# Optimization of *Aspergillus versicolor* culture and aerosolization in a murine model of inhalational fungal exposure_dataset

## *Materials and Methods*

### Culture of *Aspergillus versicolor*

*Aspergillus versicolor* (Vuillemin) Tiraboschi, ATCC 9577 (American Type Culture Collection, Manassas, VA, USA) was inoculated onto Malt Extract Agar (MEA) using a sterile inoculating loop and incubated at 23˚C with 98% humidity for 7 days. Conidia were recovered from the MEA with a sterile inoculating loop in sterile distilled water and diluted to a concentration of $2.5x10^6$ conidia/ml in 5 ml. The suspension of conidia was then inoculated onto 10 g of autoclaved (132 ˚C for 30 min) Mahatma white rice (Riviana Foods Inc., Houston, TX, USA) in 100 mm petri dishes and grown at 23 ˚C, 98% humidity for 7-10 days. Heat-inactivated *A. versicolor* conidia were used throughout these studies as a biological particle control. Rice cultures were baked in a laboratory oven (Thermo Fisher, Waltham, MA, USA) at 80 ˚C for 4 hours to produce heat-inactivated *A. versicolor* conidia. Viability was tested by plating 100 viable or heat-inactivated *A. versicolor* spores on MEA plates and counting colony growth after 72 hours. Viable and heat-inactivated *A. versicolor* rice cultures were desiccated in vacuum-sealed chambers for 3-5 days or 7-10 days where noted.

### A. versicolor protein extract & SDS PAGE Analysis

*A. versicolor* was inoculated onto MEA as described above, and conidia were recovered from the surface of MEA plates into sterile distilled water using an inoculating loop. Viable or heat-inactivated *A. versicolor* conidia were recovered from rice grains by washing and agitation in sterile distilled water. *A. versicolor* conidia were frozen at -80°C, and then lyophilized. Lyophilized conidia were disrupted with 0.5 mm glass beads using a Bead Mill 24 Homogenizer (Thermo Fisher). Conidia and beads were washed in PBS, centrifuged at 400xg, and protein supernatant was collected. Protein concentration was determined via Pierce BCA Assay (Thermo Fisher) and 30 ug was resuspended in Laemmli buffer with β-mercaptoethanol. Suspensions were heated at 95°C for 5 minutes. Samples were resolved on 4-20% polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and stained with Imperial Protein Stain (Thermo Fisher) according to manufacturer's recommendation. Protein distribution was compared.

### Fungal metabolite quantification

*Aspergillus versicolor* was cultured on rice, as described above. Following desiccation, spore-laden rice was mixed with PBS and viable or heat-inactivated conidia were liberated and collected, then quantified using hemocytometer. Aliquots of $5x10^5$ cells were lyophilized. Samples were shipped on dry ice and

stored frozen until analysis by mass spectroscopy. The lyophilized spores were extracted for 90 min with 200 µL of acetonitrile/water/acetic acid in a 79/20/1 ratio.

Detection and quantification were performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance, CA, US. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the respective retention time ±27 s and ±42 s in positive and in negative mode, respectively. The target scan time was set to 1s. The settings of the ESI-source were as follows: source temperature 550°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage −4,500 V and +5,500 V, respectively, collision gas (nitrogen) medium. The preparations were screened for a total of 662 analytes. One preparation contained traces of *Penicillium* and was therefore excluded from analysis.

## Sterigmatocystin quantification

Single grains of conidia laden rice were weighed to acquire the mass. Forty nanograms of isotopically-labeled $^{13}C$ sterigmatocystin (as internal standard) in acetonitrile was spiked onto non-inoculated grains and acetonitrile was allowed to evaporate. One milliliter of extraction solution (a combination of 99% acetonitrile and 1% acetic acid by volume) was then added into a glass tube with the grain. Sterigmatocystin was extracted from fungal rice cultures by shaking for 90 minutes in extraction solution. After shaking, the tube was centrifuged for 3 minutes at 1962 xg and 700 µl of the supernatant was transferred to an injection vial that was then dried under gentle nitrogen stream. Once dried, 800 µl of injection solution (a combination of 69% water, 30% methanol, and 1% acetic acid by volume) was added to the injection vial and vortexed for 30 seconds. Finally, 10 µl of the sample extract was injected to Ultra Performance Liquid Chromatograph coupled with Tandem Mass Spectrometer (UPLC/MSMS, Acquity H Class UPLC and Acquity Xevo TqD Quadrupole Tandem Mass-Spectrometer, Waters, Massachusetts, USA). Gradient mobile phase starting with 90% aqueous and 10% organic was pumped at a flow rate of 0.2 ml/min when sample extract was injected. The aqueous solvent with 10 mM of ammonium acetate was composed of 99% super pure water and 1% acetic acid (by volume) and the organic solvent with 10 mM

ammonium acetate was composed of 99% methanol and 1% acetic acid (by volume). The percentages of the mobile phase were changed over time to allow organic solvent to be dominant (97%) at 5.7 minutes so that the sterigmatocystin successfully eluted. Extraction of sterigmatocystin from air filters used the same procured with the exception that 200µl of injection solution was used to reconstitute dried extract prior to identification using UPLC/MSMS. Sterigmatocystin (molecular weight 324.1 g/mol) was identified and quantified with a precursor ion (325.159 dalton (Da), $[M+H]^+$) and two product ions with molecular weights of 310.086 and 280.964 Da at collision energy of 30 and 26 kV, respectively. The cone voltage suitable for this ionization was 50 kV.

## Endotoxin analysis

Endotoxin potency (EU: endotoxin unit) of each sample was determined using kinetic chromogenic *Limulus* amoebocyte lysate (LAL; Associates of Cape Cod, Inc., Falmouth, MA, USA) assay method. Reference standard endotoxin (lot # HOK354, *E. coli* O113:H10 strain) was purchased from the US Pharmacopeia (Rockville, MD, USA). All glassware used for the assay was baked at 260 °C for 2-2.5 hours to remove endotoxin and a sterile technique was applied to prevent cross contamination during the assay. Prior to the assay, 46.7 to 58.0 mg of conidia laden rice grains or filters were extracted in 2.5 ml or 3.0 ml of triethylamine phosphate buffer, respectively, by sonicating in an ultrasonic water bath sonicator (Bransonic® Model 5510, Branson Ultrasonics Corp., Danbury, CT, USA) for 1 hour. Serial dilutions were prepared for all sample extracts and standards in LAL reagent water (LRW). A 50 µl aliquot of each of the serial dilutions and negative controls were loaded onto a 96 well microplate in duplicate, and 50µl of LAL reconstituted with Glucashield® buffer (Associates of Cape Cod, Inc., Falmouth, MA, USA) was added to all standards, samples, and negative controls. Immediately after loading, the microplate was placed in a pre-incubated plate reader (ELx808IU™, Biotek Instruments, Inc., Winooski, VT, USA) at 37 °C and shaken for 30 seconds. The optical density (OD) was recorded at 405 nm every 30 seconds for 120 minutes. The onset time was defined for each well as the time when the OD reading for the well exceeded and remained higher than the threshold (OD=0.02). A parallel-line estimation method [31] was used to estimate endotoxin potency relative to reference standard endotoxin for each sample.

## Conidia aerosolization

The inhalation exposure system used in these studies was previously adapted from the Pitt 3 generator and utilized by NIOSH to allow nose-only exposure of mice to fungal conidia [24, 32, 33]. Clumps of *A. versicolor* covered rice grains were broken apart by hand in an antistatic bag (LJY, Brighton, CO, USA) and

placed in an acoustical particle generator. The acoustical generator system (AGS) produced sound energy to vibrate the conidia-laden-rice inside a polycarbonate tube (12-inch diameter x 18-inch height) that had both ends capped with thin latex rubber. Vibrations were controlled by custom software that delivered a 15 Hz sine wave to an amplifier. This resulted in the aerosolization of the conidia off the rice, which then mixed with air (7 L/min) and passed into the nose-only exposure chamber and pods. An empty nose-only exposure pod was used to collect samples onto 25 mm 0.2 µm pore size Whatman polycarbonate filters (Whatman/GE Healthcare, Chicago, IL, USA) for field emission electron microscopy, mycotoxin quantification, as well as to determine the aerodynamic size of the particles (APS, TSI, Inc., Shoreview, MN, USA). Additionally, a light scattering device, DataRAM4 (ThermoElectron Co., Franklin, MA, USA), was used to provide a real-time estimate of the mass concentration of *A. versicolor* spore within the chamber. Gravimetric determinations (37 mm cassettes with 0.45 µm pore-size Teflon filters, 1.5 l/min. sample flow) were used to calibrate and verify the Data RAM4 readings during each exposure run. The temperature and humidity were monitored and recorded in real time during each exposure using a probe (HMP60, Vaisala Inc., Louisville, CA, USA) that was adapted to an empty mouse pod. Images of conidia recovered from filters were acquired using a Hitachi S-4800 Field Emission Scanning Electron Microscope (Hitachi, Tokyo, Japan).

## Murine nose-only inhalation exposures

Five- to 6-week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed in HEPA filtered, plastic cages. Mice had *ad libitum* access to Rodent NTP-2000 diet (Zeigler Bros., Inc., Gardners, PA, USA) and water. All procedures performed on animals were approved by the National Institute for Occupational Safety and Health (NIOSH) Animal Care and Use Committee. Several experimental designs were utilized in this manuscript, as described below, and indicated in the corresponding result text.

### *Conidia deposition studies*

C57BL/6 mice were exposed to either HEPA-filtered air, viable *A. versicolor* conidia resulting in an estimated ($1x10^5$), or heat-inactivated *A. versicolor* conidia ($3.4x10^4$) spores deposited in the alveolar region of the mouse lung over a single 60 minute duration exposure. Animals were euthanized immediately following the completion of the exposure, via administration of a final dose of 100-300 mg/kg pentobarbital via intraperitoneal injection. As a secondary method of euthanasia, exsanguination was performed, and blood was collected via cardiac puncture of the right ventricle and placed in serum collection tubes. Lungs were inflated with neutral buffered formalin using a catheter inserted into the

trachea, tied off, and the lung was removed with the trachea intact. The tissue was then placed in a 15 mL tube containing 10 mL of 10% formalin for 24-48 hours.

### *Modified murine local lymph node assay (LLNA)*

For the modified local lymph node assay, five groups of C57BL/6 mice were exposed to either HEPA-filtered air, viable *A. fumigatus* conidia ($1x10^5$), or viable *A. versicolor* conidia ($1x10^4$, $1x10^5$, $5x10^5$) for 60 minutes for three consecutive days, followed by two days with no exposure. Mice were then injected intravenously via the lateral tail vein with 20 µCi 3h-thymidine (Dupont NEN, Waltham, MA; specific activity 2 Ci/mmol) 48 hours following the final exposure, then euthanized via $CO_2$ inhalation after five hours. The mediastinal lymph nodes were removed, homogenized between frosted microscope slides, and cell suspensions were prepared. Samples were incubated with 5% trichloroacetic acid overnight at 4°C and then mixed with scintillation fluid and counted using a Tri-Carb 2500TR liquid scintillation counter (PerkinElmer, Waltham, MA, USA). Stimulation indices (SIs) were calculated by dividing the mean disintegrations per minute (DPM) of the conidia-exposed mice by the mean DPM obtained from the air only control mice. Concentrations of *A. versicolor* that elicited a stimulation index (SI) greater than 3 were selected for sub-chronic exposures.

### *Dosimetry studies*

For the 4-week dosimetry studies, groups of C57BL/6 mice were exposed twice weekly for a total of 8 exposures, to doses per exposure of viable *A. versicolor*, or HEPA-filtered air. The per exposure doses included were $1x10^4$, $3x10^4$, $1x10^5$, or $5x10^5$ number of conidia deposited in the alveolar region of each mouse lung. Twenty-four hours following the final exposure, animals were humanely euthanized and dissected, as described below. Mice were weighed and euthanized using an administered dose of 100-300 mg/kg pentobarbital via intraperitoneal injection. As a secondary method of euthanasia, exsanguination was performed, and blood was collected via cardiac puncture of the right ventricle and placed in serum collection tubes. Following blood collection, tissues were collected for either histology or flow cytometry. In the mice used for histology, the right lung was inflated with 1 mL neutral buffered formalin using a catheter inserted into the trachea, tied off, and the lung was removed with the trachea intact. The tissue was then placed in a 15 mL tube containing 10 mL of 10% formalin for 24-48 hours. For flow cytometric analysis, bronchioalveolar lavage and lungs were collected. The BAL was collected by making a small nick in the trachea and using a catheter to wash the 2 mL total sterile PBS. The lung was then removed for flow cytometry processing as described below.

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## Histopathology

Right lungs from control and *A. versicolor* exposed mice were formalin-fixed and submitted to HistoTox Labs (Boulder, CO, USA) for routine processing. Each lung was paraffin embedded in a single block and one slide per block was sectioned and stained with hematoxylin and eosin (H&E). Glass slides were evaluated by a board-certified veterinary pathologist using light microscopy. Images were captured using an Olympus BX41 microscope with an Olympus SC180 camera. Lesions were given a severity score 0-5 (0 = not present/normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) [34].

## GMS staining

At necropsy, lungs were fixed by instillation with 10% neutral-buffered formalin, processed into paraffin blocks and sectioned at 5 microns. Lung sections were stained with Grocott's methenamine silver stain (GMS) for evaluating fungal deposition. GMS images were captured using an Olympus BX53 microscope in conjunction with an Olympus DP73 camera.

## Serum immunoglobulin quantification

Serum was collected from whole blood following cardiac puncture. Serum concentrations of total IgG, IgA, IgM, and IgE were quantified using Invitrogen ELISA kits, per the manufacturer's instructions (Thermo Fisher).

## Flow Cytometry

Whole lungs from control and *A. versicolor*-exposed mice were minced and digested in DMEM (Thermo Fisher) with 5% Fetal bovine serum (Thermo Fisher), 1mg/ml Collagenase/Dispase (Roche Diagnostics, Mannheim, Germany), and 30µg/ml Deoxyribonuclease I (Thermo Fisher) for 25 minutes at 37°C. Following digestion, a single cell suspension was obtained, and cells were incubated with normal rat sera (MilliporeSigma) and stained using rat anti-mouse CD16/32 (clone 2.4G2, BD Biosciences, San Jose, CA, USA) in PBS for 5 min at room temperature. Cells were then stained for 25 min at 4°C with an antibody cocktail containing PBS, 5% BSA, 2 mM EDTA and the following fluorochrome-conjugated antibodies: CD11b (clone M1/70), CD11c (clone N418), Ly6C (HK1.4), Ly6G (1A8), CD49b (clone DX5), CD3 (clone 17A2) (Biolegend, San Diego, CA, USA), Siglec F (clone E50-2440), CD103 (clone M290), CD19 (clone 1D3) (BD Biosciences). Antibody staining cocktail was washed away with FACS buffer. Cells were centrifuged (400xg, 5 min at 4°C) then fixed in the BD Cytofix Fixation Buffer (BD Biosciences) for 10 min at room temperature and washed FACS buffer and centrifuged. Cells were resuspended in FACS buffer and data were acquired

using LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).