauthoz N, Amighi K. Lactose characteristics and the generation of the aerosol. Adv Drug Deliv Rev 2012;64:233–56. doi:10.1016/j.addr.2011.05.003.
[22] Alhajj N, O'Reilly NJ, Cathcart H. Leucine as an excipient in spray dried powder for inhalation. Drug Discov Today 2021;26:2384–96. doi:10.1016/j.drudis.2021.04.009.
[23] Broeckx G, Vandenheuvel D, Claes IJJ, Lebeer S, Kiekens F. Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. Int J Pharm 2016;505:303–18. doi:10.1016/j.ijpharm.2016.04.002.
[24] Martins E, Cnossen DC, Silva CRJ, Cezarino JC, Nero LA, Perrone IT, et al. Determination of ideal water activity and powder temperature after spray drying to reduce Lactococcus lactis cell viability loss. J Dairy Sci 2019;102:6013–22. doi:10.3168/jds.2019-16297.
[25] Su Y, Zheng X, Zhao Q, Fu N, Xiong H, Wu WD, et al. Spray drying of *Lactobacillus rhamnosus GG* with calcium-containing protectant for enhanced viability. Powder Technol 2019;358:87–94. doi:10.1016/j.powtec.2018.09.082.
[26] Buttini F, Balducci AG, Colombo G, Sonvico F, Montanari S, Pisi G, et al. Dose administration maneuvers and patient care in tobramycin dry powder inhalation therapy. Int J Pharm 2018;548:182–91. doi:10.1016/j.ijpharm.2018.06.006.
[27] Buttini F, Quarta E, Allegrini C, Lavorini F. Understanding the importance of capsules in dry powder inhalers. Pharmaceutics 2021;13:1936. doi:10.3390/pharmaceutics13111936.

[28] Ong T, Ramsey BW. Cystic fibrosis: a review. JAMA 2023;329:1859–71. doi:10.1001/jama.2023.8120.
[29] Rizzo A, Losacco A, Carratelli CR, Di Domenico M, Bevilacqua N. *Lactobacillus plantarum* reduces *Streptococcus pyogenes* virulence by modulating the IL-17, IL-23 and Toll-like receptor 2/4 expressions in human epithelial cells. Int Immunopharmacol 2013;17:453–61. doi:10.1016/j.intimp.2013.07.005.
[30] Sengupta R, Altermann E, Anderson RC, McNabb WC, Moughan PJ, Roy NC. The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators Inflamm 2013;2013:237921. doi:10.1155/2013/237921.
[31] Kiekens S, Vandenheuvel D, Broeckx G, Claes I, Allonsius C, De Boeck I, et al. Impact of spray-drying on the pili of *Lactobacillus rhamnosus GG*. Microb Biotechnol 2019;12:849–55. doi:10.1111/1751-7915.13426.
[32] Taktikos J, Lin YT, Stark H, Biais N, Zaburdaev V. Pili-induced clustering of *N. gonorrhoeae* bacteria. PLoS One 2015;10:e013766. doi:10.1371/journal.pone.0137661.
[33] Rajas O, Quirós LM, Ortega M, Vazquez-Espinosa E, Merayo-Lloves J, Vazquez F, et al. Glycosaminoglycans are involved in bacterial adherence to lung cells. BMC Infect Dis 2017;17:1–14. doi:10.1186/s12879-017-2418-5.
[34] Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory carcinoma cell lines MUC genes and glycoconjugates. Am J Respir Cell Mol Biol 1999;20:500–10. doi:10.1165/ajrcmb.20.3.3383.
[35] Jamalifar H, Rahimi HR, Samadi N, Shaverdi AR, Sharifian Z, Hosseini F, et al. Antimicrobial activity of different Lactobacillus species against multi-drug resistant clinical isolates of *Pseudomonas aeruginosa*. Iran J Microbiol 2011;3:21–5.
[36] Dubourg G, Elsawi Z, Raoult D. Assessment of the in vitro antimicrobial activity of Lactobacillus species for identifying new potential antibiotics. Int J Antimicrob Agents 2015;46:590–3. doi:10.1016/j.ijantimicag.2015.05.011.