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

3
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34

35 **Abstract**

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

52 **Keywords**

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

55

56 **Introduction**

57 Several non-pathogenic or pathogenic agents present the glycan galactose- α -1,3-galactose
58 (α -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 α -Gal,
60 but can naturally produce high levels of anti- α -Gal antibodies (Abs) against the in response to

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

80 Oral administration of the bacterium *Escherichia coli* O86:B7 with high α -Gal content
81 elicited an anti- α -Gal Abs response associated with protection against malaria transmission in
82 α 1,3GT-deficient mice (XXX). In another study, probiotic formulations including *Aeromonas*
83 *veronii* and *Pseudomonas entomophila* with high content of α -Gal, were biosafe, increased the
84 levels of anti- α -Gal IgM and protect against *M. marinum* in a zebrafish model (XXX). :
85 Protective mechanisms that regulate immunity and metabolism activated in response to
86 probiotics included modification of the gut microbiota composition, B-cell maturation, anti- α -Gal
87 antibodies-mediated control of mycobacteria, while they induced innate immune responses,
88 beneficial effects on nutrient metabolism, and reduced oxidative stress (XXX). In addition, recent
89 research showed that oral administration of *E. coli* O86:B7 preventing turkeys from developing
90 aspergillosis after an experimental infection with *A. fumigatus* (REF). However, the protective
91 effect of *E. coli* O86:B7 was not associated with an increase in circulating anti- α -Gal IgY levels,
92 but with a striking reduction of anti- α -Gal IgA in the lungs of infected turkeys (REF). Animals
93 treated with *E. coli* O86:B7 experienced lower lung inflammation, which was associated with no

94 activation of pro-inflammatory cytokine genes (i.e., *IFN* γ , *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 α -Gal immunity for the control of
97 infectious diseases in fish, birds and humans (XXXX).

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

106

107 **Materials and Methods**

108

109 **Study population**

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

117

118 **Probiotic bacteria used in the study**

119 Animals in the vaccine group received orally the *Escherichia coli* Nissle 1917 contained in
120 gastro-resistant hard capsules (2.5 - 25 x 10⁹ viable cells (CFU) per capsule) of the product
121 Mutaflor (DSM 6601, Pharma-Zentrale GmbH, Herdecke, Germany). Animals in the placebo
122 group were given vegetable empty capsules made with pullulan. These capsules can hold

123 approximately 680ul for liquid solution or 270-680mg for powder antigens (YourSupplements,
124 Brebury, Stockport, UK).

125

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

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

137

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

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

153 Idexx Laboratories, Inc., Westbrook, Maine 04092 USA). Serum was also extracted, and stored
154 until further processing.

155 The supernatants obtained from the EDTA-blood + perchloric acid tubes were stored in
156 cryotubes (Thermo Fischer Scientific, 91140 Les Ulis, France) at -20°C. Once the three sampling
157 sessions were completed, samples were thawed and 3-hydroxybutyrate (3-OHB) concentration
158 was measured using the RX Daytona+® instrument with Randox® reagents (Randox
159 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
163 tomography imaging (PHILIPS Brilliance 64 CT Scanner) at two time points (T-1mo, T3mo)
164 (Table 1). Penguins were placed in a 40cm-high, 18cm-large PVC-tube fixed on a rigid plate.
165 During the first session, 13 penguins underwent conscious, standing scanning using a device
166 inspired by Rivas et al. (Figure 1), while the remaining seven underwent conscious CT scanning
167 in dorsal recumbency. During the two sessions, only two penguins were too fat to fit the PVC-
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
170 (Supplementary Table S2). Six reading categories were considered: right lung nodules, left lung
171 nodules, air sac nodules, air sac plaques, trachea nodules, and trachea plaques. For each category,
172 the presence (1) or absence (0) of lesions was recorded for each penguin at T-1mo and T3mo. CT
173 scans were blindly read (vaccination status not available), separately by two ECVDI diplomates.
174 Both readings were then harmonized to a collegiate, unique score for each CT scan. Size (mm)
175 and number of each lesion were also recorded.

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 µm

183 diameter nylon cell strainer (ClearLine Dominique Dutscher, Brumath, France), washed by
184 centrifugation at 3500× g for 10 min, resuspended in with 0.01% tween and then counted using a
185 Malassez counting chamber. *Aspergillus fumigatus* suspension containing 4×10^7 conidia was
186 resuspended in 500 µl PBST. All reagents used for the fungus preparation were apyrogenic.

187

188 ***Escherichia coli* strains culture**

189 The bacterial strains *E. coli* Nissle 1917 (mutaflor strain), *E. coli* O86:B7 (ATCC 12701), *E. coli*
190 ClearColi® BL21(DE3) (Lucigen, Middleton, Wisconsin, USA), and *E. coli* BL21 (DE3,
191 Invitrogen, Carlsbad, CA, USA) were grown on 20 mL of Luria Broth (Sigma-Aldrich, St. Louis,
192 MO, USA), at 37 °C overnight to OD600 = 0.8. Bacteria were washed twice with phosphate
193 buffer saline (PBS): 10 mM NaH₂PO₄, 2.68 mM KCl, 140 mM NaCl, pH 7.2 (Sigma-Aldrich,
194 St. Louis, MO, USA), centrifuged at 1000× g for 5 min at 4 °C, and resuspended in 500 µl lysis
195 buffer, PBS with 1% triton (PBST, Sigma-Aldrich, St. Louis, MO, USA). All reagents used for
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 paraformaldehyde. Then, the bacteria were incubated with monoclonal mouse anti-α-Gal antibody
201 (mAb) M86 to α-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**

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

212

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

214 To evaluate the specificity of anti-turkey and anti-chicken IgY against penguin IgY, plates
215 (Nunc-Immuno™ Plate, Thermo Scientific, Waltham, MA, USA) were coated with α -Gal
216 linked to HSA (HSA- α -Gal, 50ng/well, Dextra Laboratories, Reading, UK) diluted in
217 carbonate/bicarbonate buffer (0.05 M, pH 9.6) and incubated overnight at 4 °C. Wells were
218 washed three times with 100 μ L of PBST and then blocked with 100 μ L of 1% HSA/PBS for 1 h
219 at RT. After three washes, serum samples, diluted 1:200 in PBS, were added to the wells and
220 incubated for 1 h at 37 °C. The plates were washed three times and HRP-conjugated goat anti-
221 turkey IgY (Mybiosource) or goat anti-chicken IgY (Sigma-Aldrich, St. Louis, MO, USA) Abs
222 were added at dilution 1:1500 in PBS (100 μ L/well) and incubated for 1 h at RT. Plates were
223 washed three times and the reaction was developed by adding 100 μ L ready-to-use TMB solution
224 (Promega) at RT for 20 min in the dark, and then stopped with 50 μ L of 0.5 M H₂SO₄. The OD
225 was measured at 450 nm using a Multiskan FC ELISA reader (Thermo Scientific, Waltham, MA,
226 USA). All samples were tested in triplicate and the average value of three blanks (no Abs) was
227 subtracted from the reads. The cut-off was determined as two times a mean OD value of the
228 blank controls.

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

238

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

240 To measure the levels of Abs against α -Gal, *A. fumigatus* and *E. coli* in penguin sera, plates
241 (Nunc-Immuno™ Plate, Thermo Scientific, Waltham, MA, USA) were coated with α -Gal-HAS,
242 *A. fumigatus* or *E. coli* (50ng/well) proteins, respectively. Sera collected at T-1mo, T1mo and

243 T3mo (Table 1) were diluted to 1:200 with phosphate buffered saline (PBS). Plates were blocked
244 by adding 100 μ L of 1% HSA/PBS for 1 h at RT. After three washes, samples diluted were added
245 and incubated for 1 h at 37 °C. The plates were washed three times and horseradish peroxidase
246 (HRP)-conjugated goat anti-chicken IgY or IgM (Sigma-Aldrich, St. Louis, MO, USA) were
247 added at 1:1500 dilution in 1% HSA/PBS (100 μ L/well) and incubated for 1 h at RT. The plates
248 were washed three times and the reaction was developed by adding 100 μ L ready-to-use TMB
249 solution (Promega, Madison, WI, USA) at RT for 20 min in the dark, and then stopped with 50
250 μ L of 0.5 M H₂SO₄. The OD was measured at 450 nm using Multiskan FC ELISA reader
251 (Thermo Scientific, Waltham, MA, USA). All samples were tested in triplicate and the average
252 value of three blanks (no Abs) was subtracted from the reads. The cut-off was determined as two
253 times a mean OD value of the blank controls

254

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

268

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

270 Penguin egg yolk and egg white were separated. Total egg IgY was purified with IgY
271 Purification Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's
272 instructions. Briefly, egg yolk was weighted and mixed with a volume of cold delipidation
273 reagent equal to five times the egg yolk weight. The mix was incubated for two hours at 4°C, and

274 then centrifugated 20 minutes at 10,000xg in refrigerated centrifuge. The supernatant was
275 recovered and an equal volume of cold IgY precipitation reagent was added and incubated for
276 two hours at RT. After 20 minutes of centrifugation at 10,000xg in a refrigerated centrifuge, the
277 pellet was collected and a volume of PBS equal to the original volume of the egg yolk was
278 added. Total proteins were quantified using BCA Protein Assay (Thermo Scientific, Waltham,
279 MA, USA) with BSA as standard.

280

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

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

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

299 PICRUSt2 XX was employed for predictions of functional metabolic pathways based on Kyoto
300 Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) XX. Briefly, ASVs were placed
301 into a reference tree containing 16S rRNA sequences from prokaryotic genomes, which is then
302 used to infer gene family copy numbers from ASVs dataset. After, gene family abundance per
303 sample and pathway abundances were determined. The analysis was focused on the detection of
304 the presence and abundance of α 1,3 galactosyltransferase genes and the bacterial taxa, from

305 penguin microbiome, contributing to these genes. Specifically, we tested the presence and
306 abundance of α 1,3GT genes (i.e., K02450, K02847, K03275, K03279, K03276, and K03278) in
307 the microbiomes of the selected penguin species. Sankey diagram was performed in R studio XX
308 using the networkD3 package.

309

310 **Statistical analyses**

311 Statistical differences of parameters measured in penguins of each group were evaluated using
312 paired and unpaired Student's *t*-test applied to individual paired and unpaired values,
313 respectively. Pearson correlation was used to assess the correlation between anti- α -Gal and anti-
314 *E. coli* Abs levels. All statistical analysis were performed in the GraphPad 9 Prism program
315 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when *p*
316 < 0.05.

317 **Ethics**

318 ?

319 **Results**

320

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

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

332

333 *Presence and variation of anti- α -Gal Ab in penguin sera and egg yolks*

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

345

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

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

359

360 *Oral administration of E. coli Nissle expressing α -Gal was associated with modulation of anti- α -* 361 *Gal Abs levels in penguin sera*

362 Two penguins from the vaccine group took two extra doses (penguins n°12 and n°20, Table 1),
363 while another one missed the last dose due to difficulties in animal handling (penguin n°18,
364 Table 1). These three individuals were kept into the vaccine group for statistical analyses.

365 However, one penguin (penguin n°24, Table 1) from the placebo group accidentally swallowed a
366 dose of vaccine and was excluded from statistical analysis.

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

372 Sera levels of total Abs against α -Gal were measured by competitive ELISA as M86 binding
373 inhibition % in penguins that received *E. coli* Nissle or placebo orally. Three months (T3mo)
374 after the oral vaccination (Post-V), the penguins that received *E. coli* Nissle showed a significant
375 reduction (10.19 SD \pm 12.05, 95 % IC 1.57-18.81) in total anti- α -Gal Abs compared with the
376 levels before (Pre-V) the oral vaccination (20.20 SD \pm 11.31, 95 % IC 12.11-28.29) (Student's *t*-
377 test $p = 0.03$, Figure 5A). No differences were observed in the levels of anti- α -Gal IgM (Figure
378 5B) and IgY (Figure 5C) Post-V compared with the levels Pre-V. On the other hand, the animals
379 in the placebo group showed no significant difference in the levels of total anti- α -Gal Abs
380 (Figure 5D) and anti- α -Gal IgY (Figure 5F) before and after administering the placebo (Figure
381 5D), while in the same group of animals the levels of anti- α -Gal IgM were significantly lower
382 Post-V (0.66 SD \pm 0.49, 95 % IC 0.80-1.56) compared to Pre-V (2.22 SD \pm 0.68, 95 % IC 1.69-
383 2.74) (Student's *t*-test $p = 0.003$, Figure 5E). No significant differences were found in the levels
384 of total anti- α -Gal (Figure 5G), or anti- α -Gal IgM (Figure 5H) and IgY (Figure 5I) Pre-V or Post-
385 V in any group.

386 We then asked whether oral administration of *E. coli* Nissle elicits anti-*E. coli* Abs and, if yes,
387 whether a correlation existed between anti-*E. coli* and anti- α -Gal Abs levels. No significant
388 change was found in the levels of anti-*E. coli* IgM Pre-V and Post-V (Figure 5A), while anti-*E.*
389 *coli* IgY (Figure 5B) showed a significant increase in the penguins of the *E. coli* Nissle group.
390 However, the same pattern was found in the placebo group, no significant change in anti-*E. coli*
391 IgM Pre-V and Post-V (Figure 5C) and a significant increase in anti-*E. coli* IgY (Figure 5D). The
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
394 the levels of anti-*E. coli* IgM and anti- α -Gal IgM Pre-V (Pearson coefficient $r=0.46$, $p = 0.04$,
395 Figure 5G) and anti-*E. coli* IgY and anti- α -Gal IgY in the *E. coli* Nissle group Post-V (Pearson
396 coefficient $r=0.63$, $p = 0.04$, Figure 5H) were found. There was no correlation between levels of

397 anti-*E. coli* IgM or IgY and anti- α -Gal IgM or IgY in the placebo group Post-V or between anti-
398 *E. coli* IgM and anti- α -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
403 prealbumin, albumin, α 1, α 2, β 1, β 2, and γ -globulins concentrations and albumin/globulin (A/G)
404 ratio compared with the placebo group (Figure 6). Except for prealbumin, the levels of the above
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
407 prealbumin were significantly lower Post-V (3.36 SD \pm 2.95, 95 % IC 1.09-5.64) compared with
408 the levels Pre-V (6.56 SD \pm 1.37, 95 % IC 5.50-7.62) (Student's *t*-test $p = 0.01$), but the level of
409 this plasma protein did not change significantly in the *E. coli* Nissle group (Supplementary
410 Figure S2).

411 The mean concentration of 3-OHB Post-V in penguins of the *E. coli* Nissle group was lower
412 (540.3 SD \pm 226.9 μ mol/L) than that in the placebo group (610 SD \pm 259.1 μ mol/L), but the
413 difference was not statistically significant (Student's *t*-test $P > 0.05$, Figure 7A). The levels of 3-
414 OHB before and after the oral vaccination did not change significantly in the penguins of *E. coli*
415 Nissle (Figure 7B) or placebo groups (Figure 7C). Individual values of 3-OHB per penguin are
416 available as Supplementary Table S1.

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

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 (Supplementary
428 Table S2).

429

430 Morbidity and mortality comparison

431 TO BE COMPLETED AT THE END OR DELETED

432

433 **Discussion**

434

435 **Aspergillosis and diagnostic tools in penguins**

436 Clinical presentation of aspergillosis varies from acute cases involving the lungs to
437 chronic, disseminated infections of the air sacs in infected birds. Mycelial masses called
438 aspergillomas may develop within the respiratory tract, as well as fungal plaques resulting in
439 thickening of avian air sacs. Consequently, computed tomography (CT) is an interesting
440 diagnostic modality for respiratory issues in penguins and was therefore included in the
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
443 recumbency in Humboldt penguins undergoing CT, while no difference was seen in conscious *vs.*
444 anesthetized individuals. Thus, decision was made to perform CT in non-anesthetized penguins
445 in a standing positioning, as previously described. Despite increased motion artifact in some
446 penguins, assessment of the whole respiratory tract and scoring of aspergillosis-related lesions
447 were possible in all but two penguins (Supplementary Table S2). Dorsal recumbency and/or
448 anesthesia were only used as a last resort in very few individuals (Table 1), and this technique is
449 considered safe and reliable and is routinely used for assessment of the respiratory tract of
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 β globulin fraction containing the fibrinogen and 3-OHB
455 levels. For these reasons, capillary zone electrophoresis assay and measurement of 3-OHB levels

456 were added to the diagnostic panel in this study (Table 1). Despite being a sometimes useful
457 assay in selected avian species such as psittacine birds, the α -galactomannan assay is not reliable
458 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
462 power and the oral vaccine is associated with lower progression of subclinical *Aspergillus* sp.
463 infection as reflected by lower 3-OHB values would be overstated. Mean values were below
464 previously reported values in healthy African penguins (*Spheniscus demersus*), a species closely
465 related to the Humboldt penguin. For accurate aspergillosis assessment in penguins, plasma
466 levels of 3-OHB should be interpreted together with β -globulins and α -2 globulins in order to
467 have a high specificity ($> 90\%$) and a good negative predictive value ($\geq 80\%$). No significant
468 difference was seen in these electrophoretic fractions between penguin groups (Figure 6,
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
471 throughout the study period. As a consequence, differences observed between penguin groups in
472 Abs levels Post-V were only attributable to the oral vaccine compared to the placebo.

473 However, a significant reduction of the pre-albumin fraction was observed on the
474 electrophoretic profiles of non-vaccinated penguins after the vaccination trial (Supplemental
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- α -Gal Abs in vaccinated penguins
478 and of anti- α -Gal IgM in non-vaccinated individuals (Figure 4). Consequently, a direct link
479 between globulin levels and prealbumine concentrations seems highly unlikely in our study
480 population and the exact clinico-pathological relevance of this finding remains unknown. Still, a
481 link with non-humoral immunity pathways is hypothesized. Significantly lower prealbumin
482 values have been reported in falcons with confirmed aspergillosis. In humans, a similar trend was
483 seen in fatal cases of COVID-19. As a transport protein, the human prealbumin fraction (also so
484 called transthyretin) has various physiological effects, including anti-inflammatory actions and a
485 strongly hypothesized positive effect on the cell-mediated immunity, especially on immune cells
486 from the myeloid compartment. Even if no statistical difference was seen in the prealbumin
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).

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

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

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

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

552 dose (three administrations *per* week for three weeks in turkeys (XXX) vs. two administrations
553 *per* week for three weeks in penguins), or a differential cellular location of the α -Gal epitope in
554 *E. coli* Nissle and *E. coli* O86:B7. The α -Gal epitope of *E. coli* O86:B7 is expressed on the
555 capsule or glycoprotein portion of the bacterial wall, rather than on the LPS molecules (XXX).
556 Other *E. coli* strains were found to express the glycan in the LPS molecules (XXX). These
557 findings suggest that the α -Gal epitope can be located in the LPS, capsule or glycoprotein portion
558 of *E. coli*. The cellular fraction in which *E. coli* Nissle express α -Gal is currently unknown. The
559 results of the present study suggest that *E. coli* Nissle express α -Gal in a location that is not
560 accessible to the immune system to induce a significant increase of anti- α -Gal Abs. However, the
561 positive correlation between the levels of anti- α -Gal and anti-*E. coli* IgY in the *E. coli* Nissle
562 group suggests that the immune response to α -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 α -
566 Gal in the Humboldt penguin is closely related to that of *E. coli*. The oral vaccine tested failed to
567 produce a significant increase in anti- α -Gal Abs as we suspect *E. coli* Nissle to express α -Gal in a
568 location that is not accessible to the penguin's immune system. Nevertheless, a positive effect on
569 the cell-mediated immunity of vaccinated penguins may be hypothesized, as suggested by the
570 significant decrease of the pre-albumin fraction in the placebo group only. Further study is
571 needed to assess a positive effect of *E. coli* Nissle oral probiotic on myeloid immune cells in the
572 Humboldt penguin.

573

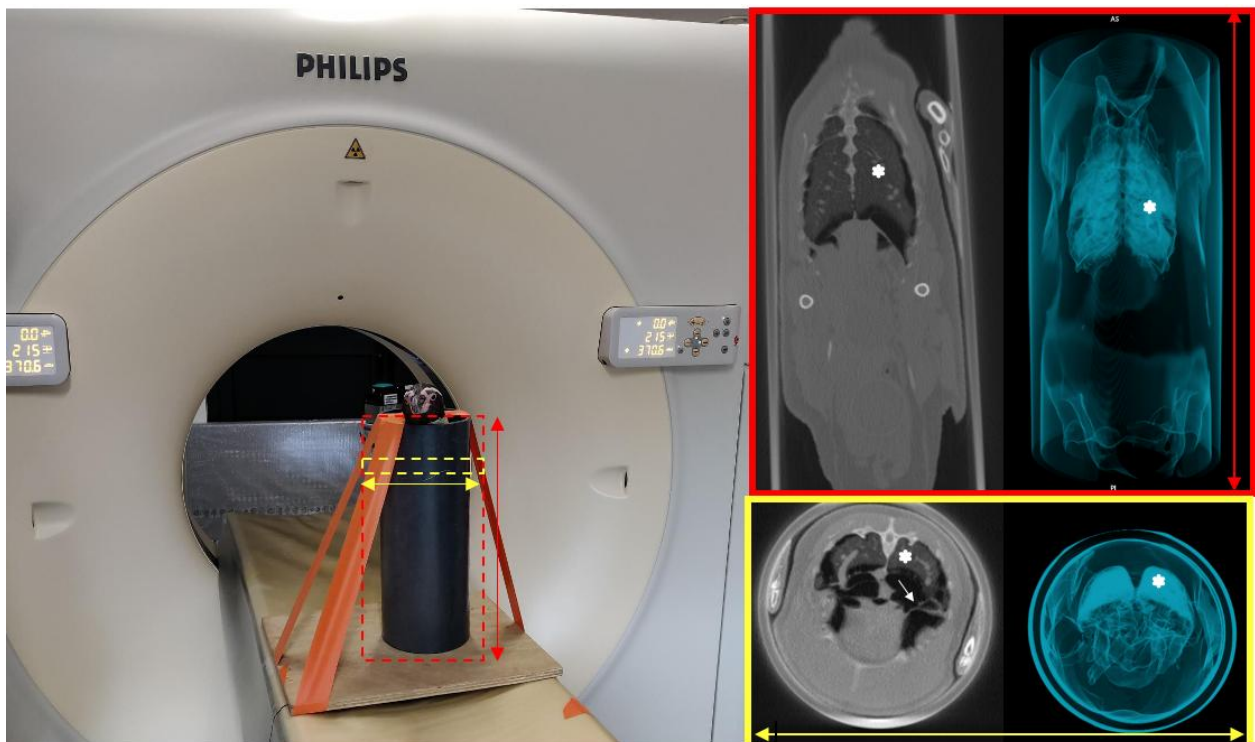
574 **References**

575

576 **Figure legends**

577

578 **Figure 1. Computed tomography in a non-anesthetized, standing Humboldt penguin (n°13,**
579 **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.

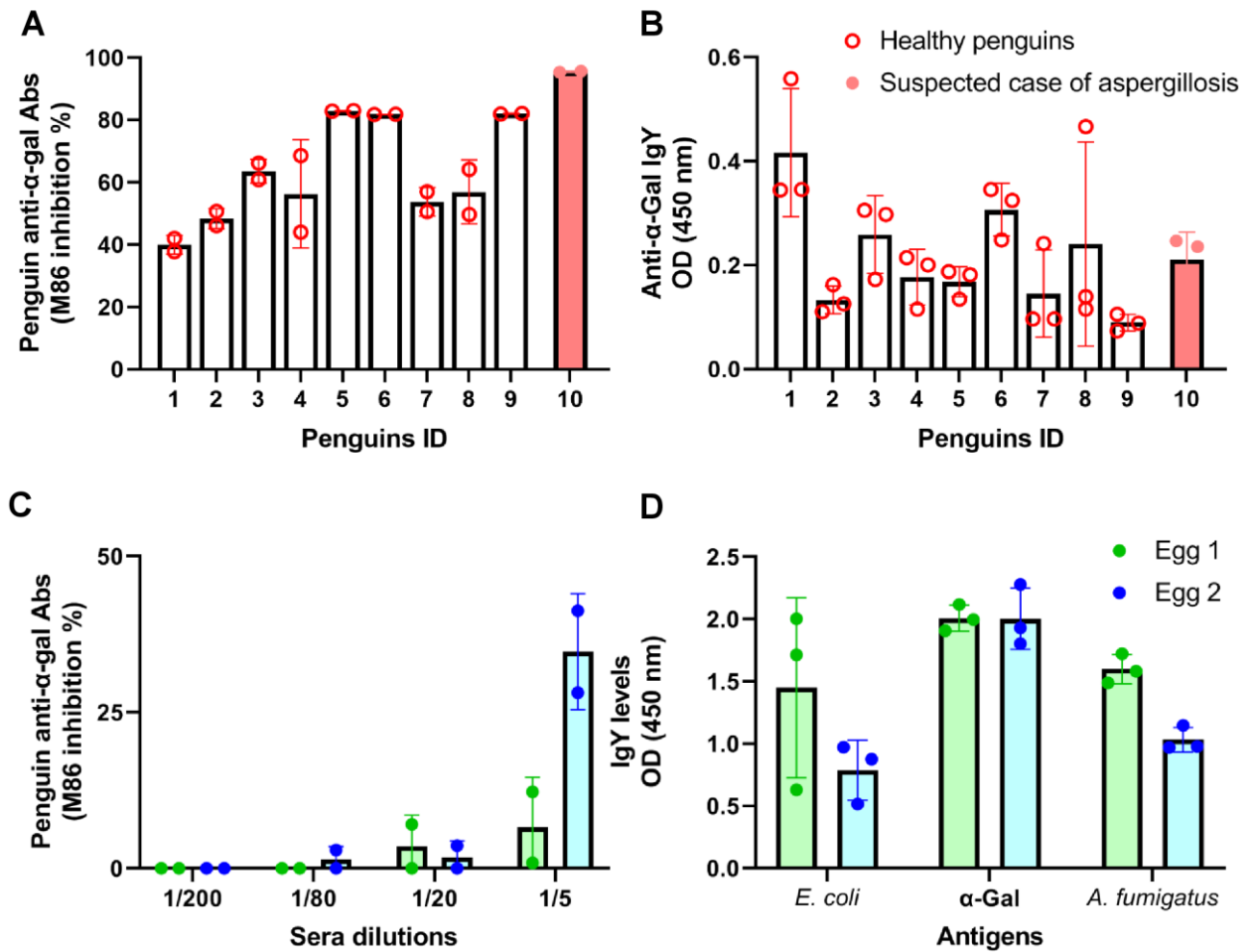


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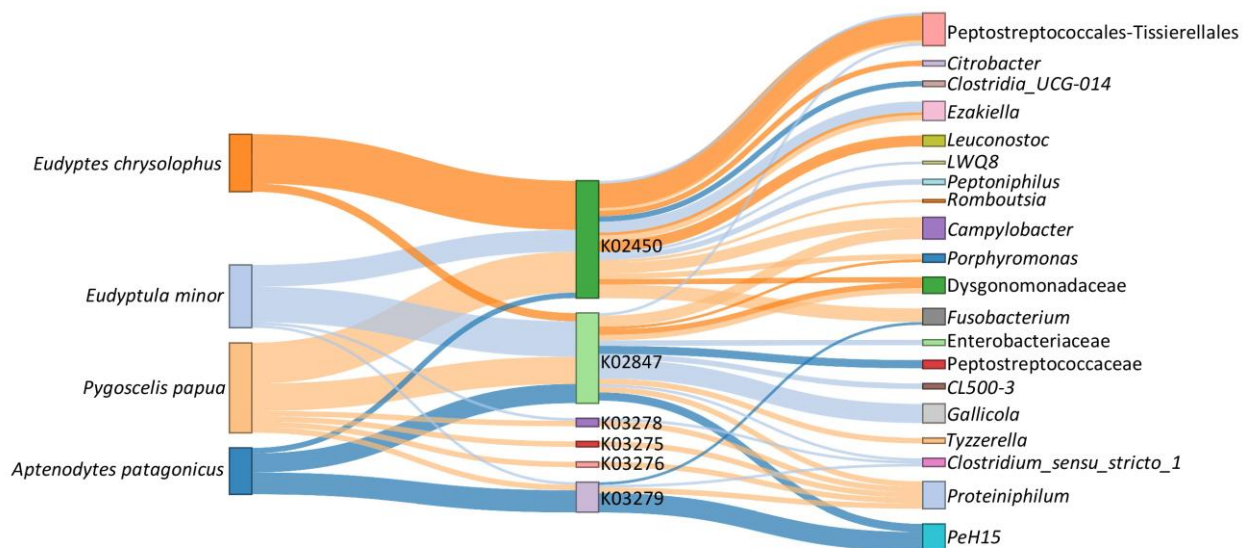
585 **Figure 2. Variation of anti- α -Gal Abs in penguin sera and eggs.** The levels of circulating total
586 anti- α -Gal Ab (A) and anti- α -Gal IgY (B) were measured by inhibition and indirect ELISA,
587 respectively. Total and IgY anti- α -Gal Abs were variable in the sera of healthy penguins (white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

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

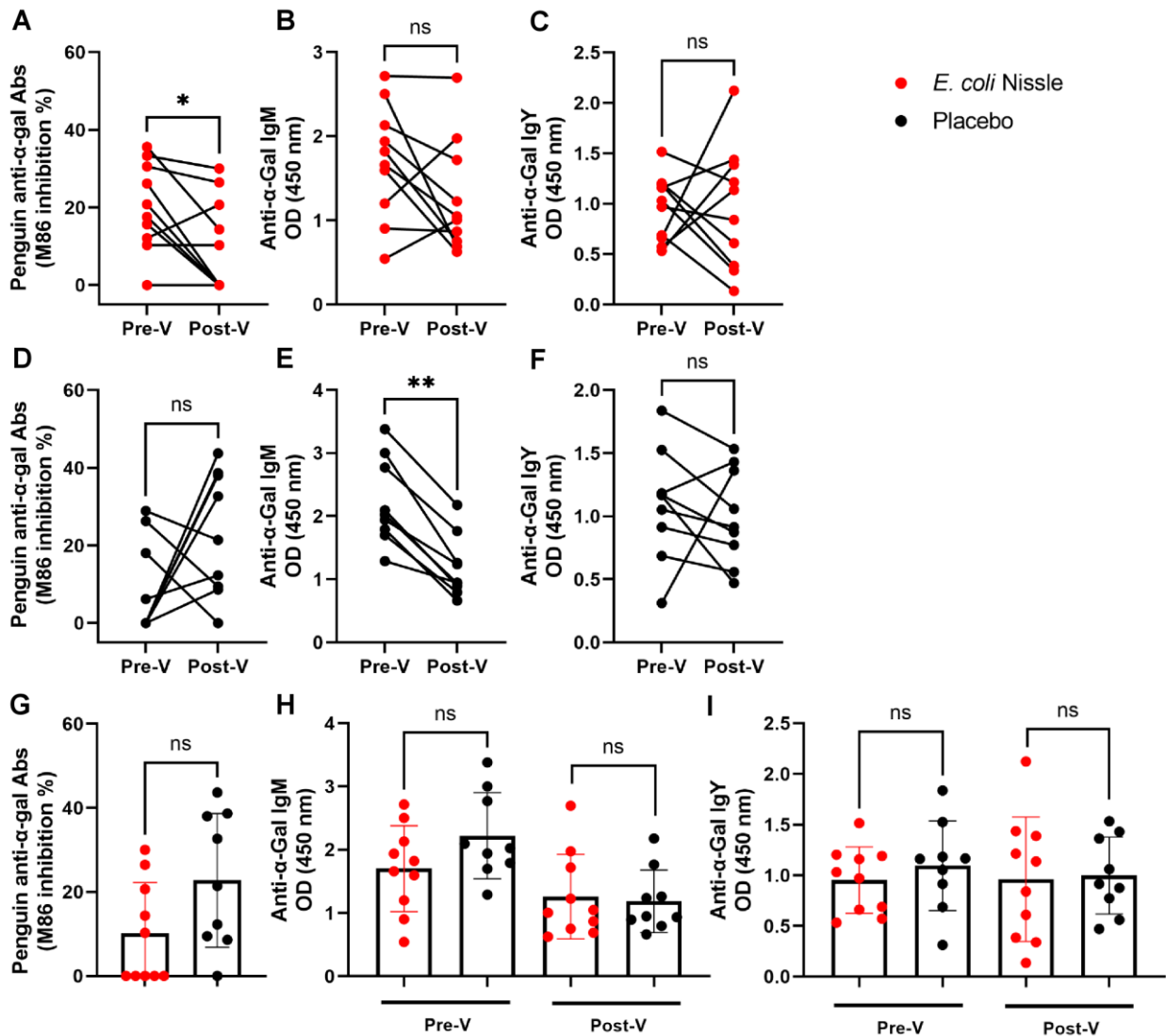


595
 596 **Figure 3. Contribution of bacterial taxa to α 1,3GT genes in the microbiome of penguins.**
 597 Sankey diagram showing the presence of α 1,3GT genes (i.e., K02450, K02847, K03275,
 598 K03279, K03276, K03278) in the microbiome of various penguin species, as well as the bacterial
 599 genera harboring these genes in each host. The presence and the contribution of taxa to α 1,3GT
 600 genes were predicted from 16S rRNA data using PICRUSt2 and the α 1,3GT genes were annotated
 601 using the KEGG orthologs (KO) database as reference. Nodes represent penguin species (left
 602 column), functional genes (middle column) and bacterial taxa (right column). The edges
 603 represent the connection between individual host, α 1,3GT genes and contributing taxon. The
 604 contribution of each taxon to different α 1,3GT genes is represented proportionally by the size of
 605 the edges. K02450: *gspA*, general secretion pathway protein A; K02847: *waaL*, *rfaL*, O-antigen
 606 ligase [EC:2.4.1.-]; K03275: *waaO*, *rfaI*, UDP-glucose:(glucosyl)LPS alpha-1,3-
 607 glucosyltransferase [EC:2.4.1.-]; K03279: *waaJ*, *rfaJ*, UDP-glucose:(galactosyl)LPS alpha-1,2-
 608 glucosyltransferase [EC:2.4.1.58]; K03276: *waaR*, *waaT*, *rfaJ*, UDP-

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



611
 612 **Figure 4. Variation in penguin anti- α -Gal Ab levels after oral administration of *E. coli***
 613 **Nissle.** The levels of circulating total anti- α -Gal Ab (A) and anti- α -Gal IgM (B) and IgY (C)
 614 were measured by inhibition (A, D and G) and indirect (B-C, E-F and H-I) ELISA in the *E. coli*
 615 Nissle (red dots) and placebo (black dots) groups before (Pre-V) and after (Post-V) the
 616 vaccination protocol. Total anti- α -Gal Abs decreased in the sera of penguins vaccinated with oral
 617 *E. coli* Nissle (A). Anti- α -Gal IgM decreased in the sera of penguins of the placebo group (E).
 618 No significant differences were observed in the levels of anti- α -Gal Ab (G), anti- α -Gal IgM (H)
 619 and IgY (I) when the two groups, *E. coli* Nissle and placebo, were compared Pre-V and Post-V.
 620 Individual (A-I) and means and standard deviation (G-I) values are shown. Values were
 621 compared by paired (A-F) or unpaired (G-I) student's *t*-test (* $p < 0.05$, ** $p < 0.005$; ns: not
 622 significant, 1 experiment, $n = 10$ in the *E. coli* Nissle group and $n = 9$ in the placebo group, and
 623 three technical replicates per sample).

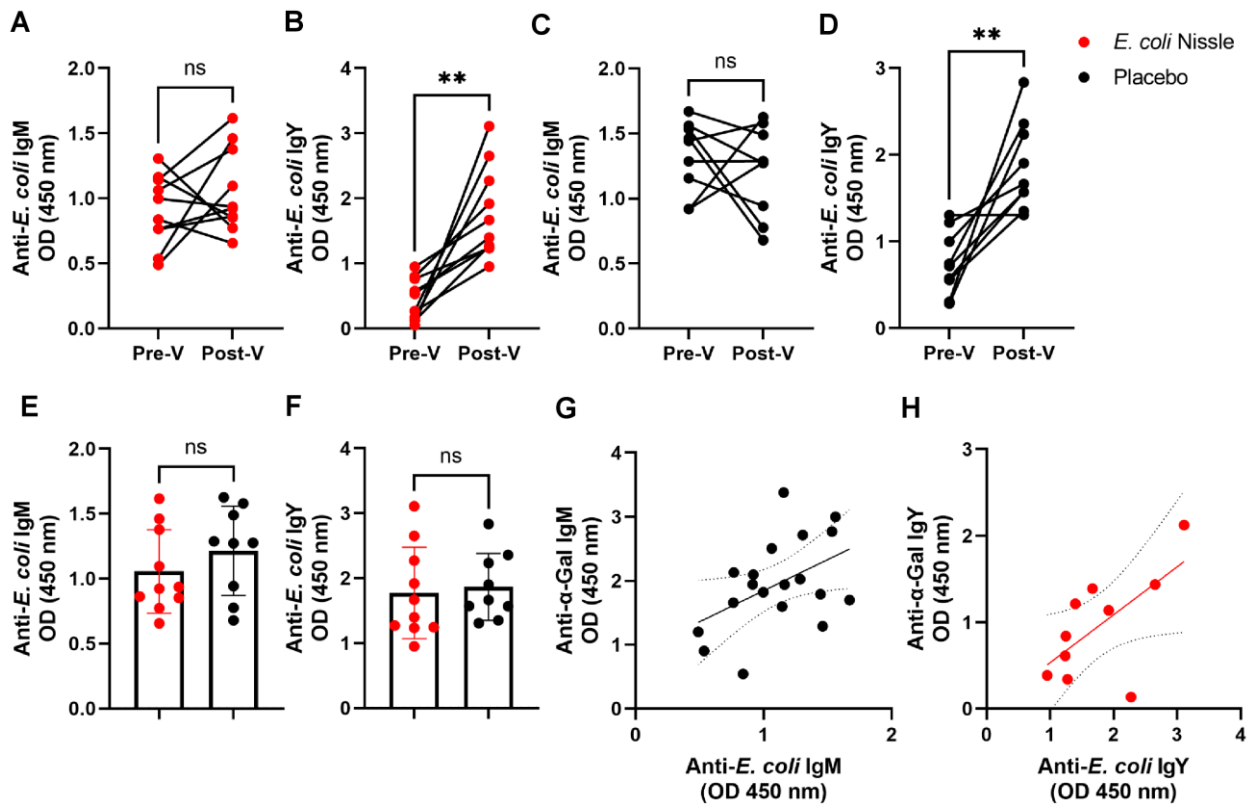


624

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

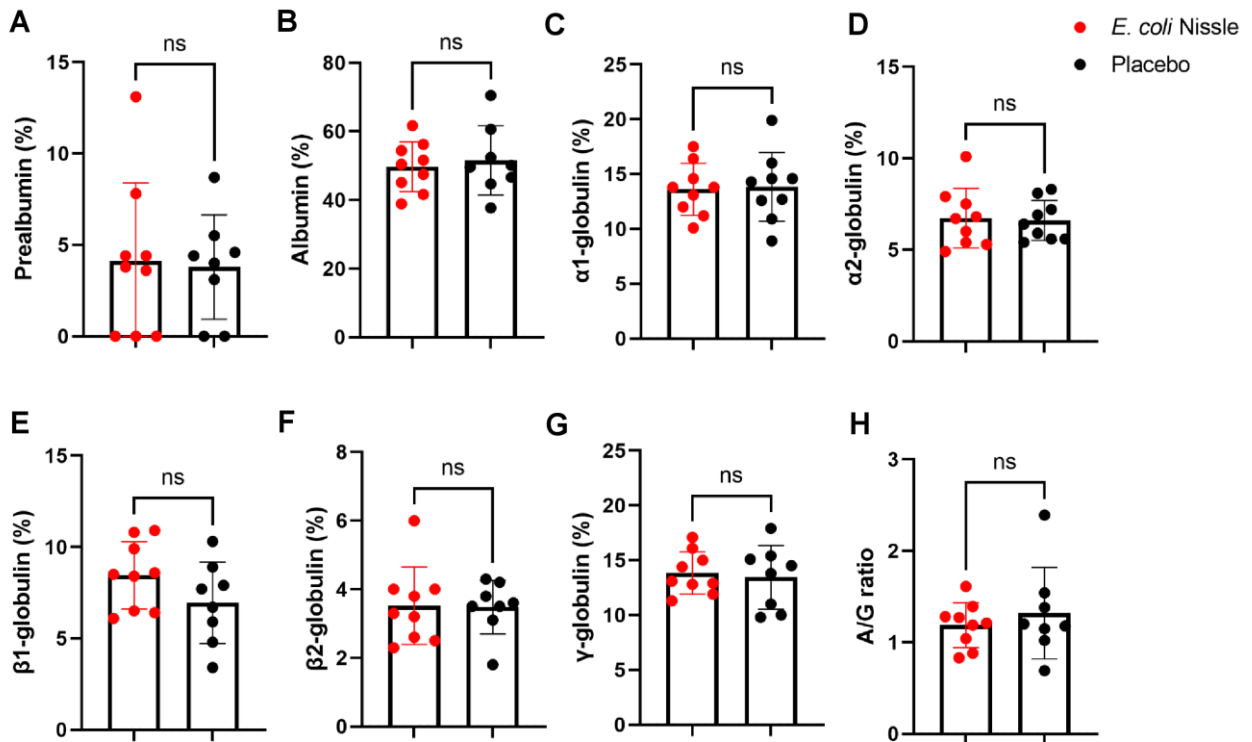
637 and three technical replicates per sample, A-F). Values of both groups placebo and *E. coli* Nissle
 638 were merged for the Pre-V anti-*E. coli*-anti- α -Gal IgM correlation analysis (n = 19). For the
 639 correlation of anti-*E. coli*-anti- α -Gal *E. coli* Nissle Post-V IgY n = 10.

640



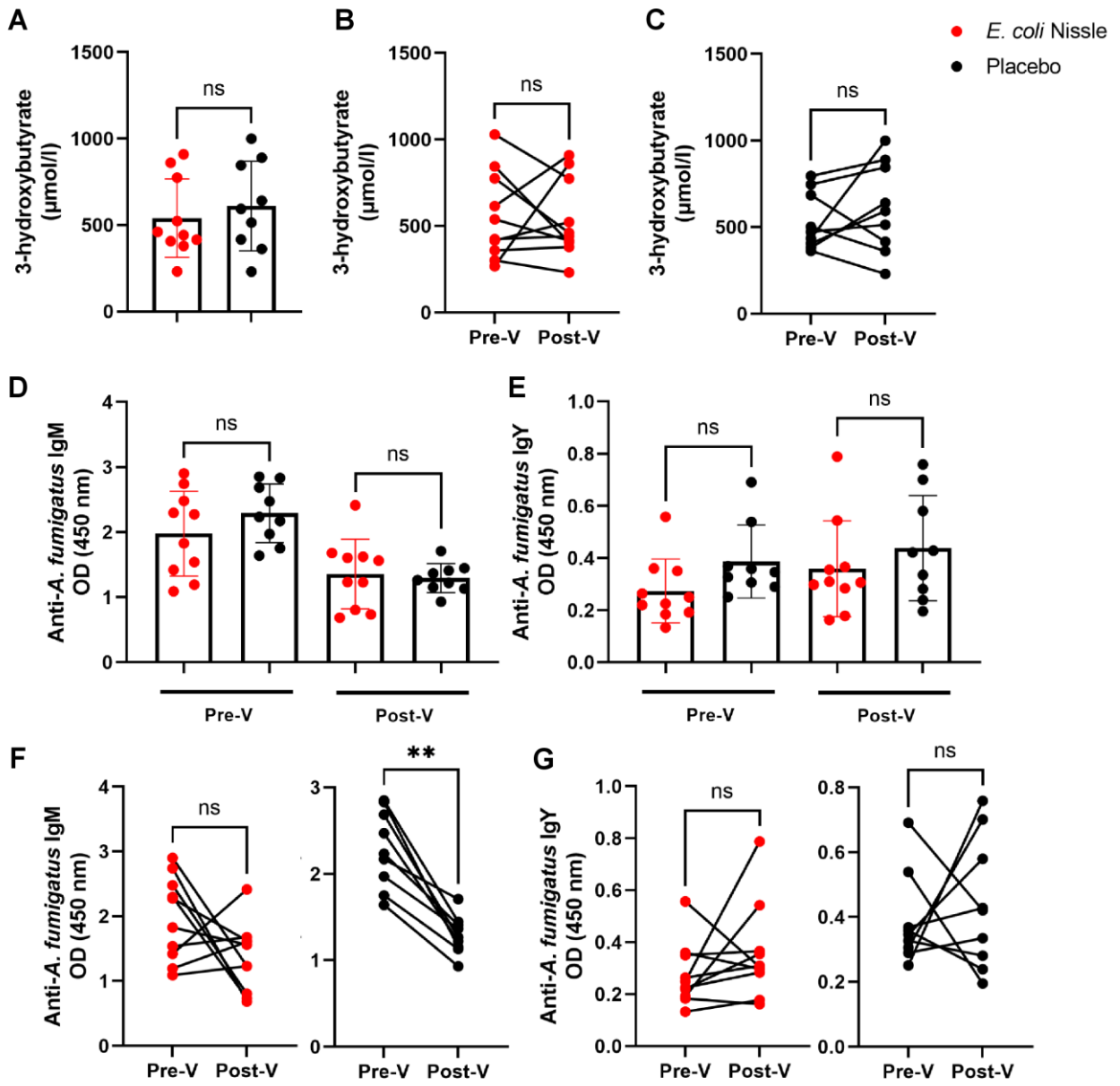
641

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



648

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



659

660 **Supplementary materials**

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

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

* 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.**

664

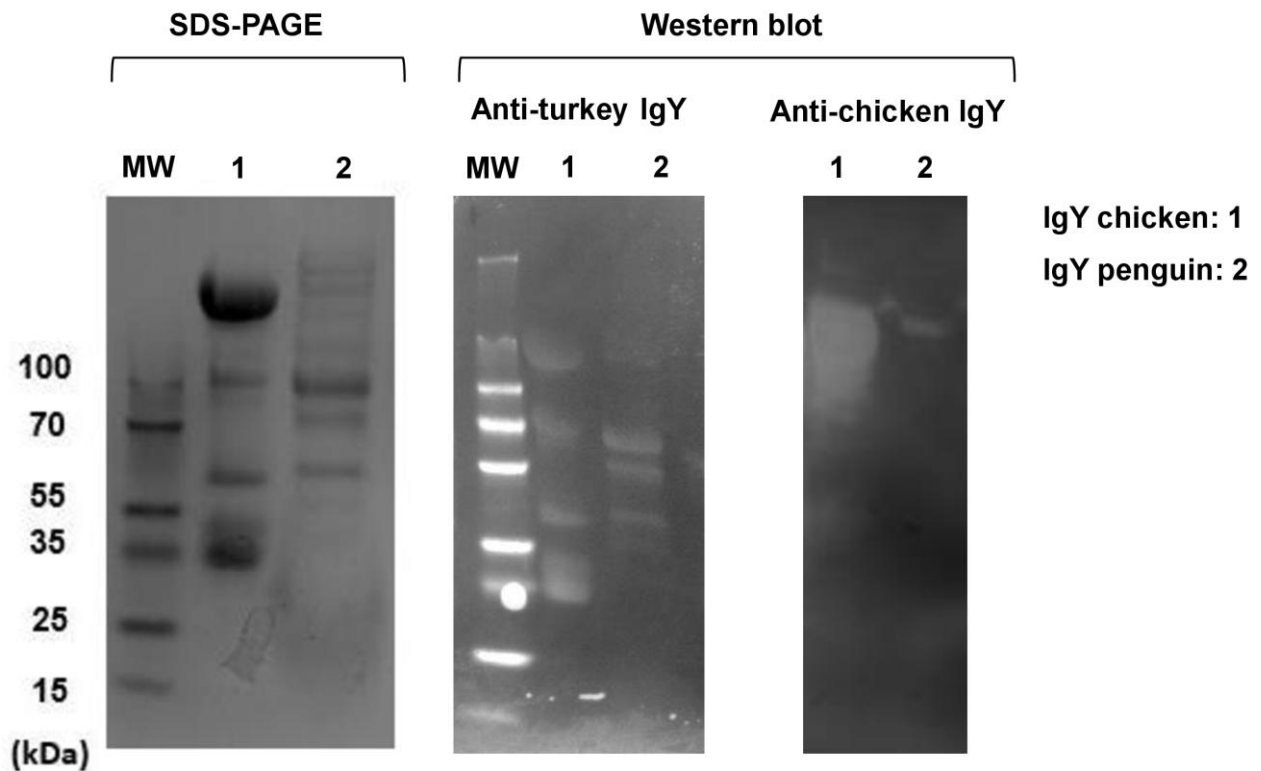
	Penguin n°	CT exam n° (date)	Right lung nodules	Left lung nodules	Air sac nodules	Air sac plaques	Trachea nodules	Trachea plaques
			Yes = 1 ; No = 0					
<i>E. coli</i> Nissle	1	23 (17/12/21)	0	0	0	0	0	0
		24 (31/03/21°)	0	0	0	0	0	0

Placebo group

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 (17/12/20)	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
	8 (31/03/21)*	-	-	-	-	-	-
18	15 (20/12/20)	0	0	0	0	0	0
	16 (31/03/21)	0	0	0	0	0	0
19	37 (16/12/20)	0	0	0	0	0	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

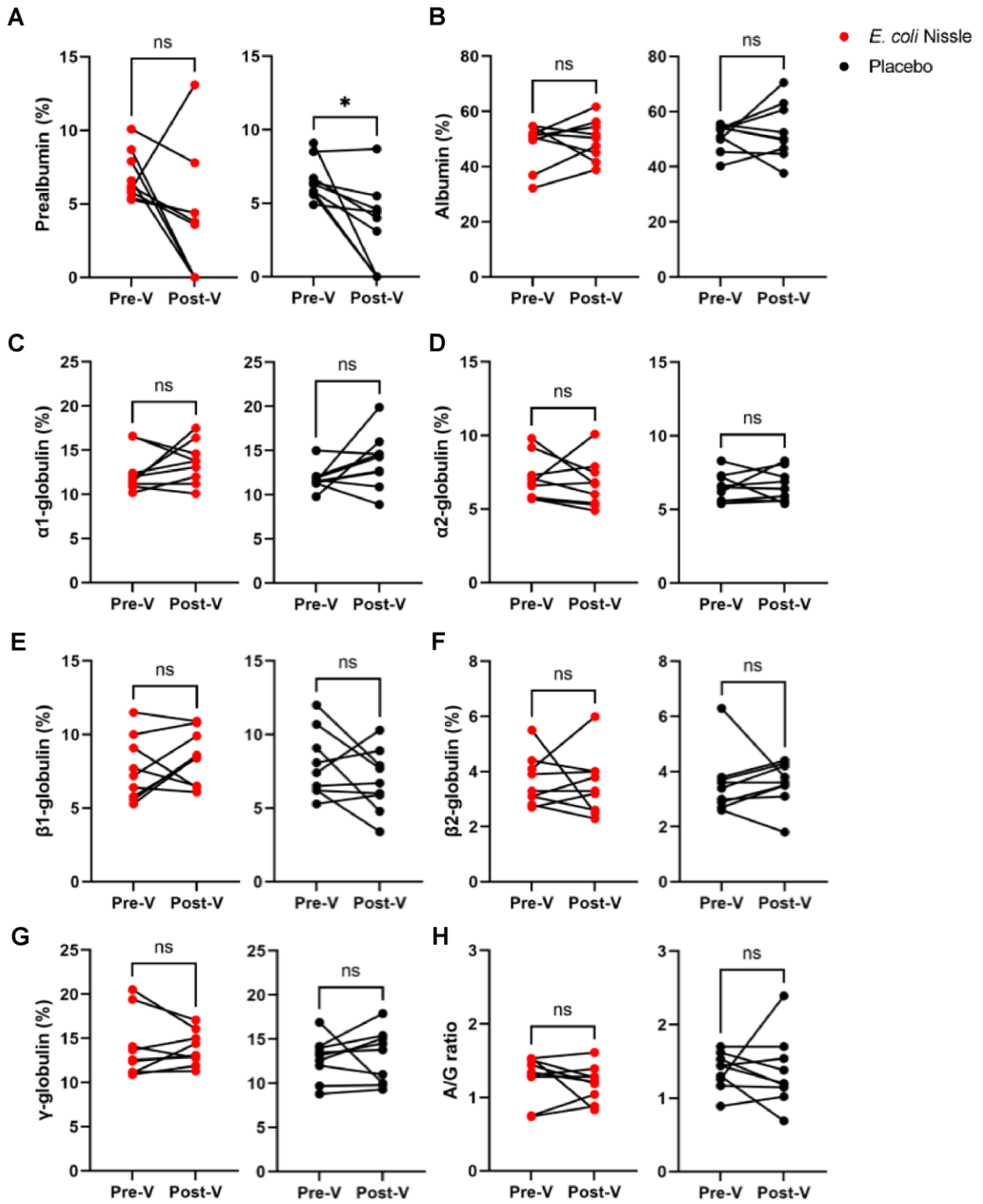
665 Interpretation of CT scans n° 6 (penguins n°15) and 8 (penguins n°17) were not possible due to excessive
666 motion artifact.

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
671 penguin IgY, while anti-chicken IgY recognizes only one band in chicken and penguin IgY.



672

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



681 **Tables**682 **Table 1. Animals included in the preliminary study, vaccination protocol, CT scans, and**
683 **blood samplings.**

Group	Penguin n°	ID	Sex	Date of sampling (frozen serum)	Clinical findings						Suspicion of aspergillosis	
Preliminary study: test of the competitive ELISA assay	1	QRD16-00616	F	16/10/2017	Suspicion of ocular aspergillosis on 29/05/2016						Mild	
	2	QRD16-01010	M	16/10/2017	Suspicion of aspergillosis on 10/09/2016						Mild	
	3	QRD15-05130	F	16/10/2017	-						Weak	
	4	QRD16-00603	F	16/10/2017	Suspicion of ocular aspergillosis on 18/07/2017						Mild	
	5	QRD16-00994	F	16/10/2017	-						Weak	
	6	QRD15-05124	F	16/10/2017	-						Weak	
	7	QRD16-01002	M	16/10/2017	-						Weak	
	8	QRD16-00615	M	16/10/2017	-						Weak	
	9	MIG12-17845737	M	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	
				Restraint sessions	Vaccine sessions						Restraint sessions	
Group	Penguin n°	ID	Sex	1 st Blood samplings #1 + CT #1 « T-1mo »	1 st Monday 21/12/20 PO	2 nd Wednesday 23/12/20 PO	3 rd Monday 28/12/20 PO	4 th Wednesday 30/12/20 PO	5 th Monday 04/01/21 PO	6 th Wednesday 06/01/21 PO	2 nd Blood samplings #2 « T1mo »	3 rd Blood samplings #3 + CT#2 « T3mo »
Vaccine group	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
	14	QRD14-03648	M	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	15	22197091	M	16/12/2020*	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
	16	14923345	M	16/12/2020* [®]	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	M	17/12/2020	ok	ok	ok	ok	ok	no	15/01/2021	31/03/2020
	19	QRD17-03052	M	17/12/2020	ok	ok	ok	ok	ok	ok	20/01/2021	31/03/2020* [®]
	20	24773872	F	16/12/2020	ok	ok	ok	2 doses	ok	ok	14/01/2021	31/03/2020
Placebo group	21	27535275	F	16/12/2020	ok	ok	ok	ok	ok	ok	15/01/2021	31/03/2020
	22	MIG12-20638625	M	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	M	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	M	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	M	16/12/2020	ok	ok	ok	ok	ok	ok	14/01/2021	31/03/2020
30	QRD15- 04789	M	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 © flash isoflurane anesthesia (facemask) was performed, as these penguins were too fat to be placed
687 appropriately into the PVC tube

688

689 **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
<i>E. coli</i> Nissle	1536	9417	Test strain	N/A**
	143	130	Negative control for <i>E. coli</i> Nissle*	
<i>E. coli</i> O86:B7	634	5374	Positive control of α -Gal production	REF
	123	17	Negative control for <i>E. coli</i> O86:B7*	
<i>E. coli</i> clean	218	839	Negative control for α -Gal production. This strain lacks LPS.	REF
	200	581	Negative control for <i>E. coli</i> clean*	
<i>E. coli</i> BL21	128	47	Negative control for α -Gal production. This strain produces low levels of α -Gal.	REF
	124	38	Negative control for <i>E. coli</i> BL21*	

690 * FITC background value resulting from the secondary antibody alone.

691 ** Not applicable