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# Assessment of the safety and the anti-α-Gal immune response to an oral vaccine in captive-bred Humboldt penguins (*Spheniscus humboldti*)

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#### 35 Abstract

Oral administration of Escherichia coli O86:B7 expressing high levels of galactose-α-1,3-36 galactose (a-Gal) was previously shown to protect turkeys from aspergillosis caused by 37 Aspergillus fumigatus. In the present study, we first tested the basal level of anti- $\alpha$ -Gal antibodies 38 39 (Abs) in healthy Humboldt penguin (Spheniscus humboldti) sera and penguin egg yolks. We then conducted a trial to test whether modulation of α-Gal immunity by oral administration of the 40 probiotic bacterium Escherichia coli Nissle could prevent Humboldt penguins from aspergillosis. 41 To this aim, E. coli Nissle was administered orally to ten penguins and a placebo was 42 concomitantly given to a control group of ten other penguins. An aspergillosis diagnostic panel 43 (CT scans, capillary zone electrophoresis, 3-hydroxybutyrate levels, anti-A. fumigatus Abs 44 measurements) was implemented before, during and after the interventional procedure in both 45 46 penguin groups. As results, healthy penguins produced natural anti- $\alpha$ -Gal Abs that were also detected in the egg yolks. Surprisingly, oral administration of *E. coli* Nissle was associated with 47 48 lower levels of anti-a-Gal and anti-A. *fumigatus* Abs compared with the control group. Still, a correlation was seen between anti-E.coli Abs (IgM/IgY) and anti-a-Gal Abs (IgM/IgY), only in 49 50 the vaccinated penguins. E. coli Nissle is a safe probiotic to use in Humboldt penguins with potential protective effect against aspergillosis to be further tested. 51

#### 52 Keywords

Anti-α-Gal immunity, Aspergillosis, *Aspergillus fumigatus*, *Escherichia coli* Nissle, penguins,
Sphenisciformes, *Spheniscus humboldti*, vaccine, zoo

55

#### 56 Introduction

57 Several non-pathogenic or pathogenic agents present the glycan galactose- $\alpha$ -1,3-galactose 58 ( $\alpha$ -Gal) epitope at their surface, including *Aspergillus fumigatus*, *Plasmodium* sp. or 59 *Mycobacterium marinum*. Birds, as well as humans or fish, lack endogenous expression of  $\alpha$ -Gal, 60 but can naturally produce high levels of anti- $\alpha$ -Gal antibodies (Abs) against the in response to

antigenic stimulation from the microorganisms of the gut microbiota (REF). (REF). Then, one 61 could legitimately assume that boost of the antibody production following the vaccination with 62  $\alpha$ -Gal antigen provided by massive input of digestive bacteria could thus prevent from the 63 aforementioned debilitating infectious diseases. Indeed, captive-bred penguins are at risk to 64 develop life-threatening *Plasmodium* sp. infections (**REF**); mycobacteriosis occurs sporadically 65 in colonies of captive penguins (**REF**), and aspergillosis is often acknowledged as the main cause 66 of death in zoological institutions. Regarding the latter, yearly fatal cases concern approximately 67 10% of the colony at ZooParc de Beauval in France and can represent 27% of all cause-mortality 68 69 in some penguin colonies. Fungal loads in the environmental of captive-penguin settings are often quite high in, making immunosuppressed or stressed individuals particularly at risk of 70 71 developing clinical aspergillosis, especially during the summer season (July-September) in the 72 northern hemisphere. Treatment of aspergillosis in clinically infected penguins relies primarily 73 on azole drug-based therapy. Alternatives include terbinafine, amphotericin B, or other antifungals. However, clinical signs are non-specific, the diagnosis in laboratory is challenging, 74 75 and all together the initiation of the adequate antifungal therapy is consequently often delay. Besides, voriconazole toxicity has been described in penguin species and resistance of 76 Aspergillus sp. strains to azole molecules is reported from low to high prevalence depending on 77 the zoological institutions. For all these reasons, medical prophylaxis against aspergillosis is 78 paramount in penguins. 79

Oral administration of the bacterium Escherichia coli O86:B7 with high α-Gal content 80 elicited an anti-a-Gal Abs response associated with protection against malaria transmission in 81 al,3GT-deficient mice (XXX). In another study, probiotic formulations including Aeromonas 82 veronii and Pseudomonas entomophila with high content of  $\alpha$ -Gal, were biosafe, increased the 83 84 levels of anti-a-Gal IgM and protect against M. marinum in a zebrafish model (XXX). : Protective mechanisms that regulate immunity and metabolism activated in response to 85 86 probiotics included modification of the gut microbiota composition, B-cell maturation, anti-α-Gal antibodies-mediated control of mycobacteria, while they induced innate immune responses, 87 88 beneficial effects on nutrient metabolism, and reduced oxidative stress (XXX). In addition, recent research showed that oral administration of E. coli O86:B7 preventing turkeys from developing 89 90 aspergillosis after an experimental infection with A. *fumigatus* (**REF**). However, the protective effect of E. coli O86:B7 was not associated with an increase in circulating anti- $\alpha$ -Gal IgY levels, 91 92 but with a striking reduction of anti-α-Gal IgA in the lungs of infected turkeys (**REF**). Animals treated with E. coli O86:B7 experienced lower lung inflammation, which was associated with no 93

94 activation of pro-inflammatory cytokine genes (i.e., *IFN* $\gamma$ , *IL6*, *IL2*) in *A. fumigatus*-infected 95 turkeys. In the light of this evidence, probiotic-based vaccines have been proposed as an 96 alternative to activate protective mechanisms associated to the  $\alpha$ -Gal immunity for the control of 97 infectious diseases in fish, birds and humans (XXXX).

98 Developing an anti- $\alpha$ -Gal oral vaccine opens the perspective to protect Sphenisciformes against aspergillosis, malaria and mycobacteriosis. (**REF**). In this study, we used the probiotic *E*. 99 coli Nissle to modulate the anti-a-Gal immunity in penguins at risk of natural A. fumigatus 100 infection at ZooParc de Beauval. The specific objectives of this study were to determine the 101 102 baseline levels of anti-α-Gal Abs in Humboldt penguins and penguin eggs, to test whether oral administration of *E. coli* Nissle is safe and if it modulates the anti-α-Gal immunity in penguin. 103 104 Besides, we compared aspergillosis-related morbidity and mortality rates between vaccinated vs. non-vaccinated penguins during a one-year period post-vaccination. 105

106

#### 107 Materials and Methods

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#### 109 Study population

Sera of ten Humboldt penguins (penguins n°1-10, Table 1) were used to develop the serological assay. For the vaccine trial, twenty adult penguins were randomly chosen in the colony of 123 individuals from ZooParc de Beauval (Table 1), and assigned to either the vaccine group (n = 10, penguins n°11-20) or the placebo group (n = 10, penguins n°21-30). Penguins were individually identified with color rings. All study animals were considered healthy at inclusion, based on medical records, initial complete clinical examination and baseline plasma electrophoretic profile.

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#### 118 **Probiotic bacteria used in the study**

Animals in the vaccine group received orally the *Escherichia coli* Nissle 1917 contained in gastro-resistant hard capsules  $(2.5 - 25 \times 10^9 \text{ viable cells (CFU) per capsule)}$  of the product Mutaflor (DSM 6601, Pharma-Zentrale GmbH, Herdecke, Germany). Animals in the placebo group were given vegetable empty capsules made with pullulan. These capsules can hold approximately 680ul for liquid solution or 270-680mg for powder antigens (YourSupplements,Bredbury, Stockport, UK).

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#### 126 Vaccination trial design and oral administration of bacteria

Before starting the vaccine trial, its safety was preliminarily assessed using two supplemental 127 penguins (not included in either aforementioned study groups). One of them was given an oral 128 dose of vaccine and the other one the placebo, and both individuals were closely monitored by 129 zookeepers for three weeks. No adverse effect was noticed. Oral administration of both the 130 131 vaccine and the placebo were considered safe in the Humboldt penguin and the trial was started. The drug or the placebo was orally administrated every Monday and Wednesday for three 132 consecutive weeks (Table 1). Free-food sessions in the large enclosure of the Humboldt penguins 133 were used to give the vaccine and placebo to study animals. Penguins from the vaccine group 134 were individually given a vaccine-baited thawed sprat, while penguins of the other groups were 135 136 given the placebo similarly.

137

#### 138 Blood samplings, and blood specimen analyses

Three sampling sessions were performed at T-1mo (1 month before the beginning of the 139 vaccination protocol), T1mo (1 month after), and T3mo (3 months after) (Table 1). Penguins 140 were manually restrained and 2-to-2.5ml of blood were sampled from either the medial 141 metatarsal vein or the dorsal coccygeal vein using a 2.5ml plastic syringe and a 22-g needle. For 142 143 each penguin, 0.5ml of whole blood was immediately transferred to EDTA-tubes (Tube BD® Vacutainer® Silicone EDTA 4ml), homogenized, then transferred to a dry tube containing 0.5ml 144 of perchloric acid, homogenized again and stored at 4°C until further processing. Another 0.5ml 145 was also immediately transferred to heparin-lithium tubes (Tube BD® Vacutainer® Silicone 146 lithium heparin 4ml) and homogenized. The remaining amount of fresh blood (1-to-1.5ml) was 147 then transferred into a plastic tube for serum with coagulation activator (Tube BD® Vacutainer® 148 Silicone dry tube 4ml). All tubes were centrifuged at 5000rpm for 15 minutes twice. Capillary 149 zone electrophoretic profiles were obtained from the fresh heparin-lithium plasma samples using 150 the Minicap® automated system (Sebia, Lissex, 91008 Evry Cedex, France). For each plasma 151 sample, total protein concentration was determined using a chemistry analyzer (Catalyst One, 152

Idexx Laboratories, Inc., Westbrook, Maine 04092 USA). Serum was also extracted, and storeduntil further processing.

The supernatants obtained from the EDTA-blood + perchloric acid tubes were stored in cryotubes (Thermo Fischer Scientific, 91140 Les Ulis, France) at -20°C. Once the three sampling sessions were completed, samples were thawed and 3-hydroxybutyrate (3-OHB) concentration was measured using the RX Daytona+® instrument with Randox® reagents (Randox Laboratories, Kearneysville, WV 25430, USA) as previously described.

160

#### 161 Computed tomography

162 The twenty penguins of the trial were brought to the on-site veterinary hospital for computed tomography imaging (PHILIPS Brilliance 64 CT Scanner) at two time points (T-1mo, T3mo) 163 164 (Table 1). Penguins were placed in a 40cm-high, 18cm-large PVC-tube fixed on a rigid plate. During the first session, 13 penguins underwent conscious, standing scanning using a device 165 166 inspired by Rivas et al. (Figure 1), while the remaining seven underwent conscious CT scanning in dorsal recumbency. During the two sessions, only two penguins were too fat to fit the PVC-167 168 tube and were anesthetized with isoflurane delivered by facemask to perform a dorsal recumbent 169 CT scan (Table 1). A binary scoring system was created to assess aspergillosis-related lesions (Supplementary Table S2). Six reading categories were considered: right lung nodules, left lung 170 nodules, air sac nodules, air sac plaques, trachea nodules, and trachea plaques. For each category, 171 the presence (1) or absence (0) of lesions was recorded for each penguin at T-1mo and T3mo. CT 172 scans were blindly read (vaccination status not available), separately by two ECVDI diplomates. 173 Both readings were then harmonized to a collegiate, unique score for each CT scan. Size (mm) 174 and number of each lesion were also recorded. 175

176

#### 177 Aspergillus fumigatus culture

178 The highly germinative *A. fumigatus* CBS 144.89 (CEA10) clinical strain was used. *Aspergillus* 179 *fumigatus* culture was performed on Sabouraud dextrose agar (SDA), supplemented with 180 chloramphenicol (5 mg/L) and incubated at 37 °C for 10 days. Sub-cultures were performed 181 twice a week and *A. fumigatus* colonies were grown for 2–3 days at 37 °C. Conidia were 182 subsequently harvested by resuspension in PBS with 0.01% tween (PBST), filtered in a 70  $\mu$ m diameter nylon cell strainer (ClearLine Dominique Dutscher, Brumath, France), washed by centrifugation at  $3500 \times \text{g}$  for 10 min, resuspended in with 0.01% tween and then counted using a Malassez counting chamber. *Aspergillus fumigatus* suspension containing  $4 \times 10^7$  conidia was resuspended in 500 µl PBST. All reagents used for the fungus preparation were apyrogenic.

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#### 188 Escherichia coli strains culture

The bacterial strains E. coli Nissle 1917 (mutaflor strain), E. coli O86:B7 (ATCC 12701), E. coli 189 ClearColi® BL21(DE3) (Lucigen, Middleton, Wisconsin, USA), and E. coli BL21 (DE3, 190 191 Invitrogen, Carlsbad, CA, USA) were grown on 20 mL of Luria Broth (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C overnight to OD600 = 0.8. Bacteria were washed twice with phosphate 192 buffer saline (PBS): 10 mM NaH2PO4, 2.68 mM KCl, 140 mM NaCl, pH 7.2 (Sigma-Aldrich, 193 St. Louis, MO, USA), centrifuged at 1000× g for 5 min at 4 °C, and resuspended in 500 µl lysis 194 buffer, PBS with 1% triton (PBST, Sigma-Aldrich, St. Louis, MO, USA). All reagents used for 195 196 bacterial preparation were apyrogenic.

197

#### **198** Determination of α-Gal on *E. coli* strains by flow cytometry

199 Different *E. coli* strains were washed three times in PBS and fixed 30 minutes in 4% 200 paraformaldehide. Then, the bacteria were incubated with monoclonal mouse anti- $\alpha$ -Gal antibody 201 (mAb) M86 to  $\alpha$ -Gal (Enzo Life Sciences, Farmingdale, NY, USA) diluted 1:100 in PBS. FITC-202 goat anti-mouse IgM (Sigma-Aldrich, St. Louis, MO, USA) labelled antibody (diluted 1:1000 in 203 PBS 2 h at RT) was used as a secondary antibody. The mean fluorescence intensity (MFI) was 204 determined by flow cytometry and compared between test and control cells.

205

#### 206 Bacterial and fungal protein extraction

*Escherichia coli and A. fumigatus* proteins were extracted with six steel balls using the homogenizer Precellys®24 Dual (Bertin, France) at 5000× g for 20s, 2 times on lysis buffer. The homogenized was centrifuged at 200xg for 5 minutes and the supernatant were collected and quantified with BCA Protein Assay (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as standard. 212

#### 213 Evaluation of the specificity of anti-turkey and anti-chicken IgY for penguin IgY

To evaluate the specificity of anti-turkey and anti-chicken IgY against penguin IgY, plates 214 (Nunc-ImmunoTM Plate, Thermo Scientific, Waltham, MA, USA) were coated with α-Gal 215 linked to HSA (HSA- $\alpha$ -Gal, 50ng/well, Dextra Laboratories, Reading, UK) diluted in 216 carbonate/bicarbonate buffer (0.05 M, pH 9.6) and incubated overnight at 4 °C. Wells were 217 washed three times with 100 µL of PBST and then blocked with 100 µL of 1% HSA/PBS for 1 h 218 at RT. After three washes, serum samples, diluted 1:200 in PBS, were added to the wells and 219 220 incubated for 1 h at 37 °C. The plates were washed three times and HRP-conjugated goat antiturkey IgY (Mybiosource) or goat anti-chicken IgY (Sigma-Aldrich, St. Louis, MO, USA) Abs 221 were added at dilution 1:1500 in PBS (100 µL/well) and incubated for 1 h at RT. Plates were 222 223 washed three times and the reaction was developed by adding 100 µL ready-to-use TMB solution (Promega) at RT for 20 min in the dark, and then stopped with 50 µL of 0.5 M H<sub>2</sub>SO<sub>4</sub>. The OD 224 was measured at 450 nm using a Multiskan FC ELISA reader (Thermo Scientific, Waltham, MA, 225 USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was 226 subtracted from the reads. The cut-off was determined as two times a mean OD value of the 227 blank controls. 228

229 Total chicken and penguin IgY were loaded on a 4-15% Mini-protean TGS stain-free gel (Bio-Rad, Hercules, USA) and separated by SDS-electrophoresis 1h at 120V by duplicated. Proteins 230 were stained with Coomassie blue on one gel, and the other gel was transferred to Trans-Blot 231 Turbo Transfer Packs membrane (Bio-Rad, Hercules, USA). The membrane was blocked 2 hours 232 with 1% Human Serum Albumin (HSA) (Sigma)/PBS at room temperature (RT), washed three 233 times with PBST 0.05% and incubated at 4°C overnight with HRP-conjugated goat anti-turkey 234 IgY (Mybiosource, San Diego, CA, USA), or goat anti-chicken IgY (Sigma-Aldrich) antibodies 235 (Abs) dilution 1:400 in PBS. Immunoreactive proteins were visualized with TMB solution 236 (Promega, Madison, WI, USA). 237

238

#### 239 Indirect ELISA for quantification of Abs against α-Gal, A. fumigatus and E. coli

240 To measure the levels of Abs against  $\alpha$ -Gal, A. fumigatus and E. coli in penguin sera, plates

241 (Nunc-ImmunoTM Plate, Thermo Scientific, Waltham, MA, USA) were coated with  $\alpha$ -Gal-HAS,

A. fumigatus or E. coli (50ng/well) proteins, respectively. Sera collected at T-1mo, T1mo and

T3mo (Table 1) were diluted to 1:200 with phosphate buffered saline (PBS). Plates were blocked 243 by adding 100 µL of 1% HSA/PBS for 1 h at RT. After three washes, samples diluted were added 244 and incubated for 1 h at 37 °C. The plates were washed three times and horseradish peroxidase 245 (HRP)-conjugated goat anti-chicken IgY or IgM (Sigma-Aldrich, St. Louis, MO, USA) were 246 added at 1:1500 dilution in 1% HSA/PBS (100 µL/well) and incubated for 1 h at RT. The plates 247 were washed three times and the reaction was developed by adding 100 µL ready-to-use TMB 248 solution (Promega, Madison, WI, USA) at RT for 20 min in the dark, and then stopped with 50 249 µL of 0.5 M H<sub>2</sub>SO<sub>4</sub>. The OD was measured at 450 nm using Multiskan FC ELISA reader 250 251 (Thermo Scientific, Waltham, MA, USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was subtracted from the reads. The cut-off was determined as two 252 253 times a mean OD value of the blank controls

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#### **255** Competitive ELISA for anti-α-Gal Abs levels determination

256 The capacity of penguin sera to inhibit the binding of mAb M86 (Enzo Life Sciences, Farmingdale, NY, USA) was measured by a competitive ELISA (ref), with modifications. 257 258 Briefly, dilutions of penguin sera were prepared (1:200, 1:80, 1:20 and 1:5) and added to  $\alpha$ -Gal antigen simultaneously with M86. Binding of penguin sera to  $\alpha$ -Gal was measured as M86 259 260 binding inhibition percentage (%). Penguin sera were mixed with mAb M86 and both Abs were added simultaneously to plates coated with  $\alpha$ -Gal-BSA (200 ng/well, Dextra Laboratories, 261 Reading, UK). The goat anti-mouse IgM-HRP (Sigma-Aldrich, St. Louis, MO, USA) was used 262 as secondary antibody and the OD was measured at 450 nm with a Multiskan FC ELISA reader 263 (Thermo Scientific, Waltham, MA, USA). Duplicates were used for each sample. The average 264 value of the blanks was subtracted from all reads before further analysis. The M86 binding 265 inhibition % was calculated as (([OD. M86 mAb alone] - [OD. M86 mAb with penguin 266 sera])/[OD. M86 mAb alone])  $\times$  100. 267

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#### 269 Total IgY purification from penguin egg yolk

Penguin egg yolk and egg white were separated. Total egg IgY was purified with IgY Purification Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, egg yolk was weighted and mixed with a volume of cold delipidation reagent equal to five times the egg yolk weight. The mix was incubated for two hours at 4°C, and then centrifugated 20 minutes at 10,000xg in refrigerated centrifuge. The supernatant was recovered and an equal volume of cold IgY precipitation reagent was added and incubated for two hours at RT. After 20 minutes of centrifugation at 10,000xg in a refrigerated centrifuge, the pellet was collected and a volume of PBS equal to the original volume of the egg yolk was added. Total proteins were quantified using BCA Protein Assay (Thermo Scientific, Waltham, MA, USA) with BSA as standard.

280

#### **281** Prediction of α-1,3-galactosyltransferase genes in penguin microbiota

In humans (XX), birds (XX) and fish (XX), the production of anti- $\alpha$ -Gal Abs has associated with the presence of bacteria expressing  $\alpha$ -Gal in the gut microbiota. Based on the finding of anti- $\alpha$ -Gal Abs in healthy penguins, we hypothesized that penguin bacterial microbiota has bacteria with  $\alpha$ 1,3GT genes. To partially test this hypothesis, the presence and abundance of  $\alpha$ 1,3GT genes in the penguin microbiome was inferred from 16S rRNA data using the bioinformatics pipeline PICRUSt2 (XXX).

For the prediction of the functional profile of the microbiome of penguins, published and 288 publicly accessible 16S datasets were used. We considered to include the Humboldt penguin 289 290 (Spheniscus humboldti), the penguin species of this study; however, no dataset was found. Four species of penguins were included in this preliminary study: the Macaroni penguin (Eudyptes 291 chrysolophus), the Little penguin (Eudyptula minor), the Gentoo penguin (Pygoscelis papua) and 292 the King penguin (Aptenodytes patagonicus) from Dewar et al. 2013 XX (PRJEB3083). After 293 downloading from SRA repository XX, the raw sequences were introduced and analyzed using 294 the QIIME2 software pipeline (v. 2021.4) XX The fastq files were first demultiplexed and 295 filtered for quality control using DADA2 pipeline XX implemented in QIIME2. After, reads 296 were denoised into amplicon sequence variants (ASVs) and taxonomy was assigned to ASVs 297 using a pre-trained Naive Bayes classifier XX from SILVA database (release 138) XX. 298

PICRUSt2 XX was employed for predictions of functional metabolic pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) XX. Briefly, ASVs were placed into a reference tree containing 16S rRNA sequences from prokaryotic genomes, which is then used to infer gene family copy numbers from ASVs dataset. After, gene family abundance per sample and pathway abundances were determined. The analysis was focused on the detection of the presence and abundance of  $\alpha 1,3$  galactosyltransferase genes and the bacterial taxa, from penguin microbiome, contributing to these genes. Specifically, we tested the presence and abundance of  $\alpha$ 1,3GT genes (i.e., K02450, K02847, K03275, K03279, K03276, and K03278) in the microbiomes of the selected penguin species. Sankey diagram was performed in R studio XX using the networkD3 package.

309

#### 310 Statistical analyses

Statistical differences of parameters measured in penguins of each group were evaluated using paired and unpaired Student's *t*-test applied to individual paired and unpaired values, respectively. Pearson correlation was used to assess the correlation between anti- $\alpha$ -Gal and anti-*E. coli* Abs levels. All statistical analysis were performed in the GraphPad 9 Prism program (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when *p* < 0.05.

- 317 Ethics
- 318 ?
- 319 **Results**
- 320

#### 321 Specificity of anti-chicken and anti-turkey Abs for penguin Abs

Considering the lack of commercially available anti-penguin Abs to perform ELISA assays, we 322 323 assessed the specificity of anti-chicken and anti-turkey Abs for penguin Abs. Western blot analysis showed that anti-chicken IgY recognizes bands of similar size in chicken and penguin 324 IgY (Supplementary figure S1). In contrast, anti-turkey IgY recognized several bands in chicken 325 and penguin IgY (Supplementary figure S1). An indirect ELISA was used to measure the levels 326 of anti-α-Gal Abs using anti-chicken and anti-turkey IgY as secondary Abs. In agreement with a 327 non-specific recognition of anti-turkey IgY for penguin Abs, the OD values using the anti-turkey 328 IgY were higher than those using the anti-chicken IgY (data not shown). Based on the western 329 330 blot and ELISA results, we decided to use anti-chicken secondary Abs in the indirect ELISA assays to measure the levels of penguin IgM and IgY. 331

332

#### 333 Presence and variation of anti- $\alpha$ -Gal Ab in penguin sera and egg yolks

Two egg yolks and nine sera samples from healthy animals were included for testing the 334 occurrence of natural anti-a-Gal Abs in penguins. Characteristics of penguins included in the 335 study are listed in Table 1. The presence of total anti- $\alpha$ -Gal Abs was measured by competitive 336 337 ELISA. Total anti- $\alpha$ -Gal Abs were detected in sera samples from healthy animals, and also in a penguin with a suspected natural Aspergillus fumigatus infection (Figure 2A). The levels of total 338 anti-a-Gal Abs were higher in the serum of the penguin with suspected aspergillosis compared 339 with that of the healthy animals (Figure 2A). The levels of penguin anti- $\alpha$ -Gal IgY was also 340 variable in this group of healthy animals (Figure 2B). However, the penguin with suspected 341 342 aspergillosis did not show the highest level of anti- $\alpha$ -Gal IgY. Total anti- $\alpha$ -Gal Abs (Figure 2C) and anti-α-Gal IgY (Figure 2D) were detected in two penguin eggs. Antibodies IgY specific to 343 344 Escherichia coli and the pathogen A. fumigatus were also detected in the egg yolks (Figure 2D).

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#### 346 Prediction of $\alpha 1,3$ -galactosyltransferase genes in penguin microbiota

347 More than twenty bacterial taxa harbor  $\alpha 1,3GT$  genes in the microbiome of the penguin species analyzed (Figure 3). However, the abundance of some of these taxa was low and thus contributed 348 349 little to the abundance of  $\alpha 1.3GT$  genes in the microbiome. The taxa with the highest contribution (above 8%) represented 8 % of the total number of taxa with these genes. All the 350 351 al,3GT genes were present in P. papua while in E. chrysolophus, E. minor and A. patagonicus, only some genes were found (i.e., K02450, K02847, K03278, K03279) (Figure 3). Among the 352 genes, the most frequently found were K02450 and K02847, especially in the Little penguin 353 (Eudyptula minor) which is the closest of the four species analyzed to the Humboldt penguin 354 from the vaccine trial, from a taxonomical approach. The taxa that most contributed to α1,3GT 355 genes in these birds were the bacterial genera Gallicola, PeH15, Fusobacterium and the bacterial 356 357 family Peptostreptococcales-Tissierellales. These results suggest a wide distribution of a1,3GT genes in penguin microbiota and we consider these results valid in S. humboldti by extension. 358

359

# 360 Oral administration of E. coli Nissle expressing α-Gal was associated with modulation of anti-α361 Gal Abs levels in penguin sera

Two penguins from the vaccine group took two extra doses (penguins  $n^{\circ}12$  and  $n^{\circ}20$ , Table 1), while another one missed the last dose due to difficulties in animal handling (penguin  $n^{\circ}18$ , Table 1). These three individuals were kept into the vaccine group for statistical analyses. However, one penguin (penguin n°24, Table 1) from the placebo group accidentally swallowed a
dose of vaccine and was excluded from statistical analysis.

The  $\alpha$ -Gal glycan was detected in protein extracts of *E. coli* Nissle and *E. coli* O86:B7 as assessed by flow cytometry (Table 2). This was not the case for *E. coli* 'clean', and *E. coli* BL21 strains, used as negative controls of  $\alpha$ -Gal production (Table 2). The average fluorescein isothiocyanate (FITC) absorption values were higher for *E. coli* Nissle and *E. coli* O86:B7 compared to those of *E. coli* 'clean' and BL21 strains (Table 2).

- Sera levels of total Abs against  $\alpha$ -Gal were measured by competitive ELISA as M86 binding 372 inhibition % in penguins that received E. coli Nissle or placebo orally. Three months (T3mo) 373 after the oral vaccination (Post-V), the penguins that received E. coli Nissle showed a significant 374 reduction (10.19 SD±12.05, 95 % IC 1.57-18.81) in total anti-α-Gal Abs compared with the 375 376 levels before (Pre-V) the oral vaccination (20.20 SD±11.31, 95 % IC 12.11-28.29) (Student's ttest p = 0.03, Figure 5A). No differences were observed in the levels of anti- $\alpha$ -Gal IgM (Figure 377 5B) and IgY (Figure 5C) Post-V compared with the levels Pre-V. On the other hand, the animals 378 in the placebo group showed no significant difference in the levels of total anti- $\alpha$ -Gal Abs 379 (Figure 5D) and anti- $\alpha$ -Gal IgY (Figure 5F) before and after administering the placebo (Figure 380 5D), while in the same group of animals the levels of anti-α-Gal IgM were significantly lower 381 Post-V (0.66 SD±0.49, 95 % IC 0.80-1.56) compared to Pre-V (2.22 SD±0.68, 95 % IC 1.69-382 2.74) (Student's *t*-test p = 0.003, Figure 5E). No significant differences were found in the levels 383 of total anti-α-Gal (Figure 5G), or anti-α-Gal IgM (Figure 5H) and IgY (Figure 5I) Pre-V or Post-384 V in any group. 385
- We then asked whether oral administration of E. coli Nissle elicits anti-E. coli Abs and, if yes, 386 whether a correlation existed between anti-E. coli and anti- $\alpha$ -Gal Abs levels. No significant 387 change was found in the levels of anti-E. coli IgM Pre-V and Post-V (Figure 5A), while anti-E. 388 coli IgY (Figure 5B) showed a significant increase in the penguins of the E. coli Nissle group. 389 However, the same pattern was found in the placebo group, no significant change in anti-E. coli 390 IgM Pre-V and Post-V (Figure 5C) and a significant increase in anti-E. coli IgY (Figure 5D). The 391 392 levels of anti-E. coli IgM (Figure 5E) and anti-E. coli IgY (Figure 5F) in the penguins of the E. 393 *coli* Nissle group were not different to those of the placebo group. Positive correlations between the levels of anti-*E*. *coli* IgM and anti- $\alpha$ -Gal IgM Pre-V (Pearson coefficient r=0.46, p = 0.04, 394 Figure 5G) and anti-E. coli IgY and anti-a-Gal IgY in the E. coli Nissle group Post-V (Pearson 395 coefficient r=0.63, p = 0.04, Figure 5H) were found. There was no correlation between levels of 396

anti-*E. coli* IgM or IgY and anti- $\alpha$ -Gal IgM or IgY in the placebo group Post-V or between anti-*E. coli* IgM and anti- $\alpha$ -Gal IgM in the *E. coli* Nissle group Post-V.

399

400 Oral administration of E. coli Nissle was not associated with changes in markers of Aspergillus
401 infection in penguin plasma

402 Oral administration of E. coli Nissle was not associated with significant changes in the prealbumin, albumin,  $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ , and  $\gamma$ -globulins concentrations and albumin/globulin (A/G) 403 ratio compared with the placebo group (Figure 6). Except for prealbumin, the levels of the above 404 405 plasma proteins did not change significantly Post-V in the E. coli Nissle or placebo groups 406 compared with the levels Pre-V (Supplementary Figure S2). In the placebo group, the levels of prealbumin were significantly lower Post-V (3.36 SD±2.95, 95 % IC 1.09-5.64) compared with 407 the levels Pre-V (6.56 SD±1.37, 95 % IC 5.50-7.62) (Student's *t*-test p = 0.01), but the level of 408 this plasma protein did not change significantly in the E. coli Nissle group (Supplementary 409 Figure S2). 410

The mean concentration of 3-OHB Post-V in penguins of the *E. coli* Nissle group was lower (540.3 SD±226.9  $\mu$ mol/L) than that in the placebo group (610 SD±259.1  $\mu$ mol/L), but the difference was not statistically significant (Student's *t*-test *P* > 0.05, Figure 7A). The levels of 3-OHB before and after the oral vaccination did not change significantly in the penguins of *E. coli* Nissle (Figure 7B) or placebo groups (Figure 7C). Individual values of 3-OHB per penguin are available as Supplementary Table S1.

417 The levels of IgM (Figure 7D) and IgY (Figure 7E) specific to A. fumigatus were not significantly different between groups (E. coli Nissle vs. placebo) Pre-V or Post-V. Oral 418 419 administration of E. coli Nissle was not associated with significant changes in anti-A. fumigatus IgM levels before and after the treatment, while the levels of this immunoglobulin Pre-V (2.29 420 421 SD±0.45, 95 % IC 1.94-2.63) decreased significantly Post-V (1.29 SD±0.22, 95 % IC 1.12-1.46) in the penguins of the placebo group (Student's *t*-test p = 0.0001, Figure 7F). No significant 422 423 changes were observed in the levels of anti-A. fumigatus IgY Pre-V and Post-V in the E. coli Nissle or placebo groups (Figure 7G). 424

425

426 Oral administration of E. coli Nissle was not associated with pulmonary lesions in penguins

427 No pulmonary lesion was observed in any of the penguins included in the study (Supplementary428 Table S2).

429

430 Morbidity and mortality comparison

#### 431 TO BE COMPLETED AT THE END OR DELETED

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#### 433 Discussion

434

#### 435 Aspergillosis and diagnostic tools in penguins

Clinical presentation of aspergillosis varies from acute cases involving the lungs to 436 437 chronic, disseminated infections of the air sacs in infected birds. Mycelial masses called aspergillomas may develop within the respiratory tract, as well as fungal plaques resulting in 438 439 thickening of avian air sacs. Consequently, computed tomography (CT) is an interesting diagnostic modality for respiratory issues in penguins and was therefore included in the 440 441 diagnostic panel for aspergillosis assessment in the study penguins. Physiological alterations in 442 lung (density and volume) and air-sac (volume) readings have been associated with dorsal recumbency in Humboldt penguins undergoing CT, while no difference was seen in conscious vs. 443 anesthetized individuals. Thus, decision was made to perform CT in non-anesthetized penguins 444 in a standing positioning, as previously described. Despite increased motion artifact in some 445 penguins, assessment of the whole respiratory tract and scoring of aspergillosis-related lesions 446 were possible in all but two penguins (Supplementary Table S2). Dorsal recumbency and/or 447 anesthesia were only used as a last resort in very few individuals (Table 1), and this technique is 448 considered safe and reliable and is routinely used for assessment of the respiratory tract of 449 450 penguins at ZooParc de Beauval.

451 Definitive diagnosis of aspergillosis relies on definitive fungal identification (by fungal 452 culture or PCR) and typical morphological and histological lesions. *Ante-mortem* diagnosis 453 remains difficult, and a multi-tools analysis is required. Plasma electrophoresis offers valuable 454 data in infected penguins, especially the  $\beta$  globulin fraction containing the fibrinogen and 3-OHB 455 levels. For these reasons, capillary zone electrophoresis assay and measurement of 3-OHB levels were added to the diagnostic panel in this study (Table 1). Despite being a sometimes useful
assay in selected avian species such as psittacine birds, the s=galactomannan assay is not reliable
in penguins and was not used in the study.

459 Three months after the beginning of the trial, vaccinated penguins showed lower plasmatic 460 levels of 3-OHB compared to placebo penguins (Figure 7A, Supplementary Table S1), but no 461 statistical difference was seen. Whether the lack of significance is due to insufficient statistical power and the oral vaccine is associated with lower progression of subclinical Aspergillus sp. 462 463 infection as reflected by lower 3-OHB values would be overstated. Mean values were below previously reported values in healthy African penguins (Spheniscus demersus), a species closely 464 465 related to the Humboldt penguin. For accurate aspergillosis assessment in penguins, plasma 466 levels of 3-OHB should be interpreted together with  $\beta$ -globulins and  $\alpha$ -2 globulins in order to have a high specificity (> 90%) and a good negative predictive value ( $\geq$  80%). No significant 467 difference was seen in these electrophoretic fractions between penguin groups (Figure 6, 468 469 Supplementary Figure S2). These results suggest that none of the penguin had developed 470 aspergillosis during the three months of the trial, which is consistent with CT scan readings throughout the study period. As a consequence, differences observed between penguin groups in 471 Abs levels Post-V were only attributable to the oral vaccine compared to the placebo. 472

However, a significant reduction of the pre-albumin fraction was observed on the 473 electrophoretic profiles of non-vaccinated penguins after the vaccination trial (Supplemental 474 475 Figure S2). This reduction was not seen in the vaccination group. This finding suggests that 476 prealbumine concentrations decrease when globulin levels rise. The only relevant difference in 477 Abs levels Pre-V vs Post-V was a significant decrease of anti- $\alpha$ -Gal Abs in vaccinated penguins 478 and of anti- $\alpha$ -Gal IgM in non-vaccinated individuals (Figure 4). Consequently, a direct link between globulin levels and prealbumine concentrations seems highly unlikely in our study 479 population and the exact clinico-pathological relevance of this finding remains unknown. Still, a 480 link with non-humoral immunity pathways is hypothesized. Significantly lower prealbumin 481 values have been reported in falcons with confirmed aspergillosis. In humans, a similar trend was 482 seen in fatal cases of COVID-19. As a transport protein, the human prealbumin fraction (also so 483 called transthyretin) has various physiological effects, including anti-inflammatory actions and a 484 485 strongly hypothesized positive effect on the cell-mediated immunity, especially on immune cells from the myeloid compartment. Even if no statistical difference was seen in the prealbumin 486 487 values between penguin groups Post-V (Figure 6A), the significant reduction of prealbumin

488 levels Pre-V vs. Post-V in the non-vaccinated group (Supplementary Figure S2) may be 489 indicative of a reduced immunity against *Aspergillus* sp., linked to a decreased cell-mediated 490 immunity compared to vaccinated penguins. Whether the *E. coli* Nissle probiotic has a positive 491 effect on myeloid immune cells in penguins warrants further research.

492

# 493 Possible association between the occurrence of natural anti-α-Gal Abs and the gut 494 microbiome in penguins

495 ELISAs have been used to study the impact of the vaccine on the immune system of the 496 penguins. The competitive ELISA test also appears useful as a diagnostic tool in penguins and 497 could be implemented in the diagnostic panel for aspergillosis in penguins for further clinical 498 assessment. The serological assay has been specifically developed for the study, but other assays 499 already exist (XXX).

We found variable levels of total and IgY Abs specific to  $\alpha$ -Gal in healthy penguins 500 501 compared to one aspergillus-suspected penguin. Variation of anti-a-Gal levels in human sera have been found in healthy individuals (XXXX), and it has also been linked to infection by 502 503 pathogens such as *Trypanosoma cruzi* expressing  $\alpha$ -Gal on their surface (XXX). Sera of patients 504 with chronic Chagas disease caused by T. cruzi contain elevated levels of anti-a-Gal Abs that were lytic to this trypanosome species (XXXX). Based on these findings, a prophylactic  $\alpha$ -Gal-505 based glycovaccine was formulated and shown to effectively protect against murine acute 506 Chagas disease (XXXX). The presence of  $\alpha$ -Gal on the surface of A. fumigatus (XXXX) may 507 508 explain variations of anti- $\alpha$ -Gal Abs in A. fumigatus-infected birds. Differences in anti- $\alpha$ -Gal Abs may also be associated with individual bacterial composition in the gut microbiome, which have 509 510 been linked with the production of these natural Abs in humans (XXX), α1,3GT-deficient mice 511 (XXXX), and fish (XXXX). Production of anti- $\alpha$ -Gal Abs in humans is thought to be driven by exposure to microbiota bacteria of the *Klebsiella* spp., *Serratia* spp., and *E. coli* expressing α-Gal 512 (XXX). In agreement with this hypothesis,  $\alpha$ 1,3-GT genes were broadly distributed in several 513 514 bacterial species of the families Enterobacteriaceae and Pasteurellaceae as well as Haemophilus and Lactobacillus genera present in the human gut microbiome (XXX). In this study, some of 515 516 these bacterial taxa were found to contribute to the pool of  $\alpha$ 1,3-GT genes in the microbiome of the penguin E. chrysolophus, E. minor, P. papua and A. patagonicus. Due to the close 517 phylogenetic relationship of the genera Eudyptula and Spheniscus (XXXX), we propose that 518 al,3-GT genes are also broadly distributed in the S. humboldti microbiome, although this 519

remains to be experimentally tested. Individual differences in the gut microbiome of *S. humboldti* could explain the presence of anti- $\alpha$ -Gal Abs in healthy penguins. The positive correlation between the levels of anti- $\alpha$ -Gal and anti-*E. coli* IgM before the treatment further suggests a role of penguin gut microbiota in the production anti- $\alpha$ -Gal Abs.

524 It has been suggested that anti-Aspergillus Abs may be transmitted from females to penguin chicks, even if the role of these Abs in the immune defense against Aspergillus sp. 525 infection remains unclear. Interestingly, we found anti- $\alpha$ -Gal Abs in penguin egg yolks. Our 526 results show the presence and variability of anti- $\alpha$ -Gal Abs in eggs from two individual penguins, 527 528 like it was reported in Leghorn chicken (XXXX). Moreover, a functional role for these Abs has 529 been related to their binding potential to  $\alpha$ -Gal antigens in mammal tissues. Particularly, chicken 530 anti-a-Gal IgY block human anti-a-Gal Abs binding to xenograft endothelial cells and consequently block human blood complement- and antibody-dependent cell-mediated lysis 531 532 mechanisms that are responsible of hyperacute rejections in xenografts (XXXX). In addition to Abs specific to  $\alpha$ -Gal, we also found IgY specific to *E. coli* and *A. fumigatus* in penguin eggs. 533 534 Maternal IgY transported to the embryonic circulation have been suggested to participate in bird immunity (XXX). Difference in anti-α-Gal Abs in egg yolks may be associated with variations in 535 the gut microbiota and anti- $\alpha$ -Gal Abs levels of mother penguins. Whether anti- $\alpha$ -Gal Abs 536 transmitted from the mother to the eggs and offspring can protect against infectious diseases 537 remains an open question. 538

The role of microbiota in the induction of anti- $\alpha$ -Gal has been experimentally tested, and gut 539 colonization by *E. coli* O86:B7 elicited anti-α-Gal Abs in α1,3GT-deficient mice (XXX), humans 540 (XXXX), primates (XXXX), white Leghorn chicks (XXXX) and turkeys (XXX). Here we 541 showed that E. coli Nissle produce a-Gal levels similar to those found in E. coli O86:B7 and 542 543 higher than those found in α-Gal-negative bacteria, as measured by flow cytometry. In our previous study, we found that oral administration of E. coli O86:B7 elicited an IgY response to 544 545 the  $\alpha$ -Gal disaccharide (Gal $\alpha$ 1-3Gal), but decreased the IgY levels to the  $\alpha$ -Gal trisaccharide (Galα1-3Galβ1-4GlcNAc) in turkeys (XXX). However, in the present study, oral administration 546 of E. coli Nissle was not associated with significant changes in the levels of IgY specific to the a-547 Gal disaccharide (Figure 5C), but with a significant decrease in the levels of total anti- $\alpha$ -Gal Abs 548 549 in penguins of the E. coli Nissle group (Figure 5A). Notably, oral administration of E. coli Nissle 550 prevented a sharp decrease in anti-a-Gal IgM observed in the placebo group (Figure 5E). 551 Possible explanations for this are the difference in species (turkeys vs. penguins), the bacterial

dose (three administrations per week for three weeks in turkeys (XXX) vs. two administrations 552 per week for three weeks in penguins), or a differential cellular location of the  $\alpha$ -Gal epitope in 553 E. coli Nissle and E. coli O86:B7. The a-Gal epitope of E. coli O86:B7 is expressed on the 554 capsule or glycoprotein portion of the bacterial wall, rather than on the LPS molecules (XXX). 555 Other E. coli strains were found to express the glycan in the LPS molecules (XXX). These 556 findings suggest that the  $\alpha$ -Gal epitope can be located in the LPS, capsule or glycoprotein portion 557 of *E. coli*. The cellular fraction in which *E. coli* Nissle express α-Gal is currently unknown. The 558 results of the present study suggest that E. coli Nissle express a-Gal in a location that is not 559 560 accessible to the immune system to induce a significant increase of anti-α-Gal Abs. However, the positive correlation between the levels of anti-a-Gal and anti-E. coli IgY in the E. coli Nissle 561 562 group suggests that the immune response to  $\alpha$ -Gal is associated to that triggered by *E. coli*.

563

#### 564 Conclusion

565 E. coli Nissle oral probiotic is safe in the Humboldt penguin. The immune response to  $\alpha$ -Gal in the Humboldt penguin is closely related to that of E. coli. The oral vaccine tested failed to 566 567 produce a significant increase in anti- $\alpha$ -Gal Abs as we suspect *E. coli* Nissle to express  $\alpha$ -Gal in a location that is not accessible to the penguin's immune system. Nevertheless, a positive effect on 568 569 the cell-mediated immunity of vaccinated penguins may be hypothesized, as suggested by the significant decrease of the pre-albumin fraction in the placebo group only. Further study is 570 needed to assess a positive effect of E. coli Nissle oral probiotic on myeloid immune cells in the 571 572 Humbold penguin.

573

#### 574 **References**

#### 576 Figure legends

577

- 578 Figure 1. Computed tomography in a non-anesthetized, standing Humboldt penguin (n°13,
- **18806226).** Coronal (top right) and axial (bottom right) sections are presented. The pulmonary
- 580 parenchyma is easily visualized (asterisks), while walls of thoracic air sacs are also visible
- 581 (arrow). A three-dimensional reconstruction shows high definition of the lungs (in blue). No
- 582 lesions (nodules or plaques) are seen.



584

Figure 2. Variation of anti-α-Gal Abs in penguin sera and eggs. The levels of circulating total 585 anti-a-Gal Ab (A) and anti-a-Gal IgY (B) were measured by inhibition and indirect ELISA, 586 respectively. Total and IgY anti- $\alpha$ -Gal Abs were variable in the sera of healthy penguins (white 587 bars, ID 1-9). A serum sample of a penguin with suspicion of aspergillosis (pulmonary 588 589 granuloma seen on CT scan) was included in the analysis (red bar, ID 10). Total anti-α-Gal Ab 590 (C) and anti- $\alpha$ -Gal IgY (D) were measured by inhibition and indirect ELISA, respectively. Egg anti-a-Gal IgY specific to E. coli and A. fumigatus proteins were measured by indirect ELISA. 591 M86 binding inhibition % (A and C) and OD (C and D) means and standard deviation values of 592

technical replicates *per* sample are displayed. Characteristics of penguins included in the analysis 593 are listed in Table 1. 594





- 609 glucose/galactose:(glucosyl)LPS alpha-1,2-glucosyl/galactosyltransferase [EC:2.4.1.-]; K03278:
- 610 waaI, rfaI, UDP-D-galactose:(glucosyl)LPS alpha-1,3-D-galactosyltransferase [EC:2.4.1.44].



Figure 4. Variation in penguin anti-a-Gal Ab levels after oral administration of E. coli 612 613 Nissle. The levels of circulating total anti- $\alpha$ -Gal Ab (A) and anti- $\alpha$ -Gal IgM (B) and IgY (C) were measured by inhibition (A, D and G) and indirect (B-C, E-F and H-I) ELISA in the E. coli 614 Nissle (red dots) and placebo (black dots) groups before (Pre-V) and after (Post-V) the 615 vaccination protocol. Total anti- $\alpha$ -Gal Abs decreased in the sera of penguins vaccinated with oral 616 *E. coli* Nissle (A). Anti-α-Gal IgM decreased in the sera of penguins of the placebo group (E). 617 No significant differences were observed in the levels of anti-α-Gal Ab (G), anti-α-Gal IgM (H) 618 and IgY (I) when the two groups, E. coli Nissle and placebo, were compared Pre-V and Post-V. 619 Individual (A-I) and means and standard deviation (G-I) values are shown. Values were 620 compared by paired (A-F) or unpaired (G-I) student's t-test (\* p < 0.05, \*\* p < 0.005; ns: not 621 significant, 1 experiment, n = 10 in the *E. coli* Nissle group and n = 9 in the placebo group, and 622 three technical replicates per sample). 623



Figure 5. Levels of anti-E. coli Abs after oral administration of E. coli Nissle and their 625 association with anti-a-Gal Ab level. The levels of anti-E. coli IgM (A, C and E) and IgY (B, D 626 and F) were measured in penguin sera by indirect ELISA in animals of the E. coli Nissle (red 627 dots) and placebo (black dots) groups before (Pre-V) and after (Post-V) the vaccination protocol. 628 No significant differences were observed in the levels of anti-E. coli IgM within (A and C) and 629 between (E) groups. Significant differences were observed in the levels of anti-E. coli IgM 630 within (A and C), but not between (E) groups. Positive correlations between the levels of anti-E. 631 coli IgM and anti- $\alpha$ -Gal IgM Pre-V (Pearson coefficient r=0.46, p = 0.04) (G), and anti-E. coli 632 IgY and anti- $\alpha$ -Gal IgY in the *E. coli* Nissle group Post-V (Pearson coefficient r=0.63, p = 0.04) 633 (H) were found. Individual (A-H) and means and standard deviation (E and F) values are shown. 634 Values were compared by paired (A-D) or unpaired (E and F) student's *t*-test (\*\* p < 0.005; ns: 635 not significant, 1 experiment, n = 10 in the *E. coli* Nissle group and n = 9 in the placebo group, 636

and three technical replicates per sample, A-F). Values of both groups placebo and E. coli Nissle 637 were merged for the Pre-V anti-E. coli-anti- $\alpha$ -Gal IgM correlation analysis (n = 19). For the 638 correlation of anti-*E. coli*-anti-α-Gal *E. coli* Nissle Post-V IgY n = 10. 639



642 Figure 6. Levels of plasma proteins after oral administration of E. coli Nissle. The percentage of prealbumin (A), albumin (B),  $\alpha 1$  (C),  $\alpha 2$  (D),  $\beta 1$  (E),  $\beta 2$  (F), and  $\gamma$  (G) -globulins 643 644 concentrations and the albumin/globulin (H) ratio of animals in the E. coli Nissle (red dots) and placebo (black dots) groups Post-V are shown. Individual, means and standard deviation values 645 646 are shown. Values were compared by unpaired student's *t*-test (ns: not significant, 1 experiment, n = 9 in the *E*. *coli* Nissle group and n = 8 in the placebo group). 647



648

649 Figure 7. 3-hydroxybutyrate plasma levels and anti-A. fumigatus Abs after oral administration of *E. coli* Nissle. The concentration of 3-hydroxybutyrate (µmol/L) in the *E. coli* 650 651 Nissle (red dots) and placebo (black dots) groups were compared between groups (A) and within E. coli Nissle (B) or placebo (C) groups before (Pre-V) and after (Post-V) the vaccination 652 protocol. Anti-A. fumigatus IgM (D and F) and IgY (E and G) levels in penguin sera were 653 measured by indirect ELISA and compared between (D and E) and within (F and G) groups. 654 Individual (A-G) and means and standard deviation (A, D and E) values are shown. Values were 655 compared by paired (B, C, F and G) or unpaired (A, D and E) student's *t*-test (\*\* p < 0.005; ns: 656 not significant, 1 experiment, n = 10 in the *E. coli* Nissle group and n = 9 in the placebo group, 657 and three technical replicates per sample in the ELISA). 658



### 660 Supplementary materials

			3OHB (µmol/L)			
Group	Penguin n°	ID	Sex	« T-1mo »	« T1mo »	« T3mo »
	11	MIG12-16898112	F	539	617	416
	12	19152643	F	268	594	860
	13	QRD14-03650	F	302	688	232
	14	QRD14-03648	М	844	590	407
	15	22197091	М	359	332	379
Vaccine group	16	14923345	М	417	554	523
	17	QRD16-01361	F	775	517	461
	18	QRD18-04258	М	424	511	442
	19	QRD17-03052	М	616	468	909
	20	24773872	F	1029	809	774
	21	27535275	F	435	278	999
	22	MIG12-20638625	М	382	584	640
	23	18806226	F	363	224	231
	24*	19117651	М	536	208	550
	25	15226273	F	502	603	362
Placebo group	26	QRD16-00621	М	795	774	889
	27	18806231	F	685	454	417
	28	24773875	F	405	940	593
	29	QRD17-02318	М	473	391	514
	30	QRD15-04789	М	747	600	845

### 661 Supplementary Table S1. 3-hydroxybutyrate plasma levels in study penguins.

\* Penguin n°24 accidentally swallowed a dose of vaccine and was removed from statistical analyses

662

# 663 Supplementary Table S2. CT scan scorings of study penguins.

			Right lung nodules	Left lung nodules	Air sac nodules	Air sac plaques	Trachea nodules	Trachea plaques
	Penguin n°	CT exam n° (date)			Yes =	1; No = 0		
<i>coli</i> issle	1	23 (17/12/21)	0	0	0	0	0	0
$\mathbf{Z}$		24 (31/03/21°	0	0	0	0	0	0

group
lacebo

2	11 (17/12/20)	0	0	0	0	0	0
	12 (31/03/21)	0	0	0	0	0	0
3	25 (17/12/20)	0	0	0	0	0	0
	26 (31/03/21)	0	0	0	0	0	0
4	27 (16/12/20)	0	0	0	0	0	0
	28 (31/03/20)	0	0	0	0	0	0
5	21 (16/12/20)	0	0	0	0	0	0
	22 (31/03/21)	0	0	0	0	0	0
6	3 (16/12/20)	0	0	0	0	0	0
	4 (31/03/21)	0	0	0	0	0	0
7	29 (17/12/20)	0	0	0	0	0	0
	30 (31/03/21)	0	0	0	0	0	0
8	31 (17/12/20)	0	0	0	0	0	0
	32 (31/03/21)	0	0	0	0	0	0
9	19 (1712/20)	0	0	0	0	0	0
	20 (31/03/21)	0	0	0	0	0	0
10	13 (16/12/20)	0	0	0	0	0	0
	14 (31/03/21)	0	0	0	0	0	0
11	17 (16/12/20)	0	0	0	0	0	0
	18 (30/03/21)	0	0	0	0	0	0
12	33 (16/12/20)	0	0	0	0	0	0
	34 (31/03/21)	0	0	0	0	0	0
13	1 (16/12/20)	0	0	0	0	0	0
	2 (31/03/21)	0	0	0	0	0	0
14	9 (16/12/20)	0	0	0	0	0	0
	10 (31/03/21)	0	0	0	0	0	0
15	5 (17/12/20)	0	0	0	0	0	0
	6 (31/03/21)*	-	-	-	-	-	-
16	35 (16/12/20)	0	0	0	0	0	0
	36 (31/03/21)	0	0	0	0	0	0
17	7 (17/12/20)	0	0	0	0	0	0
17	0 (21/02/21)*	0	0	v	v	0	0
10	8 (31/03/21)*	-	-	-	-	-	-
18	15 (20/12/20)	0	0	0	0	0	0
	16 (21/02/21)	0	0	0	0	0	0
10	10(51/05/21)	0	0	0	0	0	0
19	57 (10/12/20)	0	0	0	0	U	0
	38 (31/03/21)	0	0	0	0	0	0
20	39 (17/12/20)	0	0	0	0	0	0
	40 (31/03/20)	0	0	0	0	0	0

- 665 Interpretation of CT scans n° 6 (penguins n°15) and 8 (penguins n°17) were not possible due to excessive 666 motion artifact.
- 667 Supplementary figure S1. Recognition of chicken and penguin IgY by anti-turkey and anti-
- 668 chicken IgY. Total IgY was purified from chicken and penguin sera. Purified chicken and
- 669 penguin IgY was run in an SDS-PAGE and transferred to membrane for the western blot reaction
- 670 using anti-turkey or anti-chicken IgY. Anti-turkey IgY recognizes several bands in chicken and
- penguin IgY, while anti-chicken IgY recognizes only one band in chicken and penguin IgY.



672

Supplementary figure S2. Levels of plasma proteins after oral administration of *E. coli* Nissle. The percentage of prealbumin (A), albumin (B),  $\alpha 1$  (C),  $\alpha 2$  (D),  $\beta 1$  (E),  $\beta 2$  (F), and  $\gamma$  (G) -globulins concentrations and the albumin/globulin (H) ratio in penguins were compared within *E. coli* Nissle (red dots) and placebo (black dots) groups before (Pre-V) and after (Post-V) treatment. Individual values are shown and were compared by paired student's *t*-test (\* *p* < 0.05; ns: not significant, 1 experiment, n = 9 in the *E. coli* Nissle group and n = 8 in the placebo group).



### 681 Tables

# Table 1. Animals included in the preliminary study, vaccination protocol, CT scans, andblood samplings.

Group	Penguin n°	ID	Sex	Date of sampling (frozen serum)	Clinical findings	Suspicion of aspergillosis
	1	QRD16- 00616	F	16/10/2017	Suspicion of ocular aspergillosis on 29/05/2016	Mild
	2	QRD16- 01010	М	16/10/2017	Suspicion of aspergillosis on 10/09/2016	Mild
Prelimary study: test of the competitive ELISA assay	3	QRD15- 05130	F	16/10/2017	-	Weak
	4	QRD16- 00603 F QRD16- 00994 F		16/10/2017	Suspicion of ocular aspergillosis on 18/07/2017	Mild
	5			16/10/2017	-	Weak
	6	QRD15- 05124	F	16/10/2017	-	Weak
	7	QRD16- 01002	М	16/10/2017	-	Weak
	8	QRD16- 00615	М	16/10/2017	-	Weak
	9	MIG12- 17845737	М	15/08/2020	Mild, recurrent bilateral corneal opacification	Mild
	10	18094427	F	27/10/2020	Suspicion of pulmonary aspergillosis (granuloma seen on the CT Scan)	Strong

Rest					Vaccine sessions						Restraint sessions	
				1 <sup>st</sup> Blood	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	5 <sup>th</sup>	6 <sup>th</sup>	2 <sup>nd</sup> Blood	3 <sup>rd</sup> Blood
Group	Penguin n°	ID	Sex	samplings #1 + CT #1	Monday 21/12/20	Wednesday 23/12/20	Monday 28/12/20	Wednesday 30/12/20	Monday 04/01/21	Wednesday 06/01/21	samplings #2	samplings #3 + CT#2
				« T-1mo »	PO	PO	PO	PO	PO	РО	« T1mo »	« T3mo »
	11	MIG12- 16898112	F	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	12	19152643	F	17/12/2020	ok	2 doses	ok	ok	ok	ok	14/01/2021	31/03/2020
	13	QRD14- 03650	F	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
Vaccine	14	QRD14- 03648	Μ	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	15	22197091	М	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
group	16	14923345	М	16/12/2020* <sup>©</sup>	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	17	QRD16- 01361	F	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	18	QRD18- 04258	М	17/12/2020	ok	ok	ok	ok	ok	no	15/01/2021	31/03/2020
	19	QRD17- 03052	М	17/12/2020	ok	ok	ok	ok	ok	ok	20/01/2021	31/03/2020* <sup>©</sup>
	20	24773872	F	16/12/2020	ok	ok	ok	2 doses	ok	ok	14/01/2021	31/03/2020
Placebo	21	27535275	F	16/12/2020	ok	ok	ok	ok	ok	ok	15/01/2021	31/03/2020
group	22	MIG12- 20638625	М	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020

23	18806226	F	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
24	19117651	М	16/12/2020	ok	ok	ok	ok + 1 dose of vaccine	ok	ok	14/01/2021	31/03/2020
25	15226273	F	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
26	QRD16- 00621	М	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
27	18806231	F	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
28	24773875	F	20/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
29	QRD17- 02318	Μ	16/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
30	QRD15- 04789	М	17/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020

684 \* dorsal recumbency was needed, as excited penguins refused to stay straight in the PVC tube when
 685 standing

686 <sup>©</sup> flash isoflurane anesthesia (facemask) was performed, as these penguins were too fat to be placed

appropriately into the PVC tube

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### **Table 2. Presence of α-Gal in** *E. coli* **Nissle measured by flow cytometry.**

<i>E. coli</i> strains	FITC (average)	FITC (SD)	Commentary	Reference		
	1536	9417	Test strain			
E. coli Nissle	143	130	Negative control for	N/A**		
			E. coli Nissle*			
	634	5374	Positive control of			
$E_{\rm col} = 0.000$			α-Gal production	DEE		
<i>E. coll</i> 080.D/	123	17	Negative control for	KEF		
			<i>E. coli</i> O86:B7*			
	218	839	Negative control for			
			$\alpha$ -Gal production.			
E seli alaan			This strain lacks	DEE		
E. coll clean			LPS.	KEF		
	200	581	Negative control for			
			<i>E. coli</i> clean*			
	128	47	Negative control for			
			$\alpha$ -Gal production.			
			This strain produces	DEE		
E. COll BL21			low levels of α-Gal.	KEF		
	124	38	Negative control for			
			E. coli BL21*			

## <sup>690</sup> \* FITC background value resulting from the secondary antibody alone.

691 \*\* Not applicable