



# **Assessment of *Aspergillus Fumigatus* Burden in Lungs of Intratracheally-Challenged Turkeys (*Meleagris Gallopavo*) by Quantitative PCR, Galactomannan Enzyme Immunoassay, and Quantitative Culture**

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**Assessment of *Aspergillus fumigatus* burden in lungs of  
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**Short Title:** *Aspergillus* pulmonary burden in an avian model

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34 **Abstract – (less than 150 words)**

35

36 *Aspergillus fumigatus* remains a major respiratory pathogen in birds, but diagnosis is still  
37 difficult. We challenged different groups of few-days-old turkeys via intratracheal  
38 aerosolisation of increasing concentrations ( $10^5$  up to  $10^8$ ) of conidia using a MicroSprayer®  
39 device. The fungal burden was assessed by real-time PCR, galactomannan dosage, CFU  
40 counting and histopathological evaluation in order to provide a comparison of these results  
41 within each inoculum groups. A significant mortality occurring in the first 96 hours after  
42 inoculation was only observed with the highest inoculum dose. Culture counts, GM index  
43 and qPCR results on one hand and inoculum size on the other hand appeared to be clearly  
44 correlated. The mean fungal burden detected by qPCR was 1.3 log<sub>10</sub> units higher than mean  
45 values determined by CFU measurement. The new model and the markers will be used for  
46 the evaluation of the efficacy of antifungal treatments that could be applied in poultry farms.

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51 **Keywords (Up to 10)**

52 Turkey, lung, aspergillosis, *Aspergillus fumigatus*, model, real-time PCR, galactomannan,  
53 culture

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## 1. Introduction

Aspergillosis is the most severe fungal disease in birds involving thermophilic mold species belonging to the genus *Aspergillus*, among which *A. fumigatus* is by far the most prevalent causative agent. In poultry production and more specifically in turkeys' fast-growing or breeding flocks, this opportunistic infection induces severe economic losses. Generally confined to the lower respiratory tract in acute cases affecting young birds, lesions may extend to many other organs via secondary haematogenous dissemination or contiguous infection. The small diameter of conidia allow them, once inhaled, to be deposited directly deep within the respiratory system particularly in posterior air sacs. Therefore, primary colonization occurs in an optimal environment for germination and mycelium growth of *Aspergillus* molds. Considering the physiological specific aspects of their highly efficient respiratory tract and the lack of efficient treatments on declared aspergillosis, the development of avian models is absolutely necessary to acquire a comprehensive knowledge of the physiopathology of a disease which may be responsible for high rates of morbidity and mortality, poor performances and subsequent economic losses in poultry farms [1, 2]. Numerous vertebrate models of this disease have been successively developed to study various aspects of the fungal development in the host, the subsequent immune response and to test antifungal drug efficacy. In contrast to rodents or rabbits experimental models, immune modulation is not a necessary prerequisite in avian challenge models [1]. Aspergillosis is readily induced in healthy birds by inhalation of aerosolized conidia in turkeys [3] or chickens [4], intravenous challenge in turkeys [5], intratracheal route in starlings [6], Japanese quails [7, 8, 9], rock pigeons and gyr-falcon hybrids [10] or by transcutaneous inoculation into air sacs in turkeys [11] or racing pigeons [12]. Besides mortality or clinical signs, the monitoring of host-pathogen interactions following experimental challenge in birds rely on gross lesions and histopathological evidences [13],

completed with either the detection of markers of the fungal extension [1] or the quantification of the inflammatory response [7]. Since many years now, non-culture based methods either used alone or in combination have been targeted to *Aspergillus* DNA by real-time PCR or cell-wall antigens in different tissues or fluids to establish a rapid diagnosis of invasive aspergillosis in humans [14] or to monitor experimental disease in several rodents models [15, 16, 17, 18, 19]. Dosages of galactomannan or 1,3- $\beta$ -D-glucans have also been tested in different bird species suffering aspergillosis [20], in turkeys following experimental infection [21] and recently on field cases affecting commercial broiler chickens and turkeys [22].

Our goal was to validate an accurate model of acute aspergillosis in intratracheally-challenged turkeys. Non-culture-based methods and quantitative culture were compared to assess the fungal burden in lungs. In further investigations this original model may be used to test antifungal treatments in poultry.

## **2. Materials and methods**

**2.1. *Aspergillus fumigatus* strain, mycological cultures and preparation of the inoculum**  
*Aspergillus fumigatus* strain CBS 144.89 was used for all experiments. This strain was initially isolated from a human patient with invasive aspergillosis in France and obtained from the Centraalbureau voor Schimmelcultuur (CBS), Utrecht, The Netherlands. All mycological cultures were done on malt agar plates supplemented with chloramphenicol (50 mg/L) and incubated at 37°C till 10 days depending on the fungal growth and species. To prepare the inoculum, the strain was grown for 10 days at 37°C. Conidia were subsequently harvested by flooding the plates with sterile phosphate-buffered saline (PBS) containing

0.01% (vol/vol) Tween 20 (PBST). The conidia were concentrated by centrifugation (at 3500 g for 30 min) and counted using a Malassez counting chamber.

## 2.2. Animals and housing conditions

Conventional male turkeys (Le Sayec, Caudan, France) of 1-day-old with an average weight of 65 to 70 g were used. Groups of approximately 20 birds were housed in minimum 2 m<sup>2</sup> cages on wood chips litter, in the biosafety 3 level sector of the animal facility of the veterinary college of Alfort, France. Animals had permanent access to fresh commercial poultry starter (Versela-Laga) and water. Photoperiod as ambient temperature was regulated and two infrared lamps provided additional heat during all the experiment.

## 2.3. Environmental contamination assessment

The fungal contamination of the environment was assessed before and during the experiments by regular sampling of conidia by sedimentation on two opened plates placed in each cage for 30 min. In parallel, 1 g of litter and feedstuff were sampled and mixed in 45 mL PBST from which 100 µl was seeded on agar plates.

## 2.4. Nebulizer system and intratracheal challenge

Two sets of experimental infections were conducted in the same conditions. For the first experiment, 36 seven-days-old turkeys were inoculated (day 0) with different concentrations of *A. fumigatus* (12 birds per inoculum): 10<sup>5</sup>, 10<sup>6</sup> or 10<sup>7</sup> conidia per bird (groups named Inoc5, Inoc6 and Inoc7, respectively). Nine turkeys were inoculated with PBST as negative control. In the second experiment, 12 five-days-old turkeys were inoculated with 10<sup>8</sup> conidia (Inoc8) and 7 turkeys were inoculated with PBST. Two birds of each group were

anesthetized immediately after inoculation in order to check the contamination level of lungs.

Turkeys were anesthetized by inhalation of 5 % isoflurane (Aerrane; Baxter) in oxygen and inoculated with 100  $\mu$ L of *A. fumigatus* suspension (or PBST) using a stainless steel 19 gauge atomizer (Microsprayer IA-1B®, Penn Century, Philadelphia, PA, USA) inserted into the trachea under visual control. No intra-operative deaths or delayed recovery (less than 2 min after inoculation) were observed. Birds were monitored at least twice daily and every signs of morbidity or mortalities were recorded.

All animal research procedures were approved by the regional ethics committee for animal experimentation at the veterinary college of Alfort.

## 2.5. Sample collection

Targeted organs of the respiratory tract from all dead and euthanized animals were removed aseptically under laminar flow hood. The trachea and left thoracic air sac were sampled with a sterile swab. The left lungs were collected for histology and the right lungs were used for fungal culture, real time PCR and galactomannan dosage.

## 2.6. Histological evaluation

Tissues collected for histology were immediately fixed in 10% neutral buffered formalin and dehydrated. Paraffin wax-embedded specimens were sectioned at 4  $\mu$ m and stained with hematoxylin-eosin-saffron (HES) to evaluate the inflammatory response. Periodic acid Schiff (PAS) and Grocott-Gomori methenamine-silver stains (MS) were used to visualize *Aspergillus* in lesions. A blind reading of 1 up to 10 slides per selected turkey of each group was conducted to evaluate the intensity of microscopic lesions.

## 2.7. Fungal culture of respiratory organs

The right lungs were weighed and grinded with a homogenizer of tissue (Bio-Gen PRO200 Homogenizer, Pro Scientific, Oxford CT, USA) in 6 mL of PBS containing 0.01% (vol/vol) Tween 20. 100 µL of the primary lung homogenates were immediately used for fungal culture, the rest was frozen at -20°C until use.

Dilutions of primary lung homogenates and swabs of trachea and air sac were seeded on malt chloramphenicol agar plates and incubated at 37°C. The count of *A. fumigatus* colonies (CFU) were assessed 1 and 2 days after plate inoculation.

## 2.8. Assessment of conidial equivalent by real time PCR

The primary lung homogenates were subjected to a secondary homogenization step. 500 µL of lung homogenate was transferred to a sterile 2 mL screw-cap centrifuge tube containing 0.5 mm-diameter glass beads (Biospec, Bartlesville, USA) and placed in a MagNa Lyser homogenizer (Roche, Meylan, France) for 3 bursts of 30 s at 3500 rpm with incubation on ice between bursts. The secondary lung homogenate was collected by centrifugation at 800 g for 5 min at 4°C and 100 µL of this homogenate was directly used for DNA extraction with the QIAamp DNA Mini Kit (Qiagen, Courtabeouf, France) according to the manufacturer's directions. Fungal DNA and secondary homogenate were stored at -20°C until use. Real-time TaqMan PCR quantified the amount of *A. fumigatus* cell nuclei by targeting the single-copy *FKS* gene and the multi-copy gene encoding the 28S rRNA. The sequences of the *FKS* primers were previously reported [23]: sense amplification primer, AFKS1 (5'-GCC TGG TAG TGA AGC TGA GCG T-3'), antisens amplification primer, AFKS2 (5'-CGG TGA ATG TAG GCA TGT TGT CC-3') and the hybridization *FKS* probe (5'-6-FAM-TCA CTC TCT ACC CCC ATG CCC GAG CC-BHQ1-3'). The 28S primers were reported by Challier *et al.* [24]: 28S-466 (5'-CTC GGA ATG TAT CAC CTC TCG G-3') and 28S-533 (5'-TCC



TCG GTC CAG GCA GG-3') and the hybridization 28S probe (5'-6-FAM-TGT CTT ATA  
GCC GAG GGT GCA ATG CG- TAMRA-3').

A standard curve was established with DNA samples extracted from uninfected lungs of  
turkeys inoculated with different doses of *A. fumigatus* conidia (from  $10^2$  to  $10^8$  conidia) and  
allowed us to quantify the *Aspergillus* DNA in the organs by calculating conidial equivalents  
(CE) [15]. DNA samples were analysed in duplicate by using the LightCycler® 480  
detection system (Roche, Meylan, France). The reaction mixture consisted in 5 µL of DNA,  
900 or 500 nM sense and antisens primers from *FKS* or 28S targets, 200 nM *FKS* probe or  
250 nM 28S probe and LightCycler® 480 Probes Master 2x (Roche, Meylan, France). All  
assays were run under the following conditions: 50°C for 2 min, 95°C for 10 min, then 50  
cycles of 15 s at 95°C and 1 min at 65°C or 60°C respectively for *FKS* or 28S primers.

## 2.9. Galactomannan (GM) quantity assessment

500 µL of primary lung homogenates were centrifuged at 2,300 g for 5 min and the  
supernatant was placed into a fresh tube. Lung supernatants (300 µl) were used for GM  
determination with the Platelia GM-EIA kits (Bio-Rad Laboratories) according to the  
manufacturer's directions. The minimum and maximum thresholds of DO detected by the  
ELISA reader (Biorad PR1300) ranged between 0 and 3 Abs. A 1:100 dilution of lung  
homogenates was realized for all groups and a 1:1000 dilution for the Inoc8 group in order  
to avoid the saturation of the signal detected in some crude lung homogenates.

## 2.10. Genotyping of *Aspergillus fumigatus* isolates

A subset of strains recovered either from the environment or from internal organs was  
collected and genotyped by Multi Locus VNTR Analysis [25].

#### 2.11. Statistical analyses

Statistical analyses were performed with the JMP 10.0 software. A log rank t test has been used to analyse the survival of birds and to evaluate the effect of the inoculum dose on mortality. A nonparametric one-way analysis of variance, Wilcoxon test, linear regression models and Spearman's rank correlation were performed for multiple comparisons and to evaluate the relationship between the different biomarkers ( $\log_{10}$  CFU/g of lung,  $\log_{10}$  CE/g of lung and the GM index) and the inoculum doses.

### 3. Results

#### 3.1. Body weight, clinical signs and mortality

A dose dependent response was observed concerning weight gain evolution when inoculated turkeys were compared to non-challenged birds in both sets. If birds from Inoc5 and Inoc6 were not affected by *Aspergillus* challenge, growth average of birds exposed to higher concentrations (Inoc7 and Inoc8) decreased significantly as soon as 2 days post inoculation (figure 1).

No clinical signs or mortality were observed in non-challenged turkeys and Inoc5. The clinical signs observed in turkeys of Inoc6, Inoc7 and Inoc8 included lethargy, ruffled feathers, progressive weight loss and respiratory distress such as open-mouthed breathing leading to lateral recumbency in severe cases. Two birds out of 9 in Inoc6 exhibited clinical signs 6 days after inoculation, but did not die before the end of the experiment. Eight turkeys out of 10 in Inoc7 presented clinical signs, which appeared between 3 and 6 days after inoculation. Among them, only 3 animals died at day 5. In set B, an acute respiratory distress was registered between 1 and 3 days post inoculation of *A. fumigatus* in all turkeys of Inoc8 (n=10), which died 24 to 48h later (figure 2).

### 3.2. Macroscopic and microscopic lesions

No macroscopic lesions were observed on non-challenged and Inoc5 birds. An increasing proportion of turkeys from Inoc6 (5/10), Inoc7 (8/10) and Inoc8 (10/10) exhibited few granulomas on thoracic air sacs and a densification of variable proportions of the pulmonary parenchyma. Histological lesions associated with *Aspergillus* development, mostly represented by multifocal either isolated or coalescing granulomas with necrotic centers, and occurrence of multinucleated giant cells (figure 3) were confirmed in lungs of Inoc6, Inoc7 and Inoc8 turkeys but were absent in Inoc5 or non-challenged birds. Percentages of altered parenchyma varying from 30 to 80% have been estimated on lungs preparation coming from different birds belonging to Inoc7 and Inoc8. However, the hyphae burden seemed to be more important in the latter group.

### 3.3. Fungal cultures from trachea, air sacs and lungs

The lower limit of the assay is approximately 300 CFU/g of lung (10 CFU/plate). All non-challenged turkeys exhibited negative cultures. Semi-quantitative numerations from trachea and air sacs showed variable levels of contamination immediately after inoculation in all challenged groups. However no *Aspergillus* could be recovered from both organs of all birds of Inoc5, Inoc6 and Inoc7 that were euthanized between 5 and 8 days post challenge. For Inoc8, a high level of contamination (>100 CFU/plate) was still present in air sacs till day 4, but not in trachea even in turkeys which died spontaneously 2 days after inoculation. Positive counts ranging from 3.28 up to 9.75 log<sub>10</sub> CFU/g have been registered on lungs of all turkeys sacrificed immediately after the inoculation. In the remaining birds, the proportion of positive samples and the mean of log CFU/g of lung established on those samples increased with the inoculum size (table 1). The mean of log<sub>10</sub> CFU/g of lung for the

13 turkeys (Inoc7 and Inoc8) that died spontaneously between 2 and 5 days pi was  $5.47 \pm 0.41$ .

### 3.4. Assessment of conidial equivalent by real time PCR

The sensitivity of real time PCR on 28S target was higher than on *FKS* gene with limits of detection varying from 5 CE (corresponding to  $10^3$  CE/g of lung) to 250 CE per reaction ( $5 \times 10^4$  CE/g of lung) respectively. Immediately after experimental infection, qPCR signals remained undetectable for the 2 birds of Inoc5, one turkey of Inoc6 but highly positive for the others (Inoc7 and 8). At day 8, no amplification at all was detected with both targets for Inoc5 and only 3/9 samples from Inoc6 were detected with the 28S target. The numbers of CE/g established of lung detected with *FKS* target and 28S target were positively correlated ( $\log_{10} \text{CE}_{\text{FKS}}/\text{g of lung} = -2.92 + 1.34 * \log_{10} \text{CE}_{28\text{S}}/\text{g of lung}$ ) when assessed on the 17 DNA samples (7/10 Inoc7 and 10/10 Inoc8) exhibiting amplification with both DNA targets (data not shown). Similarly to fungal numerations both proportions of positive amplifications and means of  $\log \text{CE}_{28\text{S}}/\text{g of lung}$  in positive results appeared to be correlated to the inoculum size (table 1).

### 3.5. Galactomannan (GM) quantity assessment

GM index obtained on crude lung homogenates presented huge variations from 0.5 to 6 (signal saturation) in all groups including non-challenged birds. GM index repartition after 1:100 dilution (figure 4) was quite similar in control, Inoc5 and Inoc6 birds ( $p=0.0084$ ). The highest variation of GM index values was observed in Inoc7 (mean GM index =  $3.99 \pm 0.90$ ). All turkeys that died spontaneously (including all Inoc8) exhibited index higher than 5. Interestingly, GM index of all birds immediately euthanized after inoculation were lower than 0.35.

### 3.6. Correlation between the different markers

The number of  $\log_{10}$  CE28S/g of lung was strongly correlated to the number of  $\log_{10}$  CFU/g of lung ( $p < 0.0001$ ) when the data of all groups were included (figure 5) but not in each groups considered separately. Both values increased significantly with inoculum size ( $p < 0.0001$ ). As featured by the regression line, the sensitivity threshold of CE values was almost 10 fold higher than CFU counts in lung samples ( $\log_{10}$  CE28S/g of lung =  $1.30 * \log_{10}$  CFU/g of lung). Birds, which died quickly, have constantly more CFU and CE in lungs than the other birds. GM index applied to diluted lung homogenates (1:1000) of Inoc8 was significantly correlated to  $\log_{10}$  CE/g ( $p = 0.0172$ ) but not with  $\log_{10}$  CFU/g ( $p = 0.526$ ) (figure 6).

### 3.7. Environmental contamination

Negligible levels of contamination by molds (*Aspergillus* spp. and Mucorales) have been detected in the litter or in the room's atmosphere prior to the installation of the birds or after inoculation (controls at day 1 and day 8). Feedstuffs remained constantly culture-negative.

### 3.8. Molecular typing of *A. fumigatus*

Six *A. fumigatus* isolates collected from the animals and the environment presented the same MLVA genotype as that of the reference strain (CBS 144.89) used for the experimental infection.

## 4. Discussion

### 4.1. Experimental model

Many experimental aspergillosis have already been published using different avian models and testing various bird species, age at inoculation, challenge routes and even the *Aspergillus* species [1]. Both field data [2] and experimental results [26] clearly demonstrate a higher susceptibility of turkeys and quails to *A. fumigatus* infection when compared to chickens, but also intraspecific differences in host responses [4, 27] highlighting the necessity to establish models on relevant species or lineages reared for meat or egg production. Different routes of *Aspergillus* inoculation have been tested in turkeys including intrapulmonary [28], intra-air-sac administration [11, 21] and finally aerosolization in confined plastic chambers [3]. With the exception of the latter method, which is difficult to standardize [4], the intratracheal inoculation, which has not been yet tested in turkeys, is the closest to the natural way of infection and only bypasses the upper airways and their putative defenses [1]. We propose a relevant model of acute aspergillosis in one-week-old turkeys following intratracheal nebulization of *Aspergillus fumigatus* conidia with a MicroSprayer® aerosolizer. The use of this device has already been validated in immunosuppressed 6-8 weeks-old Sprague-Dawley rats [16] and immunocompetent Japanese quail [7] to administrate precisely calibrated inoculum of *Aspergillus fumigatus* in the respiratory tract. The evaluation of the impact of four inoculum concentrations ( $10^5$  to  $10^8$  conidia per turkey) allowed us to record mortality following a dose response with values of 0, 0, 30 and 100% respectively. Global parameters such as the evolution of body-weight, the occurrence and severity of clinical signs and the chronology of deaths appeared strictly in concordance with the concentration of conidia administered. Lower mortality rates (respectively 16, 55, 56 and 20%) have been observed in turkeys treated with  $10^8$  conidia in the air sac [21] or submitted to dry aerosol delivering doses of  $5.10^9$  spores per animal [3] or in quails receiving  $1.5 \cdot 10^6$

[9] conidia intratracheally. A similar protocol ( $2.10^7$  conidia administered intratracheally) applied to the same species resulted in 55% [29] and 100% mortality [8]. As mentioned by Goetting *et al.* [7] who compared two modalities of inoculation, the nebulization by Microsprayer® should not be considered to be strictly equivalent to a “classical” intratracheal inoculation using an intravenous catheter after removing the stylet. Higher mortality (88% versus 33%) has indeed been observed after nebulization compared to the “classical” method after the same inoculum size ( $4.7 \cdot 10^7$  spores). This could be attributed to differences in dispersion parameters (velocity, droplet size) through the respiratory tract. Similarly to [7], the macroscopic lesions registered in our model were strictly limited to the respiratory apparatus and particularly marked in lungs but much more rarely on caudal air sacs. Extensive parenchyma necrotic damage consecutive with the presence of multifocal typical granulomas [11, 13] were seen in the present experiment in all birds of Inoc8 but also in some animals belonging to Inoc7, which have been euthanized at the end of our experiment. We cannot therefore exclude the possibility of delayed mortality (up to 50%) in the latter group if the experiment took place over a longer post-inoculation period [3].

#### 4.2. Biomarker evaluation

The relevance of three putative biomarkers (CFU, galactomannan and real-time PCR) has been concomitantly assessed on lung homogenates for the first time in turkeys. In contrast with already published models in the immunosuppressed rat [15] or Guinea pig [17, 18], our primary purpose was to study the kinetics of fungal development in several target organs but to compare the effect of variable inoculum sizes on these parameters and, in addition, we tried to establish potential correlations to characterize fungal burden states in the lung since it was the sole organ to present extensive macroscopic lesions in our model.

351 Semi-quantitative culture-based methods used to evaluate the *Aspergillus* load in organs are  
352 easy to perform and common but only detect viable organisms. Furthermore, temporal  
353 studies in rodents suggest that these tools may not be accurate when monitoring the  
354 development of multicellular filamentous fungi particularly in parenchyma submitted to  
355 mechanical homogenization [30] leading to possible underestimation [31, 32]. Except for  
356 some air sacs samples obtained from turkeys, which died in the first four days following  
357 inoculation, no fungus could be detected on the trachea or air sac swabs. This may be  
358 explained by fungal clearance, or low sensitivity of the method used on these organs.  
359 Although not strictly comparable because the interval post-inoculation differed between  
360 dead and euthanized birds, the mean lung numerations, the proportion and the homogeneity  
361 of positive results seemed clearly to increase with the inoculum dose. All dead animals  
362 presented at least  $5 \cdot 10^5$  spores/g of lung tissue which was estimated by Richard *et al.* [3] as  
363 the dose necessary to kill about 50% of 3-week-old turkeys exposed to an aerosol of  
364 *Aspergillus* conidia which appeared to be a clear threshold in younger birds unable to mount  
365 an effective innate response [4, 11, 13] in the critical phase of the first days following  
366 experimental infection. The detection of viable *Aspergillus* was possible in a high proportion  
367 of apparently healthy turkeys, which were euthanized at the end of the experiment, but may  
368 persist to least 8 weeks post-exposure [3]. However, fungal load in surviving birds as  
369 assessed by culture seemed to decrease post-inoculation as in other avian [4, 9] or mammal  
370 models [18, 19].

371 The enzyme immune assay of galactomannan, a specific cell-wall component of fungi is  
372 relatively easy to perform and is likely to reflect the progressive increase in mycelium load  
373 by tip extension of hyphae [31]. The availability of commercial tests has allowed this  
374 polysaccharide component to be measured as a valuable biomarker in the sera of different  
375 bird species suffering from aspergillosis [20]. It has been used in field studies on



376 commercial broiler chickens and turkeys [22], but also in mammals [16, 32] and turkeys  
377 [21] experimental models. As a potential diagnosis tool, contrasting results have been  
378 obtained due to variable sensitivity or specificity depending on the species tested. Additional  
379 difficulties exist in the definition of an appropriate cut-off value and the stage of the disease  
380 [20] but also to the ubiquitous presence of galactomannan in the poultry environment  
381 leading to false positives [1]. This last point may partially explain the positive GM values  
382 obtained from crude lung homogenates of control birds housed in a clean but not sterile  
383 environment. This necessitates work on dilutions to evaluate this parameter. The GM index  
384 on 1:100 dilutions were very low in turkeys submitted to the two lowest doses as for non-  
385 exposed birds but were much more variable in Inoc7. This may reflect individual differences  
386 in host-response efficiency. The high GM index systematically seen in turkeys, which died  
387 spontaneously in contrast to almost undetectable values in birds euthanized immediately  
388 after receiving variable doses of non-germinated conidia, is consistent with the relationship  
389 between the index value and the progression of the fungus [17, 31]. Furthermore, GM index  
390 correlated with the extent of infection as demonstrated by histopathological examination  
391 [18].

392 A real-time quantitative PCR assay has been developed recently in rodent models to  
393 measure the fungal load and its progression in different organs in order to evaluate the  
394 efficacy of antifungal therapy [15, 17, 18, 19]. This molecular tool allows the detection of  
395 both conidia and hyphae and can be very sensitive and specific but like the GM enzyme  
396 immunoassay, it does not indicate viability [31]. Gene targets included a monocopy gene  
397 like *FKS1* [18] or multicopy genes, in particular the ribosomal DNA genes [15] and internal  
398 transcribed spacer. The choice of multicopy genes alone or in the frame of a nested PCR  
399 [17] to improve sensitivity may not be ideal for standardization of the method [32]. To our  
400 knowledge, qPCR in avian lungs was evaluated for the first time in this study. The real-time

PCR signal from a sample of infected tissue was compared with a standard curve composed of conidia added to naïve pulmonary parenchyma and we chose to express the fungal biomass in terms of CE [15]. The performances of the monocopy *FKS* gene and the multicopy 28SrDNA amplification in turkey lungs proved to be equivalent although the sensitivity of the latter PCR was logically about 1 log<sub>10</sub>CE/g lower. Mathematical extrapolation predicted that the detection limit of this assay was approximately 10<sup>3</sup> CE/g of lung, which is quite similar to 2.10<sup>3</sup> CE/g (18S) estimated in an haematogenous mouse model of aspergillosis [15], but could further be improved with a nested qPCR [17]. As demonstrated by results on euthanized birds just after inoculation, the qPCR seemed to be less sensitive than CFU numerations, which may be explained by a low efficiency of extraction of conidia from tissue samples [19, 30]. Although no signal could be amplified in Inoc5 and there was no sign of illness, both the proportion of positive samples and mean log<sub>10</sub>CE/g tended to increase with the dose used. Finally, the onset of induced mortality coincided clearly with a peak in fungal burden in Inoc8 as observed in a mouse model [15]. A strong correlation between qPCR results and culture numerations was seen when positive data from all groups were included and the molecular method demonstrated a higher sensitivity. This relationship appears to be stronger when tissues contain hyphae rather than conidia [30]. When data from Inoc8 were compared with similar mammal models [15, 18], GM index and qPCR appeared to be more closely correlated with the rise of infection in comparison with CFU values which may partially be explained by the fact that the culture-numerations stagnates or decreases with time [19].

#### 4.3. Conclusion

We have developed a repeatable and standardized model of acute and fatal primary pulmonary pneumonia in the young, immunocompetent turkey using an atomizer, which

may be applicable to chemotherapy trials. Housing birds in groups in a controlled environment enables field conditions of production to be mimicked and to evaluate the treatment of aspergillosis by nebulized molecules such as azole compounds [2]. Besides survival, still considered the most reliable measure to assess antifungal therapy, the combination of additional markers of fungal development may contribute to an earlier and more precise detection of treatment efficiency in birds submitted to lethal or sub-lethal challenges but also to the refinement and the reduction objectives of *in vivo* experimental research [31, 32].

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439 **Table 1.** Fungal burdens in lungs of inoculated turkeys measured by CFU and real time PCR  
 440 (28S) assays.

Groups	Mean log <sub>10</sub> CFU/g of lung	Mean log <sub>10</sub> CE <sub>28S</sub> /g of lung
Inoc5	BLD <sup>a</sup>	BLD
Inoc6	2.84 ± 0.30 (5/9) <sup>b</sup>	5.32 ± 0.13 (3/9)
Inoc7	4.34 ± 0.29 (7/10)	5.73 ± 0.14 (7/10)
Inoc8	5.60 ± 0.12 (10/10)	7.17 ± 0.11 (10/10)

<sup>a</sup>BLD : below level of detection

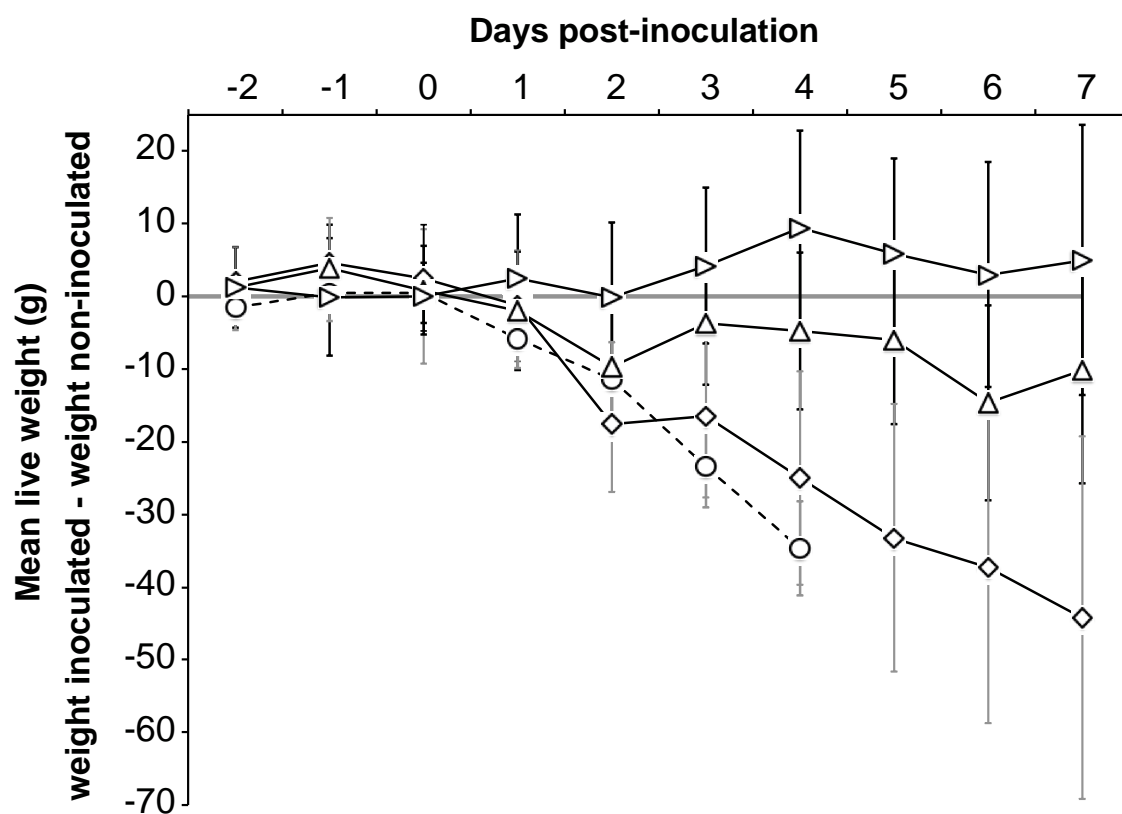
<sup>b</sup> Mean ± standard error (no. of positive values/total no. of birds tested)

441

442

443 **Figure 1.** Mean weight variations of inoculated birds (black line) compared to non-  
 444 challenged turkeys (grey line) from set A (solid line) or B (dashed line). Inoculum of  $10^5$   
 445 ( $\triangleright$ ),  $10^6$  ( $\triangle$ ),  $10^7$  ( $\diamond$ ) or  $10^8$  ( $\circ$ ) *Aspergillus fumigatus* conidia.

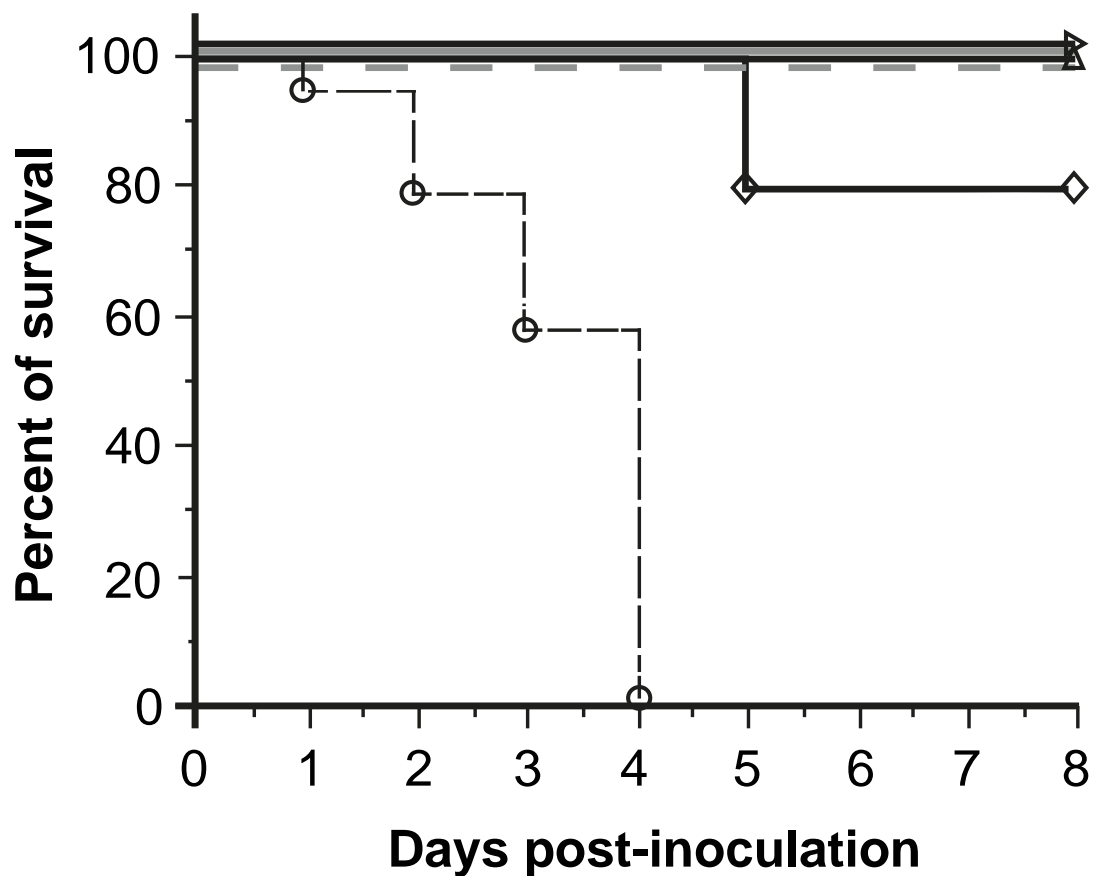
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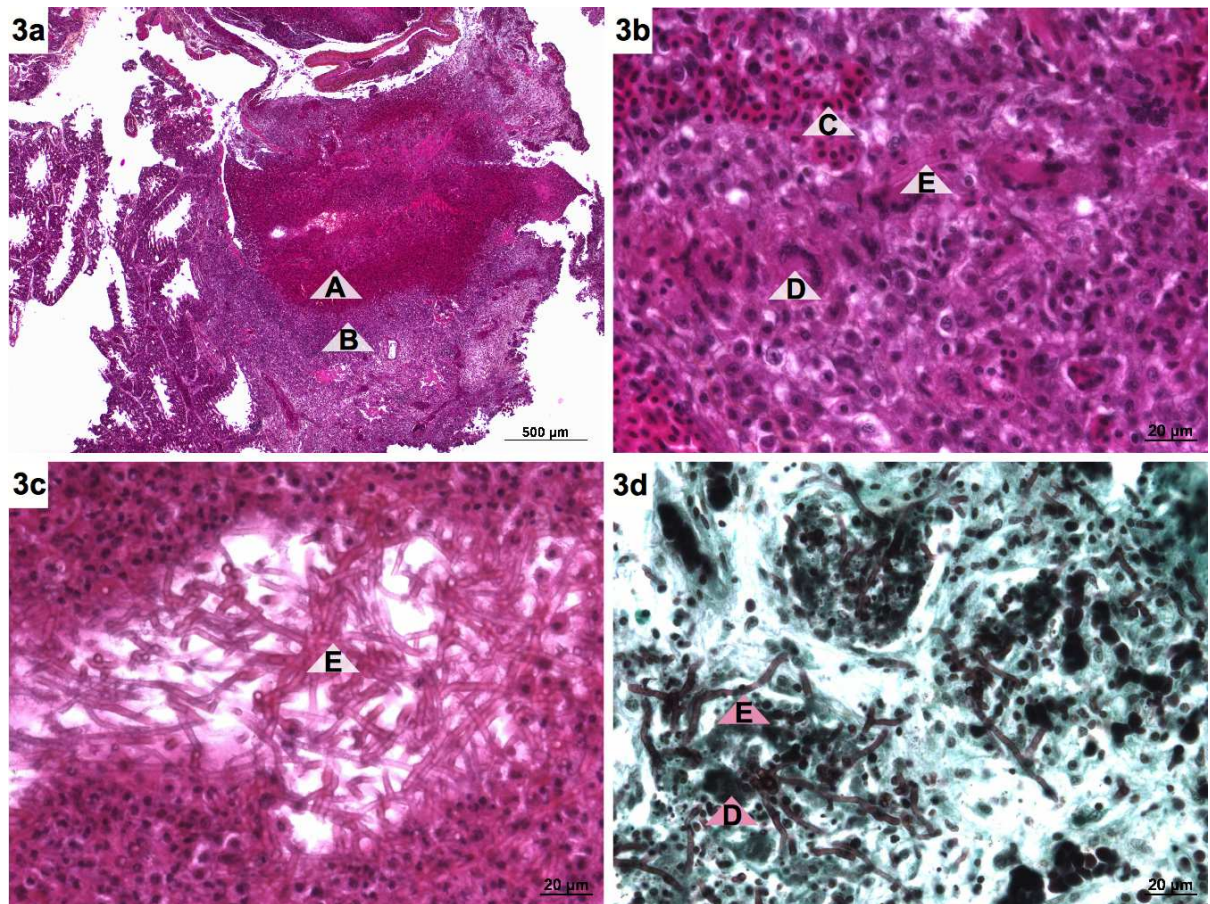
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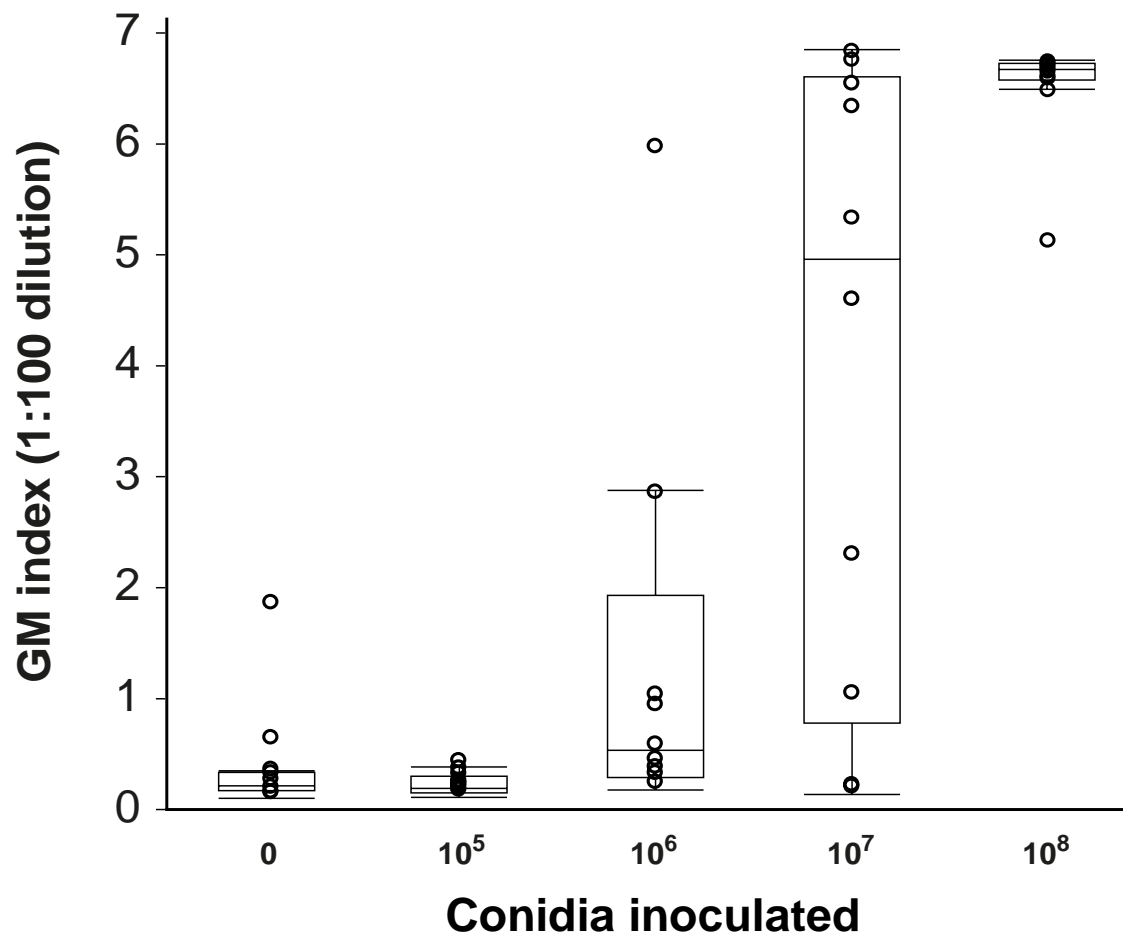
**Figure 2.** Survival time of non-challenged (grey line) and inoculated (black line) turkeys from set A (solid line) and B (dashed line) Inoculum of  $10^5$  ( $\triangleright$ ),  $10^6$  ( $\triangle$ ),  $10^7$  ( $\diamond$ ) or  $10^8$  ( $\circ$ ) *Aspergillus fumigatus* conidia.



**Figure 3.** Histological sections of lungs from 2 turkeys (Inoc8). 3a (HES stain): Voluminous granuloma with a necrotic centre. 3b, 3c (HES stain) and 3d (MS stain): Detailed of a granuloma. A: necrosis; B: granuloma; C: red blood cells in capillary; D: giant cells with multiple nuclei; E: *Aspergillus* hyphae.

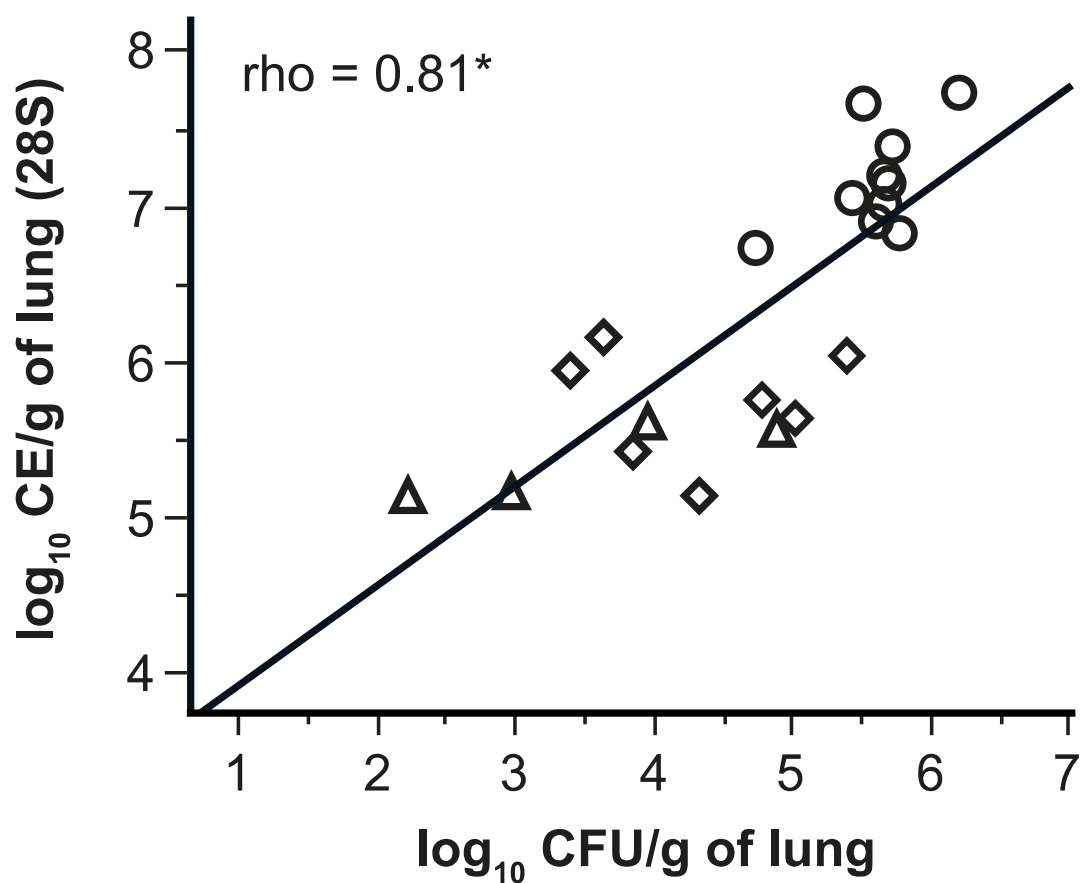


**Figure 4.** Comparison of mean Galactomannan index (1:100 dilution) in lung samples from non challenged and inoculated groups.

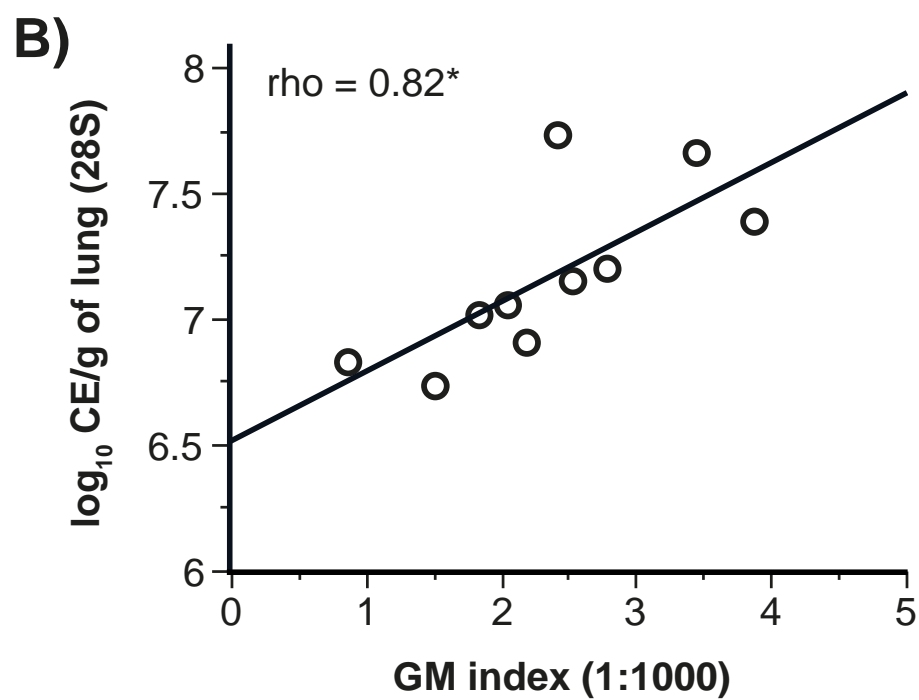
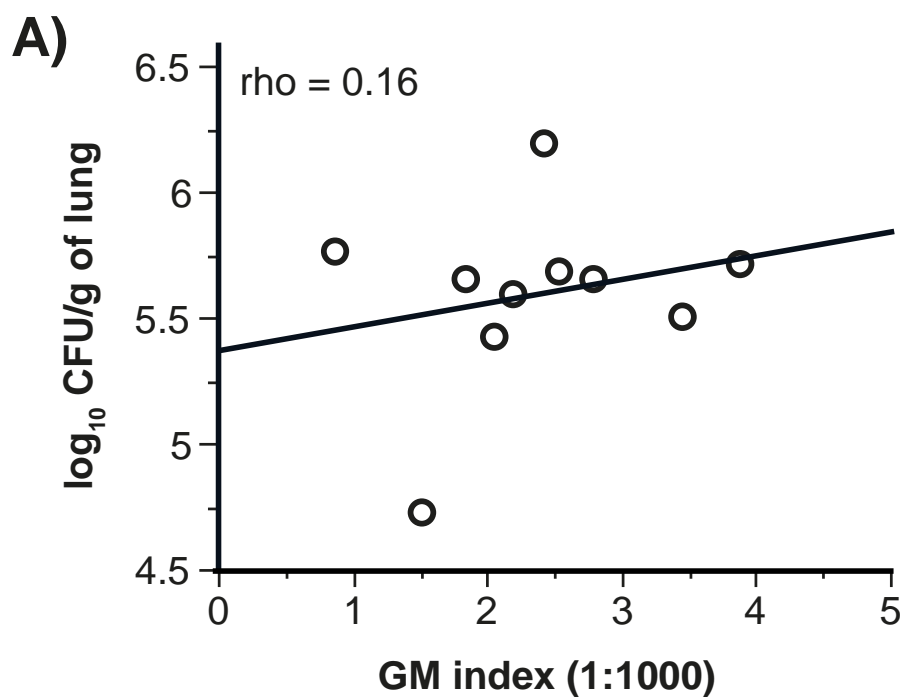




**Figure 5.** Relationship between CFU and CE ( $\log_{10}$  CE28S/g of lung =  $1.30 \times \log_{10}$  CFU/g of lung) as shown by linear regression. Turkeys inoculated with  $10^5$  ( $\triangleright$ ),  $10^6$  ( $\triangle$ ),  $10^7$  ( $\diamond$ ) or  $10^8$  ( $\circ$ ) conidia. \*Significant correlation ( $p < 0.0001$ ) between results by the Spearman rank correlation,  $n = 21$ .



**Figure 6.** Relationship between GM index and CFU (A) or CE (B) per g of lung from turkeys inoculated with  $10^8$  conidia. \*Significant correlation ( $p = 0.0038$ ) between galactomannan index and CE by the Spearman rank correlation test,  $n = 10$ .



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