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Original article

Cell-free supernatants from *Lactobacillus* strains exert antibacterial, antibiofilm, and antivirulence activity against *Pseudomonas aeruginosa* from cystic fibrosis patients

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ABSTRACT

Chronic lung infections caused by *Pseudomonas aeruginosa* play a significant role in the mortality and morbidity of cystic fibrosis (CF) patients. The widespread bacterial resistance to conventional antimicrobials demands identifying new strategies to complement or replace current antibiotic therapies. In this study, we evaluated the antibacterial, antibiofilm, and antivirulence properties of cell-free supernatants (CFS) from several *Lactobacillus* probiotic strains against *P. aeruginosa* isolated from the sputum of CF patients. A strong and fast antibacterial activity of CFS from different strains of lactobacilli was observed at acidic pH towards *P. aeruginosa*, both in planktonic and biofilm mode of growth, in conditions mimicking CF lung. Interestingly, although when adjusted at pH 6.0, CFS lost most of their antibacterial potential, they retained some antivirulence activity towards *P. aeruginosa*, largely dependent on the dose, exposure time, and the *Lactobacillus-P. aeruginosa* strain combination. *In vivo* testing in the invertebrate *Galleria mellonella* model disclosed the lack of toxicity of acidic CFS and their ability to prevent *P. aeruginosa* infection. For the first time, the results revealed lactobacilli postbiotic activities in the context of the pulmonary environment, pointing to innovative postbiotics' uses in anti-infective therapy.

1. Introduction

Pseudomonas aeruginosa is one of the major pathogens in individuals with cystic fibrosis (CF), causing severe lung infections and significantly contributing to morbidity and mortality in these patients [1]. The pathogenesis of *P. aeruginosa* infections is complex as the bacterium produces many virulence factors with multiple effects on host tissues and the immune system [2]. In addition, under stress conditions, the bacterium readily switches to the biofilm mode of growth, which allows it to evade host response and antibiotic treatment and establish persistent infections [3,4].

The World Health Organization recognizes *P. aeruginosa* as a severe threat to human health, listing it among the priority pathogens for which new control strategies, complementing or substituting antibiotic use, are urgently needed [5]. Among these strategies, the so-called bacteriotherapy, e.g., the use of beneficial bacteria or their products to treat/prevent infectious diseases, is attracting increasing consideration [6]. One of the main tools of bacteriotherapy is the administration of probiotics, e.g., bacteria that, when administered in sufficient amounts, confer a health benefit to the host [6]. Although the potential application of probiotics is continuously widening, the therapeutic use of live bacterial cells may pose safety concerns, especially in vulnerable subjects. In addition, difficulties in their standardization, reduced shelf life, and loss of vitality during the manufacturing procedures are all potential drawbacks of using live microorganisms as biotherapeutics [7]. Therefore, emerging evidence points to the use of probiotic-derived active components or metabolites, also

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referred to as "postbiotics", as a valid alternative to live bacteria [8]. A new definition of postbiotics has recently been proposed [9], but a consensus on the use of such a term still needs to be reached. Beyond definitions, non-viable microorganisms, their fragments or products released in cell-free supernatants (CFS) may provide beneficial bioactivities with pharmacological potential while exhibiting several advantages over live probiotics, including safety, ease of production and storage, and multiple mechanisms of action.

Although research on this topic is very active, further studies are needed to gain insights into the full spectrum of postbiotic activities and how they can be used and properly implemented to manage site-specific infections in humans [6]. In particular, postbiotic effects in the context of the pulmonary environment and against lung pathogens still need to be thoroughly investigated.

In this study, we aimed to evaluate the antibacterial, antibiofilm, and antivirulence potential of CFS from different lactobacilli against mucoid and non-mucoid isolates of *P. aeruginosa* from CF patients. To mimic the environmental conditions found in CF lung, antibacterial and antibiofilm assays were conducted in an artificial sputum medium (ASM) which closely resembles the composition of the thick mucus laying the lung epithelium of CF patients and reported to alter antibiotic activity [10,11]. In addition, the effects of neutralized CFS on *P. aeruginosa* virulence were thoroughly investigated at the phenotypic and genotypic levels. Finally, the most promising CFS were tested for their ability to prevent or treat *P. aeruginosa* infection in the invertebrate *Galleria mellonella* model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four strains of *P. aeruginosa* isolated from the sputum of CF patients were used throughout the study. They were identified by using a MALDI-TOF Microflex LT Mass Spectrometer (MALDI-TOF-MS) equipped with a MALDI Biotyper 3.1 software (Bruker Daltonics; Bremen, Germany) according to the manufacturer's instructions. A score $\geq$2.00 allowed identification at the species level. The Phoenix System (Becton Dickinson Italia; Milan, Italy) was used for antibiotic susceptibility testing. The main characteristics of the strains are reported in Table S1.

Six species/strains of lactobacilli were isolated from commercial products purchased from local pharmacies in Italy (e.g., dietary supplements) (Table S2). Capsules, tablets, or lyophilized powder were dissolved in water and streaked on the De Man-Rogosa-Sharpe agar (MRSA, Oxoid; Basingstoke, Hampshire, UK). Single colonies were picked up and identified at the species level by MALDI-TOF-MS as described above. Table S2 reports the strains within the species declared by the manufacturers on the product information leaflet. For three of them, which gave the best results in the present and in previous studies [11,12], strain identification was confirmed by whole genome sequencing and data analysis by Novogene (Beijing, China).

P. aeruginosa strains were grown in Luria Bertani broth or in Tryptone Soya Broth (LB, TSB, Oxoid), while lactobacilli were in De Man-Rogosa-Sharpe broth (MRSB; Oxoid). Late-log phase cultures were subdivided into aliquots and frozen at −80 °C until use. For CFU count, *P. aeruginosa* and lactobacilli were grown on Tryptone Soy Agar (TSA, Oxoid) and the MRSA, respectively.

2.2. Preparation of CFS from Lactobacillus strains

An aliquot of lactobacilli cultures from the frozen stock was inoculated in 5 ml of MRSB and incubated at 37 °C overnight in shaking conditions. After incubation, cultures were diluted 1:100 in fresh MRSB and incubated at 37 °C for 48 h. Cultures were centrifuged at 6000×*g* at 4 °C for 10 min. The supernatants were sterilized by passage through 0.22 μm pore filters, divided in aliquots, and stored at −20 °C until use in further assays.

2.3. Growth inhibitory capacity of CFS from Lactobacillus strains via the agar-well diffusion method

The agar-well diffusion method was employed to determine the growth inhibitory capacity of the CFS obtained from the different strains of lactobacilli against the clinical isolates of *P. aeruginosa*. To this aim, cultures of exponentially growing *P. aeruginosa* were diluted in TSB to reach a cell density of approximately 10^8 CFU/ml. TSA plates were then inoculated by evenly spreading 100 μl of each bacterial suspension over the agar surface. After that, wells with a diameter of 8 mm were punched aseptically with the bottom of a sterile 1 ml tip and filled with 100 μl of each sterile CFS in triplicate. The plates were incubated at 37 °C for 24 h, and examined for the presence of the inhibition zone. The diameter of the inhibition zone was measured and recorded as the mean value of three wells. Controls included sterile MRSB adjusted to pH 4 with HCl. In parallel, the same procedure was followed using CFS adjusted to pH 6.

2.4. Time kill assays

A standardized inoculum (1−2x10^5 CFU/ml) of mucoid (PA11) and non-mucoid (PA16) *P. aeruginosa* strains prepared in TSB was exposed to CFS (1:1, v/v) of *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* M tested at 1:2, 1:4 and 1:8 dilutions, at pH 4 and 6. A standardized inoculum of 1−2x10^8 CFU/ml was also tested for CFS at pH 4. Control samples were TSB with MRSB, corrected at the tested pH, and seeded with *P. aeruginosa*. During static incubation at 37 °C, aliquots were taken from the samples at prefixed intervals (0.5, 1, 2, 3, 4, 6, 8, 21, and 24 h), serially diluted in Phosphate Buffer Saline (PBS; Sigma−Aldrich; Milan, Italy), and plated on TSA. After incubation for 24 h at 37 °C, *P. aeruginosa* cell viability was evaluated by CFU counting.

For the assessment of the killing kinetics in conditions mimicking the CF lung environment, time-kill assays were performed in ASM using the formulation of Sriramulu et al. [13], which we modified with the addition of glucose at the mean concentration found in the sputum of CF patients [11]. Assays were carried out in a total volume of 500 μl containing 50 μl of exponentially growing cultures of *P. aeruginosa* (PA1, PA4, PA11, and PA16) in TSB (final cell density 1 × 10^6 CFU/ml), 125 μl of CFS from *L. plantarum* or *L. rhamnosus* M, and 325 μl of ASM. In control samples, 125 μl of CFS were replaced with 125 μl of sterile MRSB. Samples were incubated at 37 °C for 0, 10, 30, and 60 min, serially diluted in TSB, and plated on TSA for CFU enumeration.

2.5. Effect of heat treatment on the antibacterial activity of CFS

The heat treatment of CFS from *L. plantarum* and *L. rhamnosus* M was conducted by incubating them in the Eppendorf ThermoMixer® C at 70 °C or 100 °C, for 30 min. Antibacterial assays were carried out in TSB. To this aim, planktonic PA11 and PA16 (1 × 10^7 CFU/mL, in exponential phase) were incubated with untreated or heat-treated CFS (1:4). MRSB diluted 1:4 in TSB was used as control. Incubation durations were set at 1 h for the CFS of *L. plantarum* and 30 min for the CFS of *L. rhamnosus* M as these incubation times allowed to better evaluate the antimicrobial potency of the respective CFS. Following the incubation period, samples were serially diluted and plated on TSA for CFU enumeration.

2.6. Antibiofilm assays

Antibiofilm activity of CFS from *L. plantarum* and *L. rhamnosus* M was evaluated against all four strains of *P. aeruginosa*. To this aim, mature biofilms were obtained by diluting *P. aeruginosa*, grown overnight in TSB, 1:100 in ASM and incubating 100 μl of the diluted suspension in 96-well plates at 37 °C for 24 h in static conditions.

Following incubation, mature biofilms formed in ASM were gently washed 3 times with pre-warmed PBS to remove unattached bacteria and treated with 200 μl of CFS or sterile MRSB, both pre-diluted 1:4 in ASM. At 0, 2, 6, and 24 h, biofilms were washed 3 times with PBS and dislodged by scraping the bottom of the wells with a sterile tip as previously described [4,11]. Disrupted biofilms were resuspended in PBS, subjected to vigorous vortexing, and sonicated for 30 s in a water bath sonicator (Ultrasonic cleaner; VWR International, Milan, Italy) to obtain single-cell suspensions, as verified by microscopy. Serially diluted bacterial suspensions were plated on TSA and incubated for 24–48 h for CFU enumeration.

2.7. Antivirulence assays

2.7.1. Pyoverdine, pyocyanin, and protease production

Overnight grown colonies of *P. aeruginosa* PA11 and PA16 were resuspended in 5 ml LB broth to an OD_{600} of 0.1. This standardized inoculum was exposed to 5 ml of CFS at pH 6 from *L. plantarum* and *L. rhamnosus* M at different dilutions (1:2, 1:4, and 1:8) prepared in MRSB. The positive control consisted of 5 ml inoculum added with 5 ml MRSB, while the negative control was prepared by combining 5 ml LB with 5 ml MRSB. Samples were incubated at 37 °C for 48 h under static conditions, centrifuged at room temperature (15,000×*g*, 15 min), and the supernatants were collected and filtered for the quantification of pyocyanin, pyoverdine, and protease. At the end of 48 h-incubation, cell viable counts indicated no significant differences in CFUs between control and CFS-treated groups, regardless of the strain tested.

Pyoverdine was quantified by measuring OD_{400} of the supernatants. Pyocyanin was extracted from 5 ml supernatant with chloroform (Sigma–Aldrich), then with 0.2 N HCl (Sigma–Aldrich), and finally quantified by measuring OD_{520}. The results obtained were normalized to the OD_{600} of the overnight cultures.

The total proteolytic activity was determined using a skim milk assay. Briefly, *P. aeruginosa* supernatant was mixed with 2.5 % skim milk (1:1, v/v). The absorbance at 600 nm was measured till 5 min, and the results were shown as ΔA/min/ml.

2.7.2. Gene expression assays

Bacterial cells grown in LB were exposed to CFS at pH 6 at different dilutions (1:2, 1:4, and 1:8) and incubated at 37 °C for 48 h, collected by centrifugation, washed with PBS, and resuspended in 1 ml Qiazol Lysis Reagent (Qiagen; Milan, Italy) to facilitate lysis and inhibit RNases. RNA was extracted using the phenol-chloroform technique and purified by treatment with DNase I (Merck KGaA; Darmstadt, Deutschland). Two micrograms of RNA were used as a template for reverse transcription using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Italia Inc.; Milan, Italy). Next, gene expression was evaluated using 16 ng cDNA by RT-qPCR assay on QuantStudio™ 7 Pro Real-Time PCR System (Applied Biosystems Italia; Monza, Italy) using the PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific Italia Inc.). ΔΔCt method was applied to determine the relative gene expression in exposed *vs.* unexposed cells after normalizing the expression using the housekeeping *proC gene*. The expression level variations were shown as fold changes on a $\log_2$ scale.

2.8. In vivo assays

G. mellonella wax moth larvae (n = 20/group; weight: 250–350 mg) were used to evaluate CFS toxicity and for protection studies.

i) CFS toxicity. Larvae were injected in the last left proleg with 10 μl of CFS from *L. plantarum* and *L. rhamnosus* M using a Hamilton syringe. The control group was injected with MRSB only (vehicle).
ii) Protection studies. In the prophylactic challenge, larvae were first inoculated with 10 μl CFS (at pH 4 and 6) from *L. plantarum* or *L. rhamnosus* M. After 2.5 h-incubation at 37 °C, larvae were infected with *P. aeruginosa* strains, each at LD_{50} (10^5 CFU/larva for PA11; 10 CFU/larva for PA16). In the therapeutic studies, larvae were first infected with *P. aeruginosa* strains at LD_{50} and 2.5 h later administered with *L. plantarum* CFS. Control larvae group were: not challenged with CFS and *P. aeruginosa* (unexposed); administered with CFS from *L. plantarum* or *L. rhamnosus* M, and 2.5 h later with PBS (vehicle); and administered with vehicle only.

Both in toxicity and protection studies, infected and control larvae were incubated at 37 °C and monitored daily for survival over 4 days post-exposure.

2.9. Statistical analysis

Each experiment was performed in triplicate and repeated twice (n = 6) unless otherwise specified. The statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Sperman's Rho correlation test was used to assess the strength of the relationship between two variables. The data distribution was assessed by the D'Agostino & Pearson normality test. Ordinary one-way ANOVA followed by Dunnett's or Tukey–Kramer's multiple comparisons test was applied when assessing differences among 3 or more groups of unpaired data. The Student's t test was employed when comparing two groups of variables. The Log-rank Mantel–Cox test measured the statistical significance of differences in mortality rates of *G. mellonella* (n = 20/group, repeated twice). The statistical analysis assumed a confidence level ≥ 95 %, thus considering p values < 0.05 statistically significant.

3. Results

3.1. Acidifying ability of different strains of lactobacilli in MRSB

CFS was obtained from several species/strains of lactobacilli grown in MRSB for 48 h in shaking conditions at 37 °C. As shown in Fig. S1A, all the species/strains tested acidified the medium, although at different extents. The pH of the cultures spanned from 3.87 ± 0.05 in the case of *L. rhamnosus* M to 5.25 ± 0.25 in the case of *L. acidophilus* (mean values ± SEM) and correlated in a statistically significant manner (r^2: 0.8209, p < 0.01) with lactobacilli growth ability in the adopted conditions (Fig. S1B).

3.2. Growth inhibition of CFS via the agar-well diffusion method

Growth inhibitory activity of CFS was initially evaluated by the standard agar-well diffusion method against four strains of *P. aeruginosa* isolated from the sputum of CF patients. To evaluate whether an eventual inhibitory effect was due exclusively to acidity and/or other metabolites released into the extracellular environment during active growth, CFS adjusted to pH 6 with NaOH and sterile MRSB adjusted to pH 4 with HCl were included in the analysis as controls.

As shown in Fig. 1A for a representative strain (PA1) and in Fig. 1B for all 4 strains tested, CFS of different lactobacilli exerted a marked ability to inhibit the growth of *P. aeruginosa*, with the CFS of *L. rhamnosus* M and D, and *L. plantarum* being the most active.

Only minor differences were observed among different strains in the susceptibility to a given supernatant. The inhibitory capacity was completely abolished when the pH of the CFS was raised to 6.0 (Fig. 1A). Nonetheless, CFS at pH 4.0 exerted a higher inhibitory capacity than sterile MRSB adjusted to pH 4 (Fig. 1A–and B), suggesting that the inhibitory capacity might not simply be due to acidity.

Based on these results, CFS from *L. rhamnosus* M and *L. plantarum* were chosen for the subsequent experiments. CFS from *L. rhamnosus* M was selected instead of CFS from *L. rhamnosus* D as the former exhibited a slightly higher acidifying capacity than the latter (Fig. S1A).

The antibacterial and antibiofilm activity of these two CFS was tested in conditions mimicking the CF environment (i.e. in the presence of ASM) against all four strains of *P. aeruginosa* reported in Table S1. In all the other following experimental settings, one representative mucoid and one representative non-mucoid strain of *P. aeruginosa* (PA11 and PA16, respectively) were tested.

3.3. Killing kinetics via CFU count

Time-kill curve assays were initially performed in TSB. To this aim, standardized inocula of two *P. aeruginosa* strains in TSB were exposed to CFS (1:1, v/v) of *L. plantarum* and *L. rhamnosus* M tested at 1:2, 1:4, and 1:8 dilutions at pH 4 and pH 6. As shown in Fig. 2 for the mucoid strain PA11, acid but not neutralized CFS from *L. plantarum* and *L. rhamnosus* M exerted marked and fast dose-dependent killing capacity, achieving bacterial eradication after 1 h of incubation at the highest dose regardless of the inoculum size tested (10^5 and 10^8 CFU/ml). Similar results were obtained for the PA16 non-mucoid strain eradicated after as little as 30 min incubation (Fig. S2). In all the cases, the inhibitory activity of CFS was higher than that of sterile MRSB-HCl adjusted at pH 4, confirming the data of the agar-well diffusion assay.

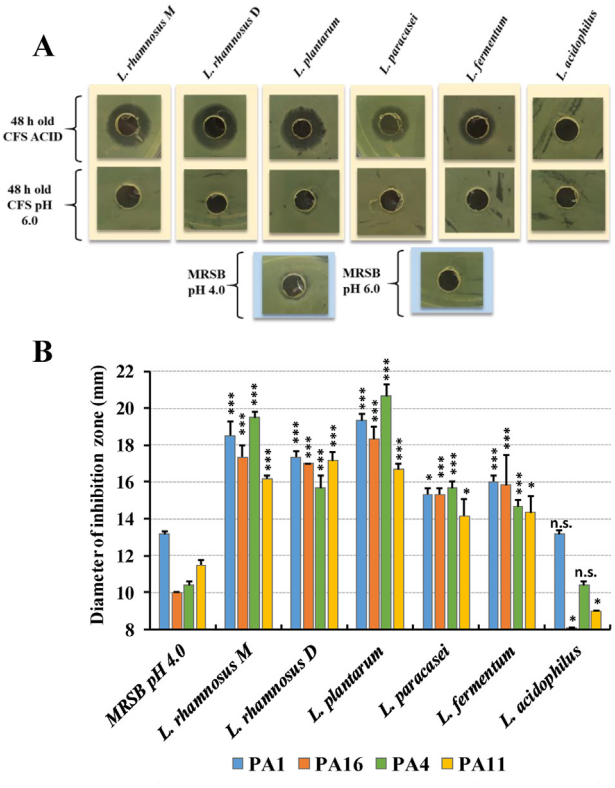

Fig. 1. Antibacterial activity of CFS from different lactobacilli against *P. aeruginosa*. **A**) Growth inhibitory ability of CFS of different *Lactobacillus* strains against *P. aeruginosa* (PA1, non-mucoid strain) via the agar well diffusion method in a representative experiment; **B**) Diameters of inhibition zone against four *P. aeruginosa* strains (mean ± SEM). Eight millimeters correspond to the diameter of the hole punched on the agar plate. Statistical significances at ANOVA followed by the Tukey–Kramer post hoc test compared to MRSB pH 4.0: *$p < 0.05$, ***$p < 0.001$, n.s. non significant.

3.4. Effect of heat on antibacterial activity of CFS

To get further insights on the nature of the active components we investigated whether the antibacterial activity of CFS was affected by heat. As shown in Fig. 3 for PA16 and PA11, following heating at both 70 °C and 100 °C for 30 min both CFS tested maintained most of their antibacterial potency as compared to the un-heated samples indicating that the antibacterial components are mainly heat-insensitive.

3.5. Antibacterial and antibiofilm activity of CFS from L. plantarum and L. rhamnosus M in conditions mimicking CF lung

To investigate CFS's antibacterial potential further, killing assays were performed against *P. aeruginosa* in planktonic form in conditions mimicking CF sputum (Fig. 4A). To this aim, CFS from *L. plantarum* and *L. rhamnosus* M were diluted 1:4 in ASM and tested at different time intervals against four strains of *P. aeruginosa*, resuspended in ASM. Even in these challenging conditions, both *L. plantarum* and *L. rhamnosus* M CFS showed rapid and marked killing effects on all the strains evident in as little as 10 min for most of the strains. In 60 min, the reduction in CFU number ranged from approximately 0.5 Log (50 %, *L. plantarum* CFS *vs.* PA4) to more than 4 Log (99.99 %, *L. rhamnosus* M CFS *vs.* PA1).

CFS from both lactobacilli also showed a marked killing effect against pre-formed biofilms, both obtained and treated in ASM (Fig. 4B). Nevertheless, against biofilms the killing kinetics appeared slower than against planktonic bacteria. Starting from 2 to 6 h of incubation, for most of the tested CFS-strain combinations the biofilm-associated viable bacterial load progressively decreased, to reach, after 24 h treatment, approximately from 3 to 7 Log reduction as compared to the corresponding un-treated controls.

3.6. Antivirulence activity of CFS from L. plantarum and L. rhamnosus M

The antivirulence potential of CFS towards PA11 and PA16 strains was evaluated phenotypically and by gene expression analysis, and results are shown in Fig. 5, S3, S4, and S5.

First, the pigment production and protease activity were measured after 48 h-exposure to CFS diluted 1:2, 1:4, and 1:8 in LB medium (Fig. 5). CFS caused effects at different extents depending on the concentration, strain, and virulence trait considered. When tested diluted at 1:2, CFS from *L. plantarum* and *L. rhamnosus* M always caused a significant reduction compared with control, except for CFS *L. rhamnosus* M *vs.* PA16 protease. It is worth noting that CFS from *L. rhamnosus* M always reduced pyocyanin production by both strains, regardless of concentration. When tested at 1:4, CFS from *L. rhamnosus* M was active in reducing pyoverdine production by both strains, whereas CFS from *L. plantarum* was active only towards PA11. CFS from *L. plantarum* caused a significant dose-dependent reduction in protease activity by the PA11 strain.

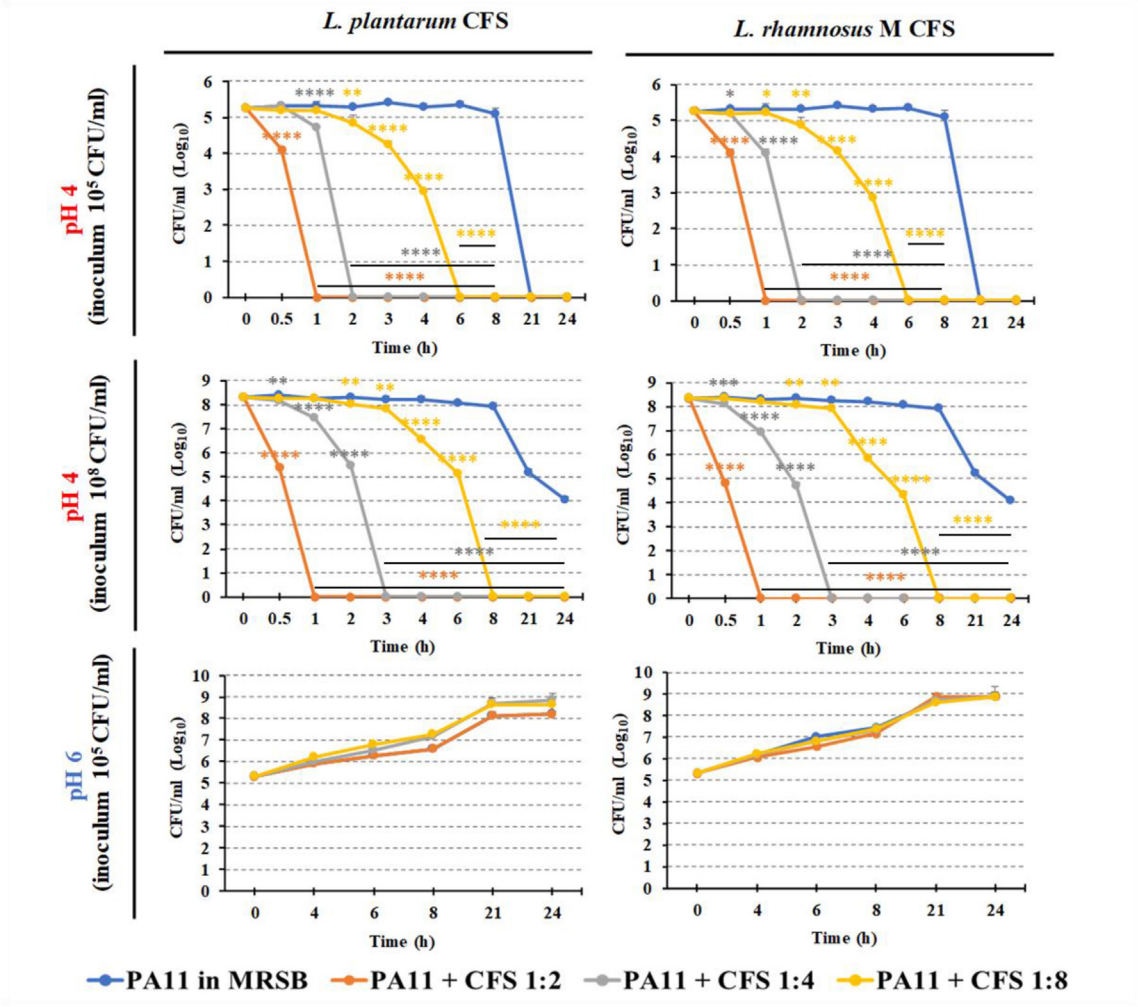

Fig. 2. Killing kinetics of CFS from *L. plantarum* and *L. rhamnosus* M *vs. P. aeruginosa* strain PA11 at pH 4 (inoculum: 10^5 CFU/ml and 10^8 CFU/ml) and pH 6 (inoculum: 10^5 CFU/ml). Results are shown as mean + SD (n = 6). Statistical significances at unpaired t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *vs.* PA11 in MRSB (pH 4 with HCl, or pH 6 blue lines).

The number of significant reductions indicated that exposure to CFS affected the virulence of the PA11 mucoid strain more than that of the PA16 non-mucoid strain (12 *vs.* 8 significant reductions, respectively). Conversely, the PA16 non-mucoid strain was more susceptible to *L. rhamnosus* M than *L. plantarum* CFS (5 *vs.* 3 reductions, respectively).

Next, the effect of CFS on the transcription levels of *algD*, *lasI*, *aprA*, *pvdS, phzH,* and *exoS* virulence genes was assessed by RT-qPCR (Figs. S3, S4, and S5). *L. rhamnosus* M CFS was the most effective, causing a down expression of *algD*, *lasI*, *aprA*, *pvdS*, and *phzH* in the PA16 strain (Fig. S3B) and *lasI* and *aprA* in the PA11 strain (Fig. S3A). However, several cases of up-expression were also observed mainly after exposure to CFS at 1:2 (e.g., *algD* and *aprA*, regardless of strain tested) (Fig. S3). *L. plantarum* CFS reduced expression only in the case of *lasI* in the PA11 strain (Fig. S4A). An opposite trend was observed for *L. plantarum* CFS which mainly caused gene upregulation both in PA11 (*algD*, *aprA*, *pvdS*, *phzH*, and *exoS*) and PA16 (*lasI*, *pvdS*, and *phzH*) strains (Figs. S4A and S4B).

Overall, our data showed that the transcriptional and phenotipic profiles were not always consistent, suggesting that postbiotics might regulate *P. aeruginosa* virulence-related responses at post-transcriptional level (Fig. S5).

3.7. *Safety and efficacy of CFS in the* G. mellonella *infection model*

First, the toxicity of CFS - at pH 4 and 6 - was assessed in *G. mellonella.* Intra-hemocoel administration of undiluted CFS from *L. plantarum* and *L. rhamnosus* M was not toxic, regardless of the pH (Fig. 6A and B).

Next, the protective effect of CFS was first tested in a prophylactic regimen by injecting larvae with undiluted CFS and, after 2.5 h incubation at 37 °C, challenging them with *P. aeruginosa* at LD_{50} (10^5 and 10 CFU/larva, respectively for PA11 and PA16 strains). Acidic CFS from both *L. plantarum* and *L. rhamnosus* M protected larvae against both the mucoid (PA11) and the non-mucoid (PA16) *P. aeruginosa* strains (Fig. 6C and D) in a statistically significant manner ($p < 0.001$ and 0.0001 for PA11 and PA16 respectively, across the entire observational period of 96 h). Conversely, *L. plantarum* and *L. rhamnosus* M CFS at pH 6 were not protective when administered in the prophylactic regimen (data not shown) and when *L. plantarum* CFS was tested in *G. mellonella* larvae previously infected with *P. aeruginosa* (therapeutic regimen) (Fig. S6).

The difference observed in the survival rate at 96 h between acidic CFS Lp (90 %) (Fig. 6A) and acidic CFS Lp + PBS (60–70 %) (Fig. 6C) was probably due to the stress caused to the larvae by the double administration in acidic CFS Lp + PBS, rather than to a

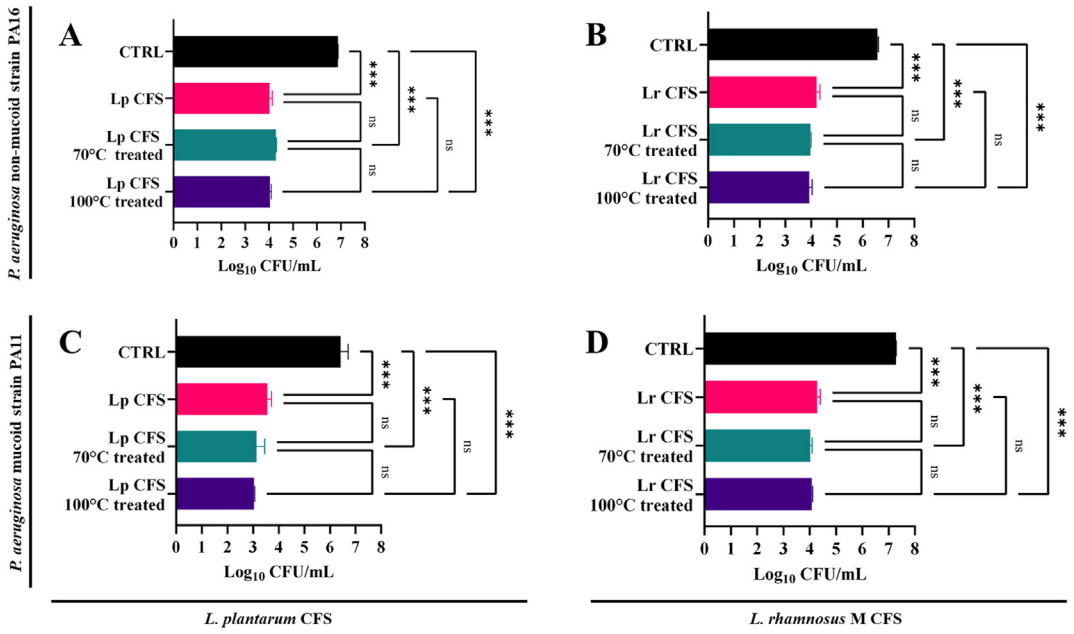

Fig. 3. Effect of heat treatment at 70 °C and 100 °C for 30 min on the antibacterial activity of CFS diluted 1:4, on planktonic *P. aeruginosa*. **A, B**) PA16; **C,D**) PA11. Incubation was carried out for 1 h for the CFS of *L. plantarum* (Lp CFS) and 30 min for the CFS of *L. rhamnosus* M (Lr CFS) as these incubation times allowed to better evaluate the antimicrobial potency of the respective CFS. Results depict the data obtained in one experiment performed in triplicate. One-way ANOVA test followed by Tukey–Kramer multiple comparisons test. ***$p < 0.001$ *vs.* control.

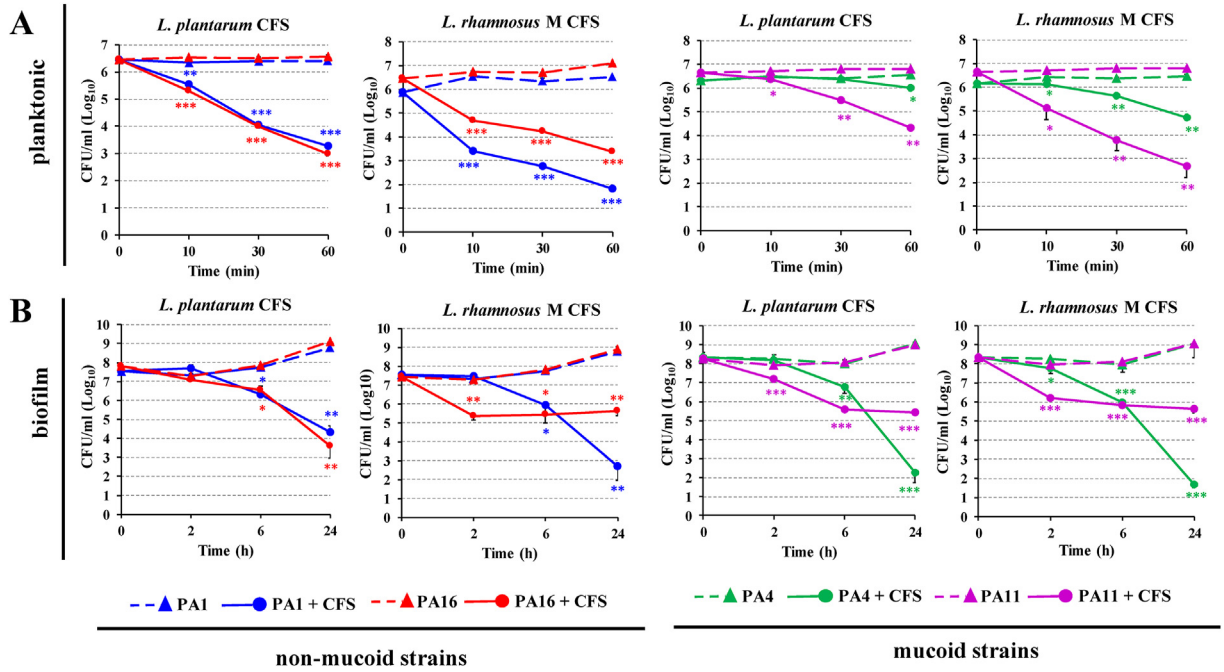

Fig. 4. Activity of CFS from *L. plantarum* and *L. rhamnosus* M against *P. aeruginosa* in conditions mimicking CF lung. **A)** planktonic bacteria; **B)** mature biofilms. All the assays were carried out in ASM. Results depict the data obtained in two experiments performed in duplicate. At each time point the CFU count of untreated samples was compared with that of the treated ones by Student's *t* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

potential killing impact of acidic *L. plantarum* CFS. The same was observed for *L. rhamnosus* CFS (Fig. 6A and D).

4. Discussion

In the present study, we aimed to evaluate the antibacterial, antibiofilm, and antivirulence activity of CFS in the context of a CF-lung environment and against CF lung isolates. With a widely adopted procedure, CFS was obtained from several commercially available lactobacilli approved for human use. As expected, following growth, all the strains tested caused acidification of the medium, although at different extents, which correlated with their growth ability in the adopted conditions. Such acidification is mainly due to the production of organic acids whose amount in the

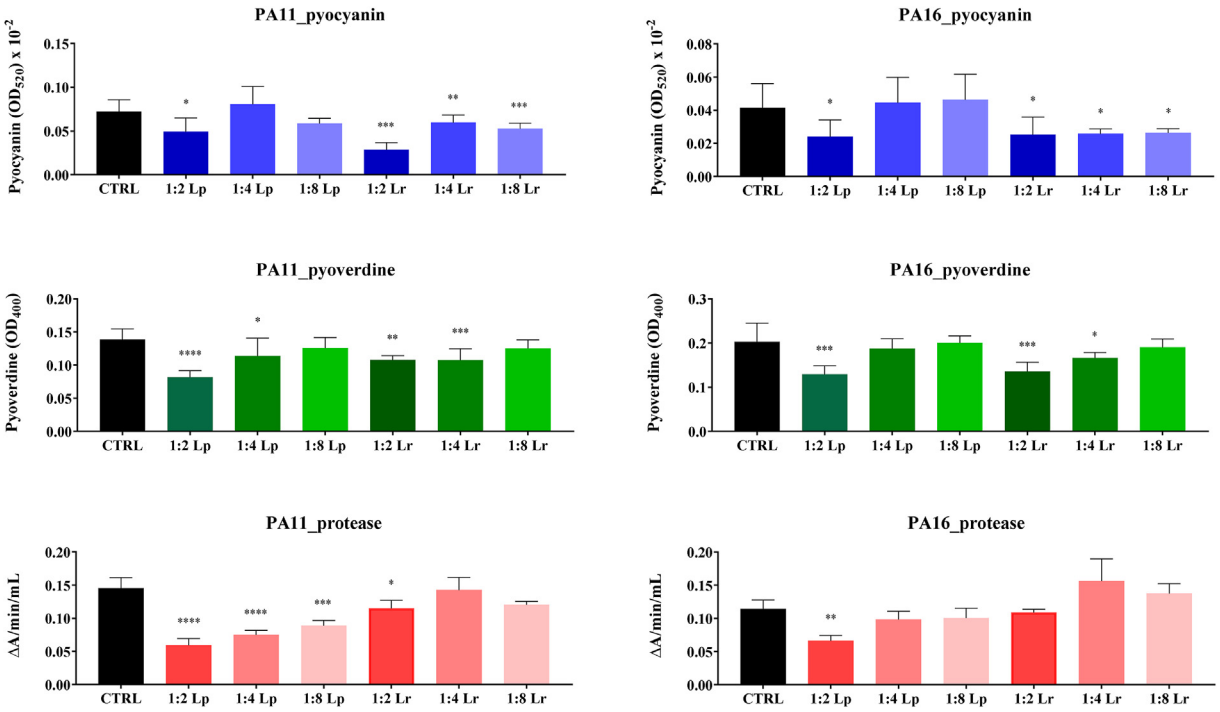

Fig. 5. *In vitro* effect of *L. plantarum* (Lp) and *L. rhamnosus* M (Lr) CFS against pyocyanin, pyoverdine, and protease production by *P. aeruginosa* PA11 and PA16. For each *P. aeruginosa* strain, several colonies were resuspended in 5 ml LB broth to an OD_{600} of 0.1 and treated with 5 ml CFS of Lp or Lr at different dilutions (1:2, 1:4 and 1:8). Control samples consisted of 5 ml bacterial suspension added of 5 ml MRS broth. After 48h-incubation at 37 °C, *P. aeruginosa* protease, pyoverdine, and pyocyanin were measured by reading the supernatants' OD_{600}, OD_{400}, and OD_{520}, respectively. Statistical significances at Dunnett's multiple comparisons test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ *vs.* untreated PA11 and PA16.

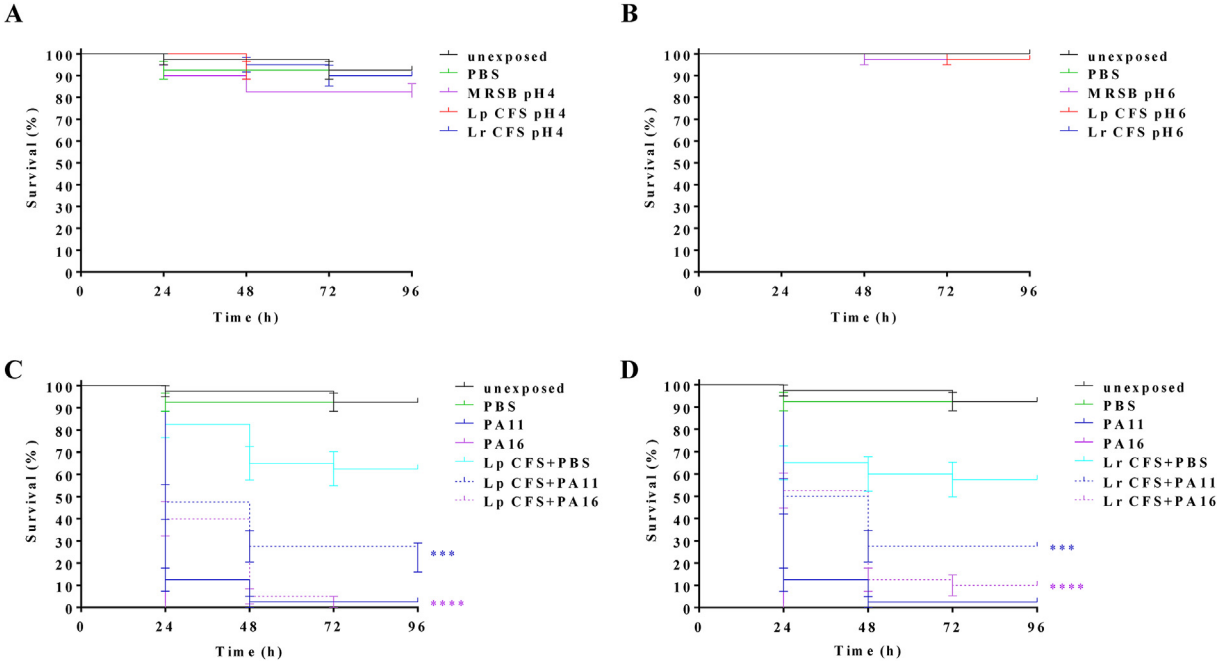

Fig. 6. A, B) *In vivo* toxicity of *L. plantarum* and *L. rhamnosus* M CFS in *Galleria mellonella*. Larvae (n = 20/group) were administered by intrahemocoel injection (i.i.) with 10 μl of *L. plantarum* (Lp) or *L. rhamnosus* M (Lr) CFS at **A**) pH 4 and **B**) pH 6, and the mortality was scored over 96 h. Control larvae were administered with MRSB corrected to the respective pH values. Negative control larvae were administered with PBS only. Results are shown as Kaplan–Meier survival plot from two experiments (n = 40). No toxic effect was observed regardless of pH tested, as assessed by Log-rank Mantel–Cox test. **C, D)** *In vivo* protection of CFS against *P. aeruginosa* infection: prophylactic studies. Larvae were administered by i.i. with **C**) Lp and **D**) Lr CFS at pH 4. After 2.5 h, larvae were infected by i.i. with PA11 and PA16 strains, each at LD_{50} (10^5 and 10 CFU/larva, respectively). Control larvae group: i) not challenged with CFS and *P. aeruginosa* (unexposed); ii) administered with CFS, and 2.5 h later with vehicle (Lp CFS + PBS, Lr CFS + PBS); and iii) administered with vehicle only (PBS). Results are shown as Kaplan–Meier survival plot from two experiments (n = 40). Statistical significances at the Log rank Mantel–Cox test: $***p < 0.001$ (Lp CFS + PA11 *vs.* PA11, and Lr CFS + PA11 *vs.* PA11); $****p < 0.0001$ (Lp CFS + PA16 *vs.* PA16, and Lr CFS + PA16 *vs.* PA16).

culture supernatant is, in turn, dependent on both the lactobacilli species and the medium type [14]. In the present study, for the preparation of CFS, lactobacilli were grown in the commonly used MRSB which contains glucose as the sole source of fermentable carbohydrates. It has been previously reported to promote the production of several organic acids by lactobacilli (e.g., lactic, acetic, formic, citric, succinic, and glutamic acid) at greater extents than other media [14]. Of note, when the different CFS were screened for their inhibitory activity against *P. aeruginosa*, the more acidic they were, the more they showed activity, suggesting that at least part of the observed growth inhibitory effect was due to the acids released in the supernatants during lactobacilli growth. Susceptibility of *P. aeruginosa* to organic acids has been previously reported, including strains resistant to multiple antibiotics [15]. Exposure to acidic pH may profoundly affect many microbial cell functions, including nutrient acquisition, energy generation, pH homeostasis, and protection of cell components [16]. In addition, in Gram-negative bacteria, lactic acid has been demonstrated to permeabilize the outer membrane, sensitizing the bacteria to the action of detergents (e.g., sodium dodecyl sulfate, Triton X-100) or lysozyme, suggesting that it may also act as a potentiator of the effects of other antimicrobials [17].

The antibacterial effect of CFS was almost completely abolished when the same CFS was adjusted to pH 6 with NaOH while it was mostly preserved following heating, supporting the organic acid-dependent inhibitory effect. Nevertheless, MRSB acidified with HCl at pH 4 exerted much milder inhibitory and killing effects than CFS at similar pH, suggesting that the acid environment *per se* is not the only one responsible for the antibacterial effect. Indeed, it is believed that the antibacterial activity of CFS from lactic acid bacteria is multifactorial and that metabolite other than organic acids may account for the inhibitory effects [18,19]. These include hydrogen peroxide, bacteriocins, or other compounds, some of which may require an acidic pH for optimal activity. For instance, some bacteriocins have been shown to increase their antibacterial activity at low pH [20]. Evidence also indicates that high lactic acid concentrations sensitize Gram-negative bacteria, normally not susceptible, to bacteriocins like nisin [17,21]. This might be due to a destabilizing effect of lactic acid on the outer membrane of Gram-negative bacteria, favoring bacteriocins reaching their main target represented by the cytoplasmic membrane [17].

The discrepancy in the antibacterial potency between acidic CFS and MRSB adjusted to a similar pH with HCl may also imply that, at a given pH, different acids exert different actions against bacteria. For instance, it has been reported that permeant weak acids like lactic acid are more effective than non-permeant strong acids like HCl, as they can pass through the membrane lipid bilayer and dissociate in the bacterial cytoplasm, lowering the internal pH [16].

To better explore the CFS therapeutic potential in conditions more closely resembling the CF lung, in the present study, we proceeded to assess the antibacterial activity of CFS from *L. plantarum* and *L. rhamnosus* M in an ASM, which mimics the nutritional environment of the CF lung better than conventional laboratory growth media, as recently reviewed by Aiyer & Manos [22]. Such medium contains mucin, DNA, amino acids, and a chelant agent and was recently slightly modified by us by the addition of glucose at the mean concentration found in the sputum of CF patients [11]. Furthermore, the pH of ASM is 6.8, resembling the mean acidic environment reported for the CF lung [23]. Many ASM components have been reported to hamper the activity of conventional antibiotics via different mechanisms [10]. For instance, mucin and extracellular DNA render the sputum highly viscous, leading to lower antibiotic diffusion. Mucins can also interact with antibiotics (e.g., aztreonam, ceftazidime, and levofloxacin) through hydrophobic interactions reducing their bioavailability. The high amino acids content of the CF sputum may lead to the selection of auxotrophic strains with mutations involving *LasR*, a transcriptional activator of *P. aeruginosa* virulence genes, also engaged in antibiotic tolerance [10,24]. Interestingly, our study demonstrated that in the presence of ASM, both CFS tested maintained a strong and fast killing activity against non-mucoid and mucoid strains of *P. aeruginosa* in planktonic form, with an evident reduction in the bacterial load starting from 10 min of incubation. A marked activity was also detected against *P. aeruginosa* biofilms, representing the preferred bacterial growth mode during chronic lung infection. In this case, the killing kinetics were slower than against planktonic bacteria, an observation compatible with the barrier effect played by the biofilm extracellular matrix that we recently demonstrated being abundantly produced in the presence of ASM [11]. In non-pathological conditions, the airway surface liquid (ASL) pH varies from 6.85 to 7.65 but, in many respiratory diseases, the pH homeostasis is altered and the ASL pH varies from ~4.5 to 8.5 [23]. Although still a matter of debate, in CF the pH of ASL appears to be acidic, especially during exacerbations (characterized by a worsening of clinical symptoms and a decline in lung function), when the decrease in pH might be even greater than in the CF stable disease (5.30 *vs*. 5.77) [23]. It is tempting to speculate that the acidic lung environment of the CF lung, which impairs the antimicrobial activity of standard-of-care antibiotics against *P. aeruginosa,* could instead help to preserve the acid-dependent CFS activity at the site of infection.

The pathogenicity of *P. aeruginosa* involves the production of several virulence factors - such as protease, pyocyanin, elastase, and exotoxins - regulated by a cell density-dependent signaling mechanism known as quorum sensing (QS) [25,26]. The global QS regulatory network consists of four interlinked circuits, i.e., *las*, *rhl*, *pqs*, and *iqs* [27]. In addition to the *las* circuit's hierarchical role in activating the other QS circuits, it modulates genes encoding elastase, proteases, and exotoxin A [28].

Recent research highlights the potential of QS quenching for an antivirulence therapeutic approach, alternative or in addition to antibiotics to control infections caused by MDR *P. aeruginosa* strains [29–31]. In this frame, a few studies have previously shown that probiotics have properties that inhibit the growth and virulence factors in *P. aeruginosa*, mainly attributable to the production of organic acids [29,32,33]. In the present study, the potential of CFS from *L. plantarum* and *L. rhamnosus* M to affect the virulence of *P. aeruginosa* was investigated at phenotypic and transcriptional levels.

Phenotypic data indicated that CFS from both probiotics significantly affects protease, pyoverdine, and pyocyanin formation by *P. aeruginosa* in a concentration-dependent manner. These findings could be particularly relevant in the management of CF patients. Pyocyanin, a blue-pigmented, redox-active phenazine compound - is relevant for acute and chronic lung infections [34,35], and its levels are negatively correlated with lung function in CF patients [36]. Pyoverdine, a siderophore for scavenging iron, which also acts as a signaling molecule for the expression of virulence factors, is necessary for the survival and virulence of *P. aeruginosa* in CF lungs [37,38].

Finally, proteases are key factors in establishing acute *P. aeruginosa* pneumonia, and elevated levels and impaired anti-protease defenses contribute to tissue destruction in the CF lung [39].

In agreement with our findings, previous studies observed that *L. plantarum* CFS possesses pro-healing and virulence factor inhibition properties (elastase, pyocyanin, biofilm, QS, rhamnolipids) in *P. aeruginosa* [40,41]. A wide variety of bioactive constituents obtained from the cell-free culture of *L. plantarum* have been identified. Notably, the postbiotic diketopiperazine 3-isobutyl 2,5

piperazinedione has been shown to interact with the QS receptors, thus preventing the binding of effector molecules to DNA and finally interfering with the transcription of virulence genes [40,42]. To our knowledge, the antivirulence findings we reported herein are the first focused on *L. rhamnosus*.

Our data on the transcriptional profile of *P. aeruginosa* revealed a complex pattern, with no evident consistent trends in the expression of the virulence genes tested, suggesting that *P. aeruginosa* strains may amend their virulence-related responses at the transcriptional levels depending on the probiotic, with the exception for *lasI* which was down-regulated in most of *P. aeruginosa*/probiotic strain combinations, thus indicating that both CFS has the potential to interfere with *lasI* QS circuit. In addition, the general discrepancy between the regulation of virulence genes and the phenotypic findings suggests a complex mechanism where postbiotics might act at a post-transcriptional level.

Previous studies used *G. mellonella* as an *in vivo* model to show the protection conferred by probiotic microorganisms against gastrointestinal pathogens [43], periodontopathogenic bacteria [44], and *P. aeruginosa* burn wound infection [45]. In the current study, for the first time, CFS from *L. plantarum* and *L. rhamnosus* M were tested for their toxicity and *in vivo* ability to prevent or treat infection caused by mucoid and non-mucoid CF *P. aeruginosa* in *G. mellonella*. Our findings showed that acidic CFS from *L. plantarum* and *L. rhamnosus* M has a protective potential against infections caused by mucoid and non-mucoid *P. aeruginosa* strains. These results are promising and, therefore, warrant additional studies to clarify the underlying mechanisms of action. No protective effect was observed after administration of *L. plantarum* CFS in *G. mellonella* larvae previously infected with *P. aeruginosa*.

The exact mechanisms of postbiotic activities demonstrated in the present study remain to be fully elucidated. Ongoing studies in our laboratories, aimed at subdividing CFS into several fractions based on the molecular weight, revealed that most of the bactericidal effect is retained in the low molecular weight fraction (<3 KDa) (our unpublished observation) which is the one likely containing small organic acids. This observation, together with the heat insensitiveness of CFS demonstrated in the present study, supports the dominant role played by organic acids in the antibacterial/antibiofilm effect, in agreement with previous reports [46,47]. Further studies performed by subjecting the different CFS fractions to heat treatment, catalase, proteolytic treatments or organic acid-acidification will help to identify the active components responsible for the antibacterial/antibiofilm effects, and whether these components differ from those responsible for the antivirulence effects.

5. Conclusions

Overall, the results presented highlight the translational potential of lactobacilli CFS against pulmonary pathogens in the context of the lung environment, supporting the current view that probiotics or their metabolites might represent a promising strategy for the prevention and/or treatment of respiratory infections [48–50].

Nevertheless, much work must be done to implement postbiotics in the clinic. For instance, a fine characterization of the bioactive substances produced by different probiotic strains, responsible for the antibacterial and/or antivirulence properties against site-specific pathogens, may help in designing optimized therapeutic cocktails. The efficacy and safety of postbiotics should also be thoroughly investigated in multicellular-based *in vitro* models - that recapitulate the aspects of the complex structure and function of the corresponding *in vivo* tissue - and animal models. Adjuvant and/or synergistic approaches with conventional antibiotics are also possible strategies to explore.

Lastly, the use of nanotechnologies to encapsulate molecules from probiotic extracts in proper polymeric matrices with the aim of improving their efficacy, delivery, and biocompatibility at the infection site holds promise in unveiling innovative postbiotics' uses in anti-infective therapy.

Author contributions

Conceptualization, AP, GM, SE, GDB, and GB; methodology, AP, EK, VL, EC, MB, GM; formal analysis and validation, AP, EK, VL, EC, MB, GM, SE, GDB and GB; investigation AP, EK, VL, EC, MB; data curation, AP, EK, VL, EC, MB, GM, SE, GDB and GB; writing-original draft preparation, AP, GDB and GB; writing-review and editing, AP, EK, VL, EC, MB, GM, SE, GDB and GB; supervision, AP, GM, SE, GDB, and GB; funding acquisition, AP and GB.

Data availability statement

The data presented in this study are available on request from the corresponding authors.

Declaration of competing interest

The authors of this article declare that they do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105301.

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