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1 **Synergy between *Lactobacillus murinus* and anti-PcrV antibody delivered in the airways to**
2 **promote protection against *Pseudomonas aeruginosa***

3
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13
14 **Running title: Airway delivery of probiotic and therapeutic antibody against *P. aeruginosa***

15
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19

20 **Abstract**

21 Therapeutic antibodies (Ab) have revolutionized the management of multiple illnesses including
22 respiratory tract infections (RTIs). However, anti-infectious Ab displayed several limitations
23 including antigen restrictiveness, narrowed therapeutic windows and limited dose in the vicinity
24 of the target when delivered by parenteral routes. Strategies enhancing further Ab-dependent
25 containment of infection is currently needed. Here we showed that a combination of inhaled anti-
26 infectious Ab and probiotics is an efficient formulation to protect against lung infection. Using a
27 mouse model of *Pseudomonas aeruginosa* -induced pneumonia, we demonstrated a synergistic
28 effect reducing both bacterial burden and pro-inflammatory response affording protection against
29 primary and secondary infections. This is the first study showing that the local combination in
30 the airways of anti-infective Ab and probiotics, subverts suboptimal potency of Ab monotherapy
31 and provides protection against respiratory pathogen.

32

33 **Introduction**

34 Respiratory tract infections (RTIs) are one of the leading causes of morbidity and mortality
35 worldwide ¹. RTIs are mostly caused by virus and bacteria and are associated with a significant
36 economic burden. On top of that, the global decline of antibiotic and antivirals effectiveness is
37 recognized as a major threat to human health, by the WHO ^{2,3}, urging the necessity to develop
38 innovating therapeutic strategies. Therapeutic antibodies (Abs) are used for the treatment of
39 various pathologies including respiratory diseases, with important clinical successes ⁴.
40 Moreover, Abs, as a prophylactic or post-exposure treatment, are considered potential game
41 changers for the containment of respiratory infections. Besides Abs, probiotics have emerged as
42 a promising strategy for treating or preventing respiratory tract infections ⁵. Probiotics are
43 defined as live bacteria that can confer health benefits to the host when administered in
44 adequate amounts ⁶. Probiotics have been proposed to reduce infection rates and the risk of
45 ventilator-associated pneumonia (VAP) in critically ill patients ^{7,8}. In fact, patients under
46 mechanical ventilation exhibit lung microbiota dysbiosis with increased and low diverse
47 commensal populations precipitating lung infection and pneumonia ⁹. Their beneficial effects
48 have been postulated to include enhanced mucosal barrier function, restoration of microbial
49 diversity in established microbiota, modulation of the host inflammatory response and
50 competitive exclusion of pathogens ^{10,11}. Historically, the lungs have been considered as a
51 sterile organ ¹² until recent high-throughput sequencing of bronchoalveolar lavage and
52 pulmonary samples revealed the presence of a core microbiota dominated by *Streptococcus*,
53 *Prevotella*, and *Veillonella* genus. Despite a low biomass, as compared to gut microbiota,
54 airway bacterial communities are involved in critical processes in respiratory health including
55 protection against infections ^{13,14}. In this context, the therapeutic potential of lung microbiota
56 correctors allowing beneficial microbes to outcompete RTI-associated pathogens may reduce
57 lung inflammation and improve respiratory health ^{15,16}. However, definitive demonstration of

58 clinical effectiveness of probiotic against respiratory pathogens is still missing while studies
59 also reported negative results¹⁷ requiring optimized treatment strategies.

60 Abs targeting microbial antigens are usually administered through systemic routes though their
61 beneficial effects are expected in the airways and only a small fraction reaches this
62 compartment after i.v. injection. Over the past decade, we have demonstrated that inhalation
63 constitutes an attractive and feasible alternative route for the delivery of full-length Abs into the
64 lungs¹⁸⁻²¹. Abs delivered through the airways – including mAb166, used in this study – exhibit
65 therapeutic responses and pass poorly and slowly into the systemic circulation²². mAb166 is a
66 murine monoclonal IgG2b, κ antibody targeting PcrV, which have been shown to prevent
67 infection in both mice and rat lung infected with *P. aeruginosa*^{23,24}.

68 Overall, our findings, supported by other groups, indicate that inhalation may have the edge
69 over other routes of administration if Abs are intended to operate into the respiratory tract to
70 fight respiratory infections²⁵⁻²⁸. Similarly, airway instead of oral application of probiotics
71 would achieve a better therapeutic index while exhibiting less systemic effects⁵. In addition,
72 combined use of probiotics with other anti-infectives may improve clinical benefit through
73 mechanistic synergy.

74 Here, we hypothesized that a combination of anti-infective Ab and probiotics delivered
75 locally into the lungs may better prevent lung infection, than Ab monotherapy. We showed that
76 airway delivery of a neutralizing Ab and *Lactobacillus murinus*, a probiotic isolated from
77 mouse lungs and displaying immunomodulatory properties²⁹, provided a synergistic protection
78 against *Pseudomonas aeruginosa*, a bacterium ranked as a high priority by the World Health
79 Organization². Overall, the combination limited both bacterial burden and pro-inflammatory
80 response. Remarkably, this combination promoted a long-lasting protection against subsequent
81 infections. This is the first study showing that the local combination **in the airways** of anti-

82 infective Ab and probiotics, subverts suboptimal potency of Ab monotherapy and provides
83 protection against respiratory pathogen.

84

85 **Results**

86 *Dose-dependent protection conferred by airway administered anti-*P. aeruginosa* antibody.*

87 We have recently demonstrated that airway administration of mAb166, a murine IgG2b
88 recognizing PcrV and neutralizing type 3 secretion system (T3SS) provided protection from a
89 lethal pulmonary infection with *P. aeruginosa*²². Here, we wanted to establish a model of
90 suboptimal mAb166 efficacy. As shown in Figure 1, mice treated with 100µg of mAb166 were
91 fully protected from this lethal dose while those treated with 50µg experienced mild symptoms,
92 including significant body-weight loss and evident signs of infection (ruffled fur, hunched
93 posture and motor impairment), and were partly protected from the infection, as outlined with
94 ~50% of animal survival (Figures 1B and 1C). Thus, this model (Figure 1A) offers a therapeutic
95 window for the evaluation of combined airway treatment to reinforce Ab efficacy.

96

97 *Intranasal application of *Lactobacillus murinus* is tolerable in mice.*

98 We previously described the immunomodulatory properties of *Lactobacillus* species
99 isolated from lung homogenates of SPF neonatal mice, which mitigated type 2 immunity and the
100 extent of allergic asthma in mice²⁹. In the perspective of using probiotics as anti-infectious
101 strategies in combination with Abs, we investigated the effects of daily intranasal applications of
102 2 strains of *L. murinus* among our collection of pulmonary bacteria (CNCM I-4967 and CNCM
103 I-4968), following the protocol described in Figure 2A. First, we analyzed the extent of
104 probiotics colonization in lungs according to the inoculum size. The two strains displayed a
105 transient, short-term persistence in the airways or in the parenchyma and only for the highest
106 bacterial load (Figures 2B and 2C). Even if strains from the *Lactobacillus* genus are Generally
107 Regarded As Safe (GRAS), we analyzed their impact after intranasal delivery into the lungs. We
108 did not observe an impact on mice body weight (Figures 2D and 2E) nor clinical symptoms (data
109 not shown), whatever the inoculum size. This agrees with the absence of inflammation and

110 cytotoxicity *in vitro* when the probiotics were co-incubated with human bronchial epithelial
111 BEAS-2B cells (Figure S1). However, we observed a significant and dose-dependent recruitment
112 of neutrophils in the airways of mice treated with the strain CNCM-I 4967 and, to a lower and
113 non-significant extent with the strain CNCM-I 4968, 24h after the last administration (Figures 2F
114 and 2G). This was accompanied with an increase of Ly6C⁺ monocytes and concomitant decrease
115 of alveolar macrophage (Figure S2). Overall, our results indicate that intranasally delivered
116 probiotics up to 10⁷ cfu were well-tolerated by mice. For the rest of the study, the lowest dose
117 (10⁵ cfu) of probiotic was selected to avoid confounding immunogenicity during its assessment
118 in combination with anti-infective Abs.

119
120 *Synergistic effect of airway-delivered Lactobacillus murinus and anti-infective antibody in P.*
121 *aeruginosa-induced pneumonia.*

122 In the absence of adverse effects, combination strategy using two antipseudomonal drugs
123 may be useful against pneumonia^{30,31}. Thus, we evaluated the combination of mAb166 and
124 probiotic strains CNCM-I 4967 or CNCM-I 4968 according to the protocol described in Figure
125 3A. Animals that received either strain CNCM-I 4967 or CNCM-I 4968 were not protected and
126 died within 1-2 days (Figure 3). Notably, mice treated with a suboptimal dose mAb166 (~40%
127 of survival) and probiotics survived better the infection. However, the animals reacted differently
128 to the infection depending on the probiotic strain. Animals that received mAb166 and strain
129 CNCM-I 4967 developed a less severe disease, as illustrated by improved survival and
130 diminished weight loss (Figures 3B and 3D), while mice treated with mAb166 and strain CNCM-
131 I 4968 had similar disease progression and severity as mAb166-only treated animals (Figures 3C
132 and 3E). This suggests different intrinsic properties of the two probiotics.

133

134 *Improved host anti-bacterial response afforded by airway-delivered Lactobacillus murinus and*
135 *anti-infective antibody.*

136 To determine the mechanisms underlying the protection associated with the combined
137 treatments, we analysed the lung bacterial load and local inflammatory response. All groups that
138 received mAb166 (alone or in combination) had a significant reduction of *P. aeruginosa* PA103
139 load in the lungs, as anticipated (Figures 4A, 4B, 4F and 4G). Based on agar-well diffusion assay
140 and competition assay, we can rule out that the control of *P. aeruginosa* burden is attributable to
141 a direct effect of probiotics on *P. aeruginosa* PA103 as CNCM-I 4967 and -I 4968 did not
142 exhibit a significant ability to inhibit *P. aeruginosa* PA103 growth *in vitro* as compared to
143 control antibiotic **or medium**, even if the probiotics were live, heat-killed or considering **soluble-**
144 **derived factors (Figure S4)**. Next, we analysed the recruitment of neutrophils – which is the main
145 leukocyte subtypes engaged in the lungs to control invading pathogens - in the airways and lung
146 parenchyma^{32,33}. After 24 hours of infection, there was significantly more neutrophils in both
147 broncho-alveolar lavage (BAL) and lungs of animals that received mAb166 and strain CNCM-I
148 4967, as compared to mAb166 alone and controls (Figures 4C and 4D). Neutrophilic-dependent
149 anti-bacterial response is necessary for controlling *P. aeruginosa* infection, however a protracting
150 and non-resolving inflammatory response may be deleterious in experimental lung infection
151 models³⁴. IL6 – a prototypal pro-inflammatory cytokine – was significantly reduced in the
152 broncho-alveolar lavage of the **CNCM-I 4967-treated groups** as compared to controls (Figure
153 4E). The better control of inflammation in mAb166 and CNCM-I 4967 strain-treated group may
154 partially account from probiotic treatment as CNCM-I 4967 significantly and dose-dependently
155 reduced TNF-induced inflammation in BEAS-B cells **(Figure S3A) which is not the case for**
156 **CNCM-I 4968 (Figure S3B)**. Interestingly, neither neutrophil recruitment nor local inflammation
157 were significantly impacted by strain CNCM-I 4968 when combined with mAb166 (Figures 4F-J
158 and **Figure S3B**). Altogether, our results suggest that the combination of probiotics and anti-

159 infective Ab administered locally in the airway improved host anti-*P. aeruginosa* immune
160 responses promoting subsequent infection control after primary exposure. This effect was
161 specific to the probiotic strain. From now, the molecular determinants accounting for this
162 protection remains to be addressed.

163
164 *The combination of airway-delivered Lactobacillus murinus and anti-infective antibody also*
165 *promoted long-term protection against P. aeruginosa.*

166 We have recently demonstrated that airway administration of the anti-*P. aeruginosa*
167 antibody mAb166 promoted genuine innate and adaptive immune responses conferring
168 protection from a secondary bacterial infection³⁵. The long-term protection was dependent on
169 the amount of mAb166 with 100 µg providing 100% animal survival after the secondary
170 infection while 50µg was suboptimal with 50% of survival (Figure S5). Here, we investigated the
171 combination of probiotics with suboptimal dose of mAb166 on the protection to secondary
172 infection, according to the protocol described in Figure 5A. All mice treated with the
173 combination of CNCM-I 4967 and mAb166 survive after a secondary infection by *P. aeruginosa*,
174 while 1 mouse over 5 in the CNCM-I 4968-mAb166 group died (Figures 5B and 5C). This
175 modest but significant improvement of survival was associated with induction of a memory anti-
176 *P. aeruginosa* humoral response, an immunological hallmark of long-term protection³⁵, with
177 mice treated with CNCM-I 4967 and mAb166 producing more anti- *P. aeruginosa* PA103 IgG
178 after the challenge – but not the priming – as compared to the other groups (Figures 5D and 5E).
179 Even if these results need to be confirmed in bigger cohort, they confirmed previously published
180 work using optimal mAb166 monotherapy³⁵.

181

182 **Discussion**

183 Despite considerable investments in research and development over recent years, only
184 few anti-infective Abs have been approved so far. Several explanations may account for Ab's
185 suboptimal capacity to treat respiratory pathogens. First, most anti-infective antibodies are
186 monovalent, binding to one antigen, which may be insufficient to neutralize pathogens
187 expressing a vast array of virulence factors or regulating the expression of their antigens during
188 infection. Secondly, the therapeutic window is critical for the efficacy of Ab immunotherapy,
189 Abs working best when they are administered immediately after diagnosis. Finally, the dose of
190 Ab reaching the infected tissue is paramount for effectiveness. Direct delivery of anti-infective
191 Abs into the lungs, by inhalation, is a promising alternative to conventional intravenous route of
192 administration of Abs for the treatment of pulmonary diseases. Our group and others have
193 demonstrated enhanced airway concentration, increased onset of action and limited systemic
194 exposure associated with Ab delivery into the lungs¹⁸⁻²¹. Moreover, airway-delivered Abs were
195 associated with better therapeutic response in various models of respiratory infections^{25,27,28,36}.
196 Despite these theoretical advantages of local administration for Ab by inhalation, few of these
197 benefits have materialized in the clinics.

198 Several evidence in the literature suggest that combining Ab treatments with other
199 therapies might strengthen pathogen containment, as illustrate for Abs and antibiotics against
200 respiratory pathogens^{31,37,38}. Here, we tested the combination of anti-infective Ab with probiotics
201 from the genus *Lactobacillus*, as they have shown to harness lung responses to promote
202 sterilizing immunity as well as inducing long-term adaptive responses³⁹⁻⁴¹. Based on the
203 literature showing the benefits of local delivery of Ab to treat respiratory infections⁴²⁻⁴⁴, both the
204 Ab and the probiotics were delivered locally into the lungs. To our knowledge, our study is the
205 first to examine the effects of intranasal administration of probiotics in combination with inhaled
206 therapeutic antibody against *P. aeruginosa* infection.

207 In this study, probiotics (CNCM-I 4967 and 4968) were commensals isolated from a
208 neonatal mouse lung²⁹. While probiotics and especially *Lactobacillus* have proven safety when
209 administered by the oral route^{45,46}, only scarce data are available after application in the
210 respiratory tract. This is of particular importance when considering the usual large dose of
211 probiotic ($\sim 10^8$ cfu) – necessary to overcome colonization resistance⁴⁷ – with the total biomass of
212 the lung microbiota, estimated to be around 10^3 - 10^5 cfu. When given intranasally, we showed that
213 *Lactobacillus* dose-dependently induced airway inflammation exemplified by the increased
214 recruitment of neutrophils and the concomitant disappearance of alveolar macrophage. For the
215 highest dose, we observed very limited amount of probiotic remaining in the respiratory tract 24h
216 after the last administration suggesting the absence of durable lung colonization. Even if
217 preclinical²⁹ and clinical data⁴⁸ showed an association between lactobacilli colonization of the
218 airways and protective effect against asthma, it remains uncertain whether the intranasal
219 administration of *Lactobacillus* may interfere with lung microbial homeostasis which could
220 induce severe outcomes^{49,50}. For these reasons, we selected a mild quantity of probiotics, to be
221 tested in combination with the Ab, that does not affect body weight nor induce any visible
222 symptoms, assuming tolerability both *in vivo* and *in vitro* and that does not provide protection
223 *per se* against pulmonary infection.

224 It is now well established that commensal microbes can control the
225 maturation/functionality of immune cells and modulate the outcome of the immune response⁵¹.
226 Despite numerous data supporting the use of probiotics in treating gastrointestinal diseases, much
227 less is known about their impact in respiratory diseases. Here, we observed a better clearance of
228 lung *P. aeruginosa* 24h after infection associated with an enhanced recruitment of neutrophils
229 and a diminished pro-inflammatory response when animals were treated with CNCM-I 4967 and
230 mAb166. Massive lung neutrophil recruitment is a shared downstream mechanism of a protective
231 anti-*P. aeruginosa* immunity and is even more important than the contribution of other immune

232 cell types ^{33,52}. Indeed, a favourable outcome of *P. aeruginosa* – and more generally lung
233 bacterial – infection necessitates a controlled neutrophil recruitment which will clear the
234 pathogen and promote survival without excessive inflammation. In fact, during pneumonia, a
235 protracted inflammation can lead to exacerbated tissue injury which is ultimately deleterious for
236 the host ⁵³. Our *in vivo* and *in vitro* results indicate that improved control of *P. aeruginosa* load
237 by combined administration in the airways of probiotic and Ab results in reduced pathogen-
238 mediated inflammation associated with improved survival. Exclusion of pathogens by probiotics
239 is known to be mediated by their ability to compete for nutrients, produce bacteriocin or
240 modulate the micro-environment ^{54,55}. In our study, we did not observe any direct bactericidal
241 effect of CNCM-I 4967 over *P. aeruginosa* nor any competitive effect suggesting that its positive
242 effect may rely on immunomodulation, as shown for other *Lactobacillus* strains ¹³.

243 We have recently demonstrated that the local delivery of Abs into the lungs was
244 associated with the promotion of long-term protection against *P. aeruginosa* ³⁵. However, the
245 vaccine-like effect mediated by mAb166 was dependent on the dose of the bacteria and Ab,
246 which is not satisfactory to translate into the clinic. The analysis of the immune response
247 suggested that the intensity/specificity of the immune response was critical for an optimal Ab-
248 mediated long-term protection against *P. aeruginosa* ³⁵. Here, we showed that intranasal
249 administration of probiotics compensates the efficacy of suboptimal concentration of mAb166
250 providing a better long-term protection against RTI-associated pathogen, than Ab monotherapy.
251 As previously observed ³⁵, this may be associated with the induction of a memory adaptive
252 humoral response against *P. aeruginosa* which composition and specific protective potency
253 remains to be determined. Increasing preclinical and clinical evidence indicate that the
254 microbiota modulates responses to Abs through interactions with the host immune system ^{56,57}.
255 Accordingly, we hypothesize that *Lactobacillus* probiotics might potentiate the immune response
256 through the improvement of neutrophil recruitment and the control of the inflammatory response

257 and act synergistically with the neutralizing anti-infective Ab. The fact that intranasal
258 *Lactobacillus* had immunomodulatory functions in lung infection models supports further this
259 hypothesis^{13,58,59}. Several studies have suggested that probiotics may activate specific T cell
260 responses against microbial antigens which either potentiate pathogen-specific immune
261 activation or may even induce heterologous protective responses⁶⁰⁻⁶³.

262 The prevention or treatment of respiratory tract infections has been complicated after
263 decades of excessive use and misuse of broad-spectrum antibiotics and the epidemic increase in
264 antibiotic resistance. This has significantly reduced the effectiveness of treatments, altering
265 patient outcomes and placing considerable pressure on overburdened healthcare systems. The
266 prevalence of *P. aeruginosa* infections has increased over the past decade and has become a
267 major concern in the hospital environment. Indeed, *P. aeruginosa* is one of the most common
268 etiologic agents of intensive care unit infections and is among the leading causes of ventilator-
269 associated pneumonia (VAP) due to mechanical ventilation, trauma or antecedent viral infections
270 with a significantly higher attributable mortality as compared to other pathogens^{64,65}. *P.*
271 *aeruginosa* is also intrinsically associated with chronic pulmonary diseases and antibiotic
272 resistance⁶⁶. Finally, resolving pneumonia requires not only to fight against airway
273 contamination by exogenous pathogen, but also against ecological dysbiosis⁹ that fuels long-
274 lived lung dysfunctions⁶⁷. In this context, the development of innovative targeted host-directed
275 immunotherapy holds great promise. Additional experiments will be necessary to provide
276 mechanistic insights on the synergistic anti-bacterial effects of probiotic and therapeutic Ab
277 administered locally into the airways after both primary and secondary infections, and to extend
278 these results to other infectious agents and Abs. However, this strategy may offer novel
279 preventive or therapeutic opportunities against pneumonia.

280

281 **Materials and methods**

282

283 *Mice*

284 Adult male C57BL/6j (B6) mice (6-8 weeks old) were obtained from Janvier (Le Genest Saint-
285 Isle, France). All mice were housed under specific-pathogen-free conditions at the PST
286 “Animaleries” animal facility (Université de Tours, France) and had access to food and water *ad*
287 *libitum*. All experiments complied with the French government’s ethical and animal experiment
288 regulations (APAFIS#7608-2016111515206630).

289

290 *P. aeruginosa* infection

291 PA103 (ATCC 33348) *Pseudomonas aeruginosa* strain, kindly provided by Pr. Teiji Sawa
292 (Kyoto University, Japan) was used in this study. The uniformity of the colonies was checked by
293 plating on *Pseudomonas* isolation agar (PIA) plates. PA103 has been transformed by
294 quadriparental mating by a mini-Tn7T transposon encoding allowing a constitutive expression of
295 the *LuxCDABE* operon. Bacteria were prepared as previously described ²⁷. Mice were
296 anesthetized with isoflurane 4% and an operating otoscope fit with intubation specula was
297 introduced to both maintain tongue retraction and visualize the glottis. A fiber optic wire
298 threaded through a 20G catheter and connected to torch stylet (Harvard Apparatus, France) was
299 inserted into the mouse trachea. Correct intubation was confirmed using lung inflation bulb test
300 and 40µL of the bacterial solution at 3.10^5 cfu was applied using an ultrafine pipette tip.
301 Inoculum size for infections were confirmed by counting colony-forming unit (cfu) on PIA
302 plates. Mortality and body weight of animals were monitored daily. In all experiments, moribund
303 animals, or animals with a weight loss of more than 20% were sacrificed for ethical reasons and
304 considered as dead animals due to the infection. For long-term experiments, an additional control

305 group of mice which were solely infected with *Pseudomonas aeruginosa* PA103 was added to
306 confirm the lethality of the inoculum.

307

308 *Antibody administration*

309 mAb166 was generated from PTA-9180 hybridoma (LGC Standards, France) and supplied as
310 sterile, non-pyrogenic, PBS solution under good manufacturing practice (BioXcell, USA).
311 mAb166 was administered 1 hour after the infection, at the dose of 100µg or 50µg (suboptimal
312 quantity) in 50µL of PBS using a Microsprayer® aerosolizer (Penn-Century, US) introduced
313 orotracheally, as described in the previous section.

314

315 *Probiotic administration*

316 Probiotics strains used in this study were previously isolated from neonatal mouse lung
317 homogenate ²⁹. They were deposited at the French National Collection of Microorganism
318 Cultures (CNCM) under the name CNCM I-4967 and CNCM-I-4968 (patented strains). Based on
319 its genomic sequence (Illumina sequencing technology provided by Eurofins), both strains were
320 identified as *Lactobacillus murinus* or *Ligilactobacillus murinus* according to the most recent
321 taxonomy ⁶⁸. The genome analysis was performed using the PathoSystems Resource Integration
322 Center (PATRIC, Version 3.5.31, <https://www.bv-brc.org/>) and Rapid Annotation using
323 Subsystem Technology (RAST, Version 2.0, <https://rast.nmpdr.org/>). In PATRIC, the services
324 Assembly, Annotation, Comprehensive Genome Analysis and BLAST were used. In RAST, the
325 tools Subsystem Feature Count, Function-Based Comparison and Sequence-Based Comparison
326 were used. Starting from frozen stock solution at -80°C, strains were resuscitated on Man,
327 Rogosa, Sharpe (MRS)-agar plate and put in culture overnight in 13mL of liquid MRS in a 14mL
328 Falcon tube at 37°C, 5%CO₂ without shaking. Bacteria were washed once in PBS diluted in

329 saline, to obtain a titer of 10^5 bacteria/40 μ L. Mice were anesthetized with isoflurane 4% and
330 40 μ L of the bacterial solution or the corresponding vehicle solution was applied intranasally
331 using an ultrafine pipette tip. Mice were left to recover for 10 s in an upright position before
332 being put back in their cage. Each inoculum was then checked for accuracy and uniformity (by
333 direct plating on fresh MRS-plates).

334

335 *Broncho-alveolar lavage, organ sampling, bacterial load assay*

336 Broncho-alveolar lavage fluid (BAL) was collected after *P. aeruginosa* infection by introducing
337 a catheter into the trachea under deep pentobarbital anaesthesia and washing sequentially the
338 lung with 1 x 0.5 mL and 2 x 1mL of 1X PBS at room temperature. The lavage fluid was
339 centrifuged at 400 g for 10 min at 4°C and the supernatant of the first lavage was stored at -20°C
340 for analysis. The cell pellet was resuspended in PBS, counted in a haemocytometer chamber, and
341 used for subsequent analysis. Lungs were perfused with 10mL of PBS1X and harvested in
342 GentleMACS C tubes (Miltenyi Biotec, Germany) containing 2mL of RPMI medium
343 (Invitrogen, France) for flow cytometry or GentleMACS M tubes (Miltenyi Biotec, Germany)
344 containing 2mL of 1X PBS for microbiology assay. Bacterial load in lung homogenates or BAL
345 (before centrifugation) was determined by plating tenfold serial dilutions on PIA-agar or MRS-
346 agar. Plates were incubated at 37°C in a 5% CO₂ atmosphere, and the cfu were counted after 24
347 hours.

348

349 *Preparation of pulmonary immune cells*

350 Lung and spleen homogenates were prepared using a GentleMACS tissue homogenizer (Miltenyi
351 Biotec, Germany). Lung pieces were then digested in a medium containing 125 μ g/mL of
352 Liberase (Roche, France) and 100 μ g/mL of DNase I (Sigma, France) for 30min at 37°C under

353 gentle agitation. After washes, contaminating erythrocytes were lysed using Hybri-Max® lysis
354 buffer (Sigma, France) according to manufacturer's instructions. Samples were sequentially
355 filtered over 100µm and 40µm nylon mesh. After final wash, cell pellets were resuspended in
356 PBS containing 2 % FCS, 2mM EDTA and 1X murine Fc-block (Becton Dickinson, France) –
357 described elsewhere as FACS buffer.

358

359 *Flow cytometry*

360 Cells were incubated in FACS buffer for 20min at 4°C. Then, cells were stained in FACS buffer
361 for 20 min at 4°C with appropriate dilutions of following antibodies: CD45-APC-Cy7 (30-F11),
362 CD11b APC (M1/70), Ly6G FITC (1A8), CD11c PE (N418), Ly6C Pe-Cy7 (HK1.4) from
363 Biolegend and Siglec-F BV421 (E50-2440) from BD. Dead cells were stained with the
364 LIVE/DEAD Fixable Aqua Dead Cell Staining kit (ThermoFisherScientific) and acquired on a
365 MACS Quant (Miltenyi Biotec) cytometer. Analyses were performed using Venturi-One
366 software (Applied Cytometry; UK). Alveolar macrophages were defined as: CD45+ CD11c+
367 CD11b- SiglecF+ Ly6G- Ly6C-, neutrophils were defined as: CD45+ CD11c- CD11b+ SiglecF-
368 Ly6G+ Ly6C-, monocytes were defined as CD45+ CD11c- CD11b+ SiglecF+ Ly6G- Ly6C+.

369

370 *BEAS-2B Human Bronchial Epithelial Cell Challenge*

371 BEAS-2B human bronchial epithelial cell line was obtained from the American Type Culture
372 Collection (ATCC CLR-9609). The protocol of co-incubation was adapted from previous
373 publication¹⁴. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal
374 bovine serum (Eurobio, Les Ulis, France), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis,
375 MO, USA) and 1% L-glutamine (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) in 75
376 cm² tissue culture flask (Sarstedt, Nümbrecht, Germany) and incubated at 37 °C, in a 5% CO₂

377 atmosphere, in a humidified incubator. Cells were passaged before reaching 80% confluency,
378 using Trypsin-EDTA (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA). Cells were
379 treated, as indicated with 1 ng/mL TNF- α , and/or living 4967 and 4968 bacteria (MOI 1:1 and
380 1:10) in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine.
381 After 6 h of co-incubation, supernatants were collected to measure cytokines, lactate
382 dehydrogenase (LDH) release (cell viability control). Production of IL-8 in BEAS-2B culture
383 supernatants was examined using a commercially human IL-8 ELISA kit (Fisher Scientific)
384 according to the manufacturer's recommendation. The Colorimetric assay that quantitatively
385 measures lactate dehydrogenase (LDH) released upon cell lysis was CytoTox 96® (Promega).

386

387 *Agar Well Diffusion Method*

388 The agar-spot method was adapted from our previous studies^{14,69,70}. Briefly, the capacity of the
389 two CNCM-I 4967 and CNCM-I 4968 strains to inhibit the growth of *Pseudomonas aeruginosa*
390 PA103 was evaluated by measuring the diameter of inhibition of the culture relative to that of
391 antibiotic (amikacin sulphate, 30 μ g/well). A layer of 40 mL MRS agar was poured into a 12 cm
392 square Petri dish and allowed to cool down, then an equal volume (20 μ L) of each CNCM-I 4967
393 and CNCM-I 4968 overnight bacterial suspension was deposited on cellulose disks. A second
394 layer of 20 mL LB agar containing *P. aeruginosa* PA103 or PA14 (McFarland = 1) culture was
395 added on top. The plates were incubated at 37°C and the growth inhibition zones were measured
396 24 h later. The inhibition diameters obtained with 4967 and 4968 smaller to that of the reference
397 antibiotic control were considered to have a negative antagonistic effect.

398

399 *Competitive growth assay*

400 PA103 was grown in lysogeny broth (LB Lennox, Invitrogen) overnight at 37°C with shaking at
401 200 rpm. CNCM-I 4967 and CNCM-I 4968 were grown in 13mL of liquid MRS overnight in a
402 14mL Falcon tube at 37°C without shaking. The following preparations were used to evaluate
403 PA103 inhibition by probiotics: (i) whole-cell culture containing 106cfu/mL of CNCM-I-4967 or
404 4968, (ii) whole-cell culture containing 106cfu/mL of CNCM-I-4967 or 4968 after being heat-
405 killed (30min at 37°C), (iii) culture supernatants from whole-cell culture containing 106cfu/mL
406 recovered following centrifugation and filtration through 0.22µm filters and buffered to pH7.
407 1mL of both competing (CNCM-I-4967 or CNCM-I-4968 from different preparations), and
408 target strains (PA103) were inoculated together for 24h at 37°C without shaking as previously
409 described⁷¹⁻⁷³. PA103 CFU were enumerated, by serial-dilution in PBS and plated on selective
410 PIA agar plates for 24h at 37°C. As controls, the growth of competing strains was systematically
411 checked on MRS-agar plates. Here are the conditions tested: PA103 alone, PA103 co-cultured
412 with live CNCM-I-4967 or CNCM-I-4968, PA103 co-cultured with heat-killed CNCM-I-4967 or
413 CNCM-I-4968 (30min at 70°C), PA103 co-cultured with buffered supernatant (pH=7) from
414 CNCM-I-4967 or CNCM-I-4968.

415

416 *Cytokines measurements and IgG measurements*

417 *P. aeruginosa* IgG titers, in serum and BALF levels were measured by sandwich ELISA. Briefly,
418 high binding Immulon 96-well plates (ThermoFischer Scientific, France) were coated with
419 0.5µg/mL of *P. aeruginosa* PA103 lysate, prepared from overnight cultures that were then
420 sonicated, and diluted in bicarbonate buffer. The plates were then washed and blocked with
421 1%BSA-PBS. Serum or BAL samples were incubated for 2 h. A biotin-conjugated goat anti-rat
422 IgG antibody (Biolegend, France) was added for 2 h. After a washing step, peroxidase-
423 conjugated streptavidin (R&D) was added for 20min. Between each step, plates were thoroughly
424 washed in 0.05%Tween20-PBS. Tetramethylbenzidine was used as a substrate, and the

425 absorbance was measured at 450 nm using a microplate reader (Tecan, Switzerland). The titer
426 was calculated by binary logarithm regression as the reciprocal dilution of the sample, where the
427 extinction was 2-fold the background extinction. Cytokines concentrations in BAL were assessed
428 using a sandwich ELISA according to manufacturer's instructions (Biotechne, France) and
429 standardized over total BAL protein.

430

431 *Statistical analysis*

432 Statistical evaluation of differences between the experimental groups was determined by using
433 one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-test (which allows
434 comparison of all pairs of groups). Log-rank test was used for survival analysis. Student's t-test
435 was used for comparison between two groups and paired t-test was used when comparing the
436 same individual before and after challenge. All tests were performed with GraphPad Prism,
437 Version 8 for Windows (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com).
438 All data are presented as mean +/- standard error of the mean (SEM). A p- value <0.05 was
439 considered significant.

440 **Keywords:** *P. aeruginosa*, pneumonia, probiotic, airway delivery

441

442 **Data Availability Statement**

443 The data generated in this study are available upon request from the corresponding author.

444

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450

451 **Author Contributions**

452 TS and NHV conceived the study. All authors substantially contributed to the acquisition,
453 analysis or interpretation of data. MT provided probiotic strains and expertise in the analysis of
454 their immunomodulatory functions. TS, MT and NHV contributed to manuscript drafting,
455 revising and critically reviewing. All authors approved the final version of this manuscript to be
456 published.

457

458 **Competing interest statement**

459 TS, MC, CB, M.T BG, AP, and CP have nothing to declare. NHV is co-founder and scientific
460 expert for Cynbiose Respiratory. In the past two years, she received consultancy fees from Eli
461 Lilly, Argenx, Novartis and research support from Sanofi and Aerogen Ltd. MT is co-founder of
462 the start-up Carembouche.

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719 **Figure Legends**

720 Figure 1: Dose-dependent protection conferred by locally administered anti-*P. aeruginosa*
721 antibody.

722 (A) C57/BL6jRj mice were infected by the orotracheal instillation of 40 μ L of *P. aeruginosa*
723 PA103 containing 3×10^5 cfu. One hour later, mice were treated or not with 50 μ g or 100 μ g of
724 mAb166 via the pulmonary route (airway). Body-weight loss (B) and survival (C) were
725 monitored over 7 days. The results correspond to 4 pooled, independent experiments (n=7-20
726 mice per group). Log-rank test was used for survival analysis, ###: $p < 0.001$ when compared to
727 Vehicle group; *: $p < 0.05$ when compared to mAb166-50 μ g group. Two-way Anova followed by
728 Tukey's post-test was used for body-weight analysis, ##: $p < 0.01$ when compared to Vehicle
729 group; *: $p < 0.05$ when compared to mAb166-50 μ g group.

730

731 Figure 2: Intranasal application of *Lactobacillus murinus* is tolerable in mice.

732 (A) C57/BL6jRj mice were daily treated or not by the intranasal administration of 40 μ L of *L.*
733 *murinus* CNCM-I 4967 or CNCM-I 4968 containing 10^5 , 10^6 or 10^7 cfu for 3 consecutive days.
734 One day later, mice were sacrificed. Probiotic load in BAL (B) and lungs (C) were determined.
735 (D and E) Body-weight loss was monitored over 3 days. (F and G) Neutrophils (CD45+ CD11c-
736 CD11b+ SiglecF- Ly6G+ Ly6C- cells) number in BAL were evaluated. The data are quoted as
737 the mean values \pm SEM. The results are representative of 2 independent experiments (n=4-5 mice
738 per group), **: $p < 0.01$ with *t* test.

739

740 Figure 3: Synergistic effect of intranasal *Lactobacillus murinus* and therapeutic antibody in *P.*
741 *aeruginosa*-induced pneumonia.

742 (A) C57/BL6jRj mice were daily treated or not by the intranasal administration of 40 μ L of *L.*
743 *murinus* CNCM-I 4967 or CNCM-I 4968 containing 10⁵ cfu for 3 consecutive days. One day
744 later, mice were infected and treated as in Figure 1A. Survival (B and C) and body-weight loss
745 (D and E) were monitored over 7 days. The results correspond to 4 pooled, independent
746 experiments (n=10-20 mice per group). Log-rank test was used for survival analysis, #: $p < 0.05$,
747 ####: $p < 0.001$ when compared to Vehicle group; ***: $p < 0.01$ when compared to mAb166-50 μ g
748 group. Two-way Anova followed by Tukey's post-test was used for body-weight analysis, ##:
749 $p < 0.01$ when compared to Vehicle group; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.01$ when compared to
750 mAb166-50 μ g group.

751
752 Figure 4: Improved host anti-bacterial response afforded by intranasal *Lactobacillus murinus* and
753 therapeutic antibody.

754 C57/BL6jRj mice were treated and infected as described in Figure 3A. One day later, mice were
755 sacrificed. *P. aeruginosa* PA103 burden in BAL (A and F) and in lungs (B and G) were
756 quantified. Neutrophils (CD45+ CD11b+ SiglecF- Ly6G+ cells) number in BAL (C and H) and
757 in lungs (D and I) were determined. IL6 in BAL (E and J) was measured and standardized over
758 total BAL protein. The data are quoted as the mean values \pm SEM. The results correspond to 2
759 pooled, independent experiments (n=10 mice per group), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$
760 with one-way Anova followed by Tukey's post-test.

761
762 Figure 5: Combined intranasal *Lactobacillus murinus* and therapeutic antibody promotes long-
763 term protection against *P. aeruginosa*.

764 (A) C57/BL6jRj mice were treated and infected as described in Figure 3A (primary infection).
765 Surviving mice were challenged, 33 days later (secondary infection) by an orotracheal instillation

766 of 40 μL of *P. aeruginosa* PA103 (3×10^5 cfu) without additional treatment. (B and C) Survival
767 was monitored over a week after the challenge. The results correspond to 4 pooled, independent
768 experiments (n=10 for CNCM-I-4967 group and n=5 for the others). Log-rank test was used for
769 survival analysis, #: $p < 0.05$, ###: $p < 0.001$ when compared to Vehicle group; *: $p < 0.05$ when
770 compared to mAb166-50 μg group. (D) The concentration of total anti- *P. aeruginosa* PA103 IgG
771 in serum was determined by ELISA at days 1, 3, 7, 14, 21 and 28 after the primary infection. The
772 data are quoted as individual values. The results correspond to 2 pooled experiments (n=4-15
773 mice per group). (E) The concentration of total anti- *P. aeruginosa* PA103 IgG in serum was
774 determined by ELISA at days -1, and +5 after the secondary infection. The data are quoted as
775 individual values. The results correspond to 2 pooled experiments (n=4 for mAb166-50 μg and
776 CNCM-I 4968-mAb166-50 μg group and n=8 mice for CNCM-I 4967-mAb166-50 μg group), *:
777 $p < 0.05$; with a paired t-test.