



Recovery from mild *Escherichia coli* O157:H7 infection in young and aged C57BL/6 mice with intact flora estimated by fecal shedding, locomotor activity and grip strength



Marco Malavolta^{a,*}, Andrea Basso^a, Robertina Giacconi^a, Fiorenza Orlando^a, Elisa Pierpaoli^a, Maurizio Cardelli^a, Francesca Leoni^b, Serena Chierichetti^b, Dorothy Bray^c, Khadija Benlhassan^d, Mauro Provinciali^a

^a Advanced Technology Center for Aging Research, Scientific Technological Area, IRCCS INRCA, Italy

^b Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Ancona, Italy

^c BHD Holding B.V., Amsterdam, the Netherlands

^d KBLH Holding B.V., Amsterdam, the Netherlands

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ABSTRACT

Escherichia coli O157:H7 is a food-borne pathogen that can cause severe complications in vulnerable populations. Mouse infection models of *E. coli* O157:H7 are usually developed under severe animal suffering classification by depleting the normal flora, in which age plays a role.

Objective: To develop a refined method for longitudinal monitoring of *E. coli* O157:H7 in young and old mice with intact flora.

Methods: We applied discriminant analysis and computed composite standardized scores from 19 variables obtained from physiological parameters, analysis of locomotor activity, grip strength measurement and fecal shedding in 16 aged and 16 young C57BL/6 mice after two mild oral challenges of *E. coli* O157:H7. The resulting scores were validated in another experiment performed in 24 aged and 24 young mice including a group (8 aged and 8 young mice) treated with oxytetracycline.

Results: We show that our scores are significantly affected in the post-infection period and that can be used to measure and compare the recovery time after a treatment. The scores are most sensitive when separately developed in young and aged mice.

Conclusions: We developed a method that minimizes the level of animal suffering and that can be applied in preclinical testing of new therapies.

1. Introduction

E. coli is a Gram-negative facultative anaerobic bacillus. Most *E. coli* strains harmlessly colonize the gastrointestinal tract of humans and animals as a normal flora. Some strains of *E. coli*, like for example O157:H7, can produce Shiga toxin (classified as a bioterrorism agent) and can cause severe diarrhea and other serious illnesses with life-threatening complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [1,2]. Because of the severity of these illnesses and the apparent low infective dose, *E. coli* O157:H7 is regarded as one of the most serious of known foodborne pathogens causing severe morbidity including kidney damage [3]. The treatment of *E. coli* O157:H7 infections is usually limited to supportive

care as there is the emerging problem of antibiotic resistance, which require a global revision of the approaches to treat bacterial infections. Moreover, treatments with poor efficacy can increase the release of toxins from the *E. coli* O157:H7, thus increasing the risk of complications [4]. Mouse infection models that mirror various aspects of *E. coli* O157:H7 pathogenesis or disease have already been developed [4]. They are usually developed depleting the normal flora to allow chronic colonization of *E. coli* O157:H7. While these models can be useful in the treatment of severe infection, they do not reflect the typical gastrointestinal environment in which *E. coli* O157:H7 is exposed after ingestion and the mild outcome displayed by most of the infected people. Moreover, these models have lethal outcome and induce severe suffering in animals. In the USA alone > 200 million episodes/year of

* Corresponding author.

E-mail address: m.malavolta@inrca.it (M. Malavolta).

infectious gastroenteritis are reported [5]. These episodes include *E. coli* and it is well known that the most vulnerable populations are the elderly, children and subjects who are immunocompromised. Such illness in humans often presents minimal clinical symptoms (nausea, stomach cramps, diarrhea, fever, loss of appetite and mild dehydration) and resolves within 6 to 14 days [6], but it contributes to the general morbidity and low quality of life in the vulnerable populations like the elderly. It is therefore essential to study models that display a similar clinical course of the infections and that are developed in the context of the intact gut flora, in which age plays a critical role. Such models may help to understand better the mechanism of such infections in populations of different age, to define strategies to minimize the consequence of *E. coli* O157:H7 infection and to develop novel treatments and prevention strategies tailored to the needs of aged people.

Our innovative approach aims to establish a refined method to measure the response to a mild *E. coli* O157:H7 infection which minimize animal suffering while being useful for testing drugs or other treatment able to counteract the pathogen “in vivo”.

Based on previous results on laboratory mice with an intact commensal flora and exposed to transient intestinal colonization by *E. coli* [4,7,8], it is expected that normal mice (C57BL/6 or BALB/c) can be suited for the development of this model. However, it is well documented that in the case of mild infection these animals do not display evident clinical signs. Hence infection is usually monitored by estimating the fecal shedding of the pathogen. However, this parameter may not be enough, as it does not take into account eventual subtle changes on sickness behavior [9] that might have a translational impact on health of individuals at high risk for morbidity and mortality when infected, such as the elderly.

In order to obtain a non-invasive and quantitative measurement of the response to infection with costs at reach of most laboratories, we included measurements of muscular strength and locomotor activity in our panel. These parameters may be sensitive to physiological changes occurring in response to infection even in the absence of other clinical signs [10,11]. The use of a panel mostly related to physical performances is based on the assumption that sickness behavior may affect both physical strength or resistance and/or the motivation to perform physical activity.

Our model was developed in experiments performed in young and old mice with intact gut flora. The data from these experiments were used to compute a score to quantitatively estimate the composite responses to infection in young and old mice. As our strain of *E. coli* was found to be sensitive to oxytetracycline, a broad-spectrum antibiotic that significantly impair fecal shedding in non-resistant pathogen [12], we additionally validated our scores in testing the response to therapy in different groups of young and old mice.

2. Material and methods

2.1. Study design

The model of mild oral infection of *E. coli* O157:H7 (10^7 - 10^8 CFU) was developed in young and aged C57BL6. The first series of experiments (experiment series 1, shown in the upper panel of Fig.1) was used to develop the model. This series of experiments included two trials (trial I and trial II) performed in a group of 32 mice equally subdivided on the basis of age and gender in a control and infected group (16 mice x group). In Trial I, infected mice received an oral inoculum of 2.5×10^7 CFU in PBS, while the control group received only PBS. After one month from the end of “Trial I” the control and infected mice group were inverted and Trial II started. The mice that were not infected in the previous trial received an oral inoculum of 2.5×10^8 CFU in PBS while the control group (mice infected in Trial I) received only PBS.

In the experiment series 2 (lower panel of Fig.1), we used another group of 48 mice (24 young male mice and 24 old male mice) equally subdivided on the basis of their age. Two groups (8 young mice and 8

old mice) received an oral inoculum of *E. coli* (5×10^7 CFU) comprised between the two doses used experiment series 1. Two groups (8 young mice and 8 old mice) received only PBS. Two groups (8 young mice and 8 old mice) received the inoculum of *E. coli* and were treated with the antibiotic oxytetracycline (0.02 mg/ml in drinking water, corresponding to a mean dose of 15 mg/Kg daily).

All experiments were performed according to the European Community Council Directives of 2010/63/UE and the protocol was approved according to current Italian law (D.Lgs. n. 26/2014) by the General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health with the authorization n° 167/2016-PR.

2.2. Bacterial strain

E. coli O157:H7 strain ATCC 35,150 (acid resistant; human feces isolate) was kindly provided by the Istituto Zooprofilattico Sperimentale dell’Umbria e delle Marche (Ancona, Italy). According to ResFinder of DTU (<https://cge.cbs.dtu.dk/services/ResFinder/>) the bacterial strain used in this study is not resistant to oxytetracycline.

2.3. Mice and treatments

C57BL/6 J mice from the Specific Pathogen Free (SPF) animal facility of INRCA (Italian National Centre on Health and Science on Aging, Ancona, Italy) were used for all the studies. Mice were individually caged in visual, auditory and olfactory contact with other mice of the same experimental group (within ventilated cabinets cage systems) as well as in presence of environmental enrichments consisting of nesting materials and wooden toys. Mice were kept in a 12 h light dark cycle with food and drinking water ad libitum and allowed to equilibrate in the phenotyping area allocated in the Scientific and Technological Pole of INRCA for approximately 1 month before starting the experiments. Animals were orally infected by pipette feeding *E. coli* in 20 μ l of PBS, while control groups received only 20 μ l of PBS. Mice were followed up after infection until stabilization of phenotype parameters.

2.4. Mouse phenotype

Non-invasive mouse phenotyping included body weight (BW, g), water intake (WI, ml), food intake (FI, g) a general aspect score (AS), ventral surface temperature (ST), grip strength and locomotor activity parameters. Mice were trained to the phenotype manipulation two-three days per week, for a total of 2 weeks before starting the experiments. Experimental data were collected the day before infection as well as at days 1, 3, 6 and 9 post-infection. The measurement of AS consisted of the sum of five observations comprised on a scale of 0, 0.5 and 1, where 1 is the optimal condition and 0 the worst, while 0.5 is an intermediate or “difficult to evaluate” condition. We evaluated reactivity to stimulus (1 = active and curious mice without any sign of pain or lethargy; 0 = non active or signs of pain or lethargy), fur aspect (1 = normal aspect with lucent shield; 0 = ruffled fur or dermatitis or loss of fur), eyes condition (1 = normal eyes; 0 = eyes recessed with signs of dehydration or presence of cataracts or conjunctivitis or any other sign of clear eyes abnormality), feces aspect (1 = normal aspect; 0 = e.g. excessive moist feces, or presence of blood) and breathing (1 = normal breathing; 0 = breathing abnormalities including dyspnea, rales or tachypnea). ST was collected with a non-contact infrared thermometer. Grip strength was measured using the gripped weights method proposed by Deacon [13] with a slight adaptation to better discriminate changes of strength in old mice. The adaptation consisted in the introduction of two intermediate weights (52.5 g and 62.5 g) around the 59 g weight, which was a critical threshold for most of our mice in preliminary experiments. This adjustment was made in order to better discriminate the changes of the strength score in old mice. Thus, the final gripped weights were 20, 33, 46, 52.5, 59, 65.5, 72, 85 g. To

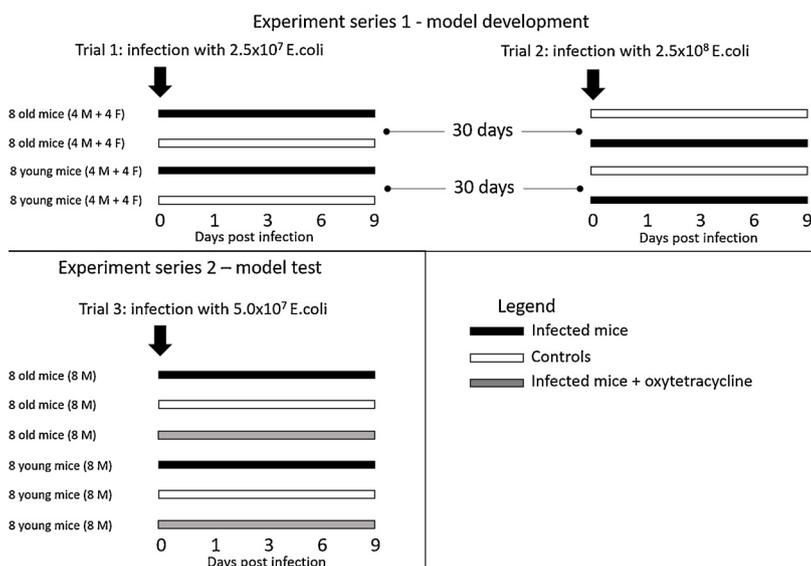


Fig. 1. Study Design.

The study is subdivided into two series of experiments. The first series of experiments (experiment series 1) is shown in the upper panel. This series of experiments include two trials (trial I and trial II) performed in the same group of 32 mice. The mice were equally subdivided on the basis of age and gender in a control and infected group (16 mice x group). In Trial I, infected mice received an oral inoculum of 2.5×10^7 *E. coli* O107:H7 in PBS while the control group received only PBS. After one month from the end of “Trial I” the control and infected mice group were switched and Trial II started. The mice that were not infected in the previous trial received an oral inoculum of 2.5×10^8 *E. coli* O107:H7 in PBS while the control group (the mice that were infected in Trial I) received only PBS. In the experiment series 2 (lower panel), we used another group of 48 mice (24 young male mice and 24 old male mice) subdivided in 6 groups (3 groups for young mice and 3 groups for old mice). Two groups (8 young mice and 8 old mice) received an oral inoculum of *E. coli* (5×10^7 CFU) comprised between the one used in Trial I and Trial II. Two groups (8 young mice and 8 old mice) received only PBS. Two groups (8 young mice and 8 old mice) received the inoculum of *E. coli* and were treated with the antibiotic oxytetracycline.

reduce the risks of cross-contaminations we used 3 identical sets of gripped weights, each one used within the same experimental group. After each experimental session, the gripping weights were placed in a boiler with 1–2% detergent, boiled for 30 min, rinsed in tap water and dried in the air. All other equipment and work surfaces that may come in contact with animals were also sanitized with Virkon followed by 70% ETOH.

The score assigned to the measurements is reported in Supplementary Table 1. The measurements yielded a score that we named Strength Score (SS).

Locomotor activity was conducted by a 5-min open field test on a white wood-chamber (72 × 72 × 30 cm) surmounted by a Xiaomi Yi Camera 16 MP 1080 P 60FPS (YI Technology) controlled WI-Fi by a Smartphone. At the conclusion of all testing for the day, as well as before changes from an experimental group to another, the arena was sanitized with Virkon followed by 70% ETOH to remove any Virkon residue.

Videos were collected in a microSD disk and the tracking was performed offline with Biobserve Viewer3 (Biobserve GmbH, Germany).

We used a Biobserve Viewer protocol to collect a list of 30 parameters for each minute of video (for a total of 180 variables). After a preliminary elaboration, we decided to include in the final analysis a restricted pool of variables excluding redundant information (e.g. mean velocity which displayed the same information of track length and zone counts which displayed a correlation of 0.979 with Rated Zone counts) and to take into account only the locomotor activity variables resulting from the whole 5 min video (as single minute parameters displayed high variability which may increase the chance of Type II errors). The final variables that were included in the analysis were: Track Length (TL, cm), Activity (AL, % the mice run at speed above 0.45 cm/s), Rated Zone Counts on a 4 × 4 grid (RZC, n), time spent at speed of 0–2 cm/s (VC1, s), time spent at speed of 2–4 cm/s (VC2, s), time spent at speed of 4–6 cm/s (VC3, s), time spent at speed of 6–8 cm/s (VC4), time spent at speed of 8–10 cm/s (VC5), time spent at speed of 10–12 cm/s (VC6), time spent at speed of 12–15 cm/s (VC7), time spent at speed of 15–20 cm/s (VC8), time spent at speed of 20–30 cm/s (VC9), time spent at speed of 30–40 cm/s (VC10). The velocity classes were also grouped into low (VC_low = VC1 + VC2), medium (VC_med = VC3 + VC4 +

Table 1

Baseline levels of phenotype parameters measured in old and young mice before infection (experiment series 1)^a.

	Young Mice				Old Mice			
	Trial I		Trial II		Trial I		Trial II	
	Control (n = 8)	Infected (n = 8)	Control (n = 8)	Infected (n = 8)	Control (n = 8)	Infected (n = 8)	Control (n = 8)	Infected (n = 8)
	Mean ± SD	Mean ± SD						
Operator collected data								
Body weight (g) (BW)	26.7 ± 3.0	26.8 ± 2.8	26.6 ± 3.2	27.1 ± 3.2	30.7 ± 5.4	30.2 ± 3.9	29.3 ± 3.7	30.3 ± 5.8
Water intake (ml) (WI)	5.5 ± 0.5	5.5 ± 0.6	5.6 ± 1	5.3 ± 0.3	5.7 ± 1.3	5.4 ± 0.7	6.8 ± 1.5	6.1 ± 1.7
Food intake (g) (FI)	3.7 ± 0.2	3.7 ± 0.3	3.7 ± 0.3	3.9 ± 0.3	3.4 ± 0.3	3.6 ± 0.4	3.8 ± 0.4	3.8 ± 0.4
Strength Score (SS)	9.3 ± 1.6	9.5 ± 2.2	8.4 ± 1.9	9.2 ± 1.1	7.0 ± 1.9	7.6 ± 2.4	6.9 ± 1.1	7.3 ± 1.6
Ventral surface temperature (°C) (ST)	32.8 ± 0.4	32.5 ± 0.3	32.9 ± 0.6	33.1 ± 0.5	32.6 ± 0.4	32.4 ± 0.8	32.8 ± 0.7	32.5 ± 0.8
Aspect Score (AS)	5.0 ± 0.1	4.9 ± 0.1	5.0 ± 0.0	5.0 ± 0.0	4.4 ± 0.5	4.5 ± 0.5	4.3 ± 0.3	4.5 ± 0.5
Locomotor activity data								
Track Length (cm) (TL)	2919 ± 769	3077 ± 1645	2474 ± 1504	3063 ± 957	2413 ± 467	2585 ± 1231	2071 ± 819	2369 ± 899
Activity (%) (AL)	86.9 ± 6.2	88.8 ± 6.2	77.7 ± 12.6	84.1 ± 8.7	84.4 ± 6.7	82 ± 11.7	74.5 ± 13.0	78.4 ± 12.2
Rated Zone Counts (n) (RZC)	405 ± 142	443 ± 340	351 ± 284	452 ± 160	340 ± 81	367 ± 205	293 ± 137	359 ± 165
Time (s) spent at speed of 0-6 cm/s (VC_low)	151 ± 28	146 ± 49	184 ± 53	156 ± 41	161 ± 29	161 ± 50	194 ± 41	183 ± 66
Time (s) spent at speed of 6-20 cm/s (VC_med)	73 ± 9	74 ± 14	54 ± 18	64 ± 17	73 ± 19	68 ± 17	51 ± 22	55 ± 31
Time (s) spent at speed of 20-40 cm/s (VC_high)	82 ± 17	86 ± 23	59 ± 26	75 ± 23	90 ± 16	85 ± 35	62 ± 20	83 ± 47

^a The control group in trial I is constituted by the same mice that are infected in trial II (performed month after trial I) while infected mice in trial I are the same mice used as controls in trial II. No significant difference is detected among the four groups within Young and Old mice by ANOVA.

Table 2
Multivariate Comparison of mean baseline levels of phenotype parameters between young and old mice.

	Female		Male		P values		
	Old Mean ± SD	Young Mean ± SD	Old Mean ± SD	Young Mean ± SD	age	gender	age*gender
Operator collected data							
Body weight (g) (BW)	26.1 ± 1.6	24.3 ± 0.9	34.1 ± 2.5	29.3 ± 1.8	< 0.01	< 0.01	< 0.01
Water intake (ml) (WI)	6.3 ± 1.3	5.5 ± 0.5	5.7 ± 1.4	5.5 ± 0.7	0.05	0.29	0.23
Food intake (g) (FI)	3.7 ± 0.4	3.7 ± 0.3	3.7 ± 0.4	3.8 ± 0.3	0.33	0.35	0.29
Strength Score (SS)	7.2 ± 1.6	9 ± 1.5	7.2 ± 1.9	9.2 ± 1.9	< 0.01	0.78	0.89
Ventral surface temperature (°C) (ST)	33.1 ± 0.4	33.0 ± 0.5	32.0 ± 0.4	33 ± 0.4	0.03	< 0.01	< 0.01
Aspect Score (AS)	4.4 ± 0.4	4.9 ± 0.1	4.4 ± 0.4	5.0 ± 0.0	< 0.01	0.45	0.91
Locomotor activity data							
Track Length (cm) (TL)	2509 ± 1044	2615 ± 1594	2175 ± 634	3152 ± 674	0.04	0.70	0.10
Activity (%) (AL)	78.8 ± 12.2	79.3 ± 10.6	80.7 ± 10.1	89.5 ± 3.9	0.04	0.70	0.10
Rated Zone Counts (n) (RZC)	368 ± 180	381 ± 311	304 ± 106	444 ± 131	0.13	0.01	0.08
Time (s) spent at speed of 0–6 cm/s (VC_low)	178 ± 56	176 ± 54	175 ± 40	142 ± 24	0.12	0.07	0.38
Time (s) spent at speed of 6–20 cm/s (VC_med)	56 ± 24	55 ± 16	66 ± 23	77 ± 8	0.21	0.54	0.19
Time (s) spent at speed of 20–40 cm/s (VC_high)	78 ± 38	69 ± 27	80 ± 25	82 ± 17	0.63	0.33	0.05

* Multivariate comparisons are computed using baseline levels (mean values from trial I and trial II). Abbreviations: Body weight (g) (BW), Water intake (ml) (WI), Food intake (g) (FI), Strength Score (SS), Ventral surface temperature (°C) (ST), Aspect Score (AS), Track Length (cm) (AL), Activity (%) (AL), Rated Zone Counts (n) (RZC), Time (s) spent at speed of 0–6 cm/s (VC_low), Time (s) spent at speed of 6–20 cm/s (VC_med), Time (s) spent at speed of 20–40 cm/s (VC_high).

VC5 + VC6 + VC7) and high (VC_high = VC8 + VC9 + VC10).

To exclude behavioral changes related to novelty of the arena, mice were exposed to the apparatus for 5 min 2 days per week for two consecutive weeks until stabilization of baseline parameters.

2.5. Fecal shedding of *E. coli*

Stool samples from uninfected and infected mice were collected from home cages at days 1, 3, 6 and 9 post-infection. Stool samples were weighed and homogenized in physiological solution. Homogenized stool samples were serially diluted and 100 µl aliquot of each fecal dilution was spread plated on MacConkey-Sorbitol agar supplemented with 50 mg/l cefixime and 2.5 mg/l potassium tellurite (CT-SMAC medium). After 24 h of incubation at 37 °C, all sorbitol nonfermenting (neutral/gray with a smoky center, 1–2 mm in diameter) or negative colonies were counted and CFU were determined per gram of stool. Negative colonies were also recovered for PCR confirmation.

Qualitative PCR was used to confirm positive colonies grew on CT-SMAC medium. Genomic DNA was extracted from bacterial colonies grew in CT-SMAC medium after an incubation at 37 °C for 24 h. A boiling method was used. Briefly, 4 bacterial colonies were picked and suspended in 100 µl of sterile distilled water. The suspension was placed in a boiling water bath for 5 min, placed in an ice bath for 2 min, and then centrifuged at 12,000 rpm for 5 min. The supernatants were stored at –20 °C until they were used for the qPCR test. Bacterial genomic DNA from faeces samples was extracted using GeneAll Exgene™ Stool DNA mini kit (TEMA Ricerca, Bologna, Italy) following the manufacturer's instructions.

PCR primers were designed based on the wzx gene region of *E. coli* O157:H7 and are reported below:

5'-GCT GCT TAT GCA GAT GCT C-3'

5'-CGA CTT CAC TAC CGA ACA CTA-3'

The PCR reaction was performed in 50 µL volumes, in which the reaction mixture contained 2 µL of DNA, 1x RBC Taq DNA Polymerase Mix (RBC Bioscience), 300 µM of dNTPs, 3 mM MgCl₂, 1.25 U Taq DNA Polymerase and 0.3 µM of each primer. The cycling protocol consisted of an initial denaturation at 94 °C for 5 min, then 35 cycles which consisted of denaturation at 94 °C for 30 s., annealing at 63.5 °C for 20 s and extension at 72 °C for 52 s. The sample was then heated at 72 °C for 7 min for a final extension. Negative controls were run with each test. Agarose gel electrophoresis was used to detect PCR products (133bp).

2.6. Statistics

Normality tests were run for the whole set of variables and within each experimental group and time. Generalized linear mixed model analysis (IBM SPSS, v.23) was used to take into account the longitudinal design. The identifier of each mouse, experimental trial and repeated measures (time) was indicated in all model tested. The linear models were developed assuming normal distribution with identity link function. For variables not following normal distribution we verified significant differences by Kruskal-Wallis or Mann-Whitney test. Scores aimed at tracking with high sensitivity the burden of infection were developed by stepwise discriminant analysis and by the sum of the standardized variables showing major changes during the infection. All statistics were computed using SPSS v. 23 (IBM).

3. Results

3.1. Baseline levels of physical and locomotor parameters during experiments series 1

All groups of mice showed no significant differences on the studied parameters collected at baseline during trial I and trial II (Table 1). Since mice infected in trial I had completely recovered from infection before starting trial II, thus justifying the use of this group of mice as controls in trial II.

An exploratory multivariate analysis performed using the whole set of baseline data from trial I and trial II identified several differences between the young and old groups (Table 2) as well as between males and females with few interactions. In particular, BW was higher while SS, AS, AL, TL, VC2, VC3 and VC10 were lower in old mice. The most significant interactions regard ST and BW, which decline in old male mice but seems to be more stable in female.

3.2. Fecal Shedding of *E. coli* after infection in experiments series 1

We monitored fecal shedding by viable bacterial counts (confirmed by qualitative PCR). Fig. 2 represents the fecal shedding of *E. coli* during the 1st series of experiments (control groups are omitted as they were all negative). *E. coli* was undetectable in the feces of young mice after day 6 post-infection while some feces samples from old mice remained positive, suggesting a slower recovery of old animals compared to young ones. However, no statistically significant differences between trials, age or gender were observed in fecal shedding.

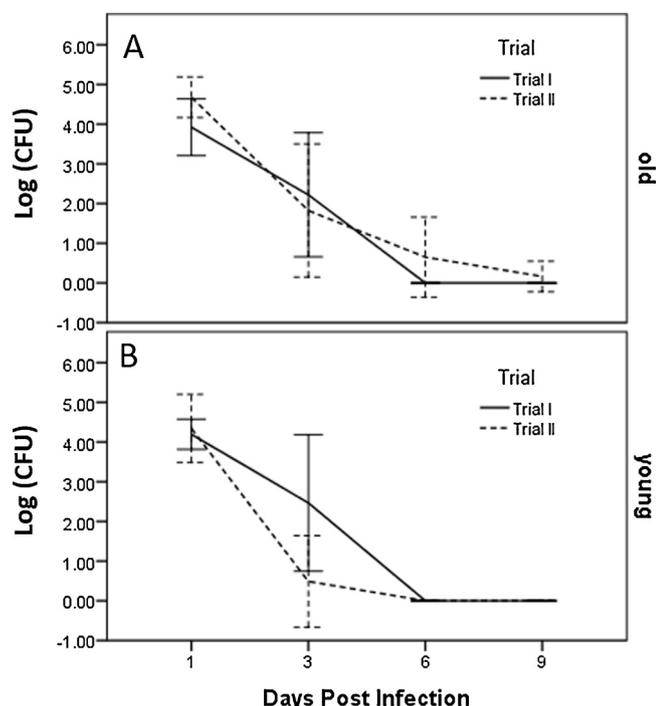


Fig. 2. Fecal Shedding post infection.

Viable bacteria, expressed as Log(CFU/g feces), recovered in the feces of old (Panel A) and young (Panel B) infected mice during the two trials of experiments series 1 (model development). CFU = colony forming units. No differences were detected between the two trials by mixed linear models.

3.3. Changes of physical and locomotor activity parameters during experiments series 1

We preliminarily checked the presence of group and gender differences in the response to infection of all parameters collected in Trial I and II by multivariate analysis (Fig. 3). We failed to observe any gender difference in the response to infection expressed as % change (% Δ) with respect to the baseline level. Conversely, we observed a significant difference in the response to infection based on age groups. In particular, there was a different response for BW (p for interaction = 0.002), AS (p for interaction < 0.001) and VC10 (p for interaction = 0.013) according to age. Additionally, a linear model computed for repeated measures on the whole post-infection detect a significant lower % Δ SS in infected old male mice compared to the adult counterpart, which suggest a delayed period to return to basal level in old male mice. Taking also into account the number of differences at baseline between young and aged mice in several physical and locomotor activity parameters we have decided to perform a separate analysis for the two age groups.

In Fig. 3 are reported the % Δ of each parameter measured in the post infection period during the 1st series of experiments. In both trials we found a significant reduction of BW at 1st and 6th day post infection in young infected mice compared to controls. Similarly, SS was significantly lower on day 1 post-infection for Trial I and on day 3 post infection for trial II in young infected mice compared to controls. Other changes in young mice involved locomotor activity parameters. In particular, % Δ RZC and % Δ VC_{high} were lower while % Δ VC_{low} was higher in the infected group compared to controls (in the period comprised from the 1st up to the 6th day post-infection). We also found significant lower % Δ AL and % Δ TL in infected mice compared to controls at 3 and 6 days post-infection. Minor changes were observed for % Δ VC_{med}. In analogy to young mice, we found a decreased % Δ SS in infected old mice compared to controls (in the period comprised from the 1st up to the 6th day post-infection). Locomotor activity parameters of old mice were also affected by the infection. The most important

changes included higher % Δ VC_{min} and lower % Δ TL, % Δ AL, % Δ RZC as well as % Δ VC_{high} in infected old mice compared to controls. However, only % Δ VC_{min} showed similar significant changes in both trial I and trial II of the crossover trial. We also detected a significantly increased water intake in infected mice compared to controls during the first day post infection (data not shown).

3.4. Computation of scores for longitudinal monitoring of bacterial burden

According to the results of experiment series I (Trial I + Trial II), differences between control and infected mice were observed up to the 6th day post infection on various phenotype parameters. We used these data to run several discriminant analysis models with the aim to identify the most important variables involved in the response to infection as well as to develop sensitive scores for the quantification of the response to infection. Although discriminant analysis may violate the assumption of independent measures, the within group correlations for repeated measures of each % Δ of phenotype variables and those of fecal shedding were remarkably low, thus suggesting that stepwise discriminant models can be applied on these data. The most representative models (Wilks' Lambda < 0.001) are reported in Table 3. In addition to fecal shedding (log(CFU)), models 1–3 included all % Δ of phenotype parameters, while models 4–5 have been computed including only % Δ data of variables measuring physical performances. Separate analysis of old and young mice appears to be important to improve sensitivity as the most important predictors were not exactly the same in old and young mice. Indeed, the most important predictors identified in young mice were % Δ RZC, % Δ SS and % Δ BW, whereas % Δ VC_{low}, % Δ SS and % Δ ST were the most important predictors in old mice. Interestingly, the discriminant model that included only variables related to physical performances still retain a significant discriminant power. Graphical representation of the scores computed with the coefficients of models 4 and 5 are reported in Fig. 4. All other models displayed very similar graphical representation (see supplementary Fig. 1), thus suggesting that the scores using the lowest number of representative variables (score 4 and score 5) have enough sensitivity to measure the response to infection. However, since the discriminant coefficients may underestimate the contribution of physical performance compared to fecal shedding, we computed two additional combined scores as the sum of the standardized values of the variables identified in model 4 and model 5. Hence, the score in young mice (Zscore 4) was computed as $Z(\% \Delta SS) + Z(\% \Delta RZC) - Z(\log CFU)$. The respective score in old mice (Zscore 5), was computed as $Z(\% \Delta SS) - Z(\% \Delta VC_{1-2}) - Z(\log CFU)$. Both scores appear to be as sensitive as the scores calculated by discriminant analysis (supplementary Fig. 1). Importantly, analysis performed with scores computed in old mice detect a significant difference between infected and control group at day 9 post-infection in the Trial II which was not detected in young mice. This difference may be due to a slower recovery from infection of old mice at the dose of pathogen tested during second trial. However, all parameters and the respective scores were normalized after day 9 post-infection (data not shown) and none of the mice died or display evident sign of suffering. It is important to compare the results based on the scores with those of fecal shedding. In fact, while fecal shedding strongly contributes to the scores during the 1st and 3rd days of infection, the remaining part of the post infection period is characterized by changes in physical parameters that are part of the overall sickness behavior of mice.

3.5. Validation of scores and estimation of effects of oxytetracycline

We tested the developed scores containing the minimum set of variables (score 4 and score 5) in a different population of old and young mice infected with 5×10^7 *E. coli* (experiment series II, see Fig. 1) and including an additional group of mice for each age group that was treated with oxytetracycline, an antibiotic that is effective

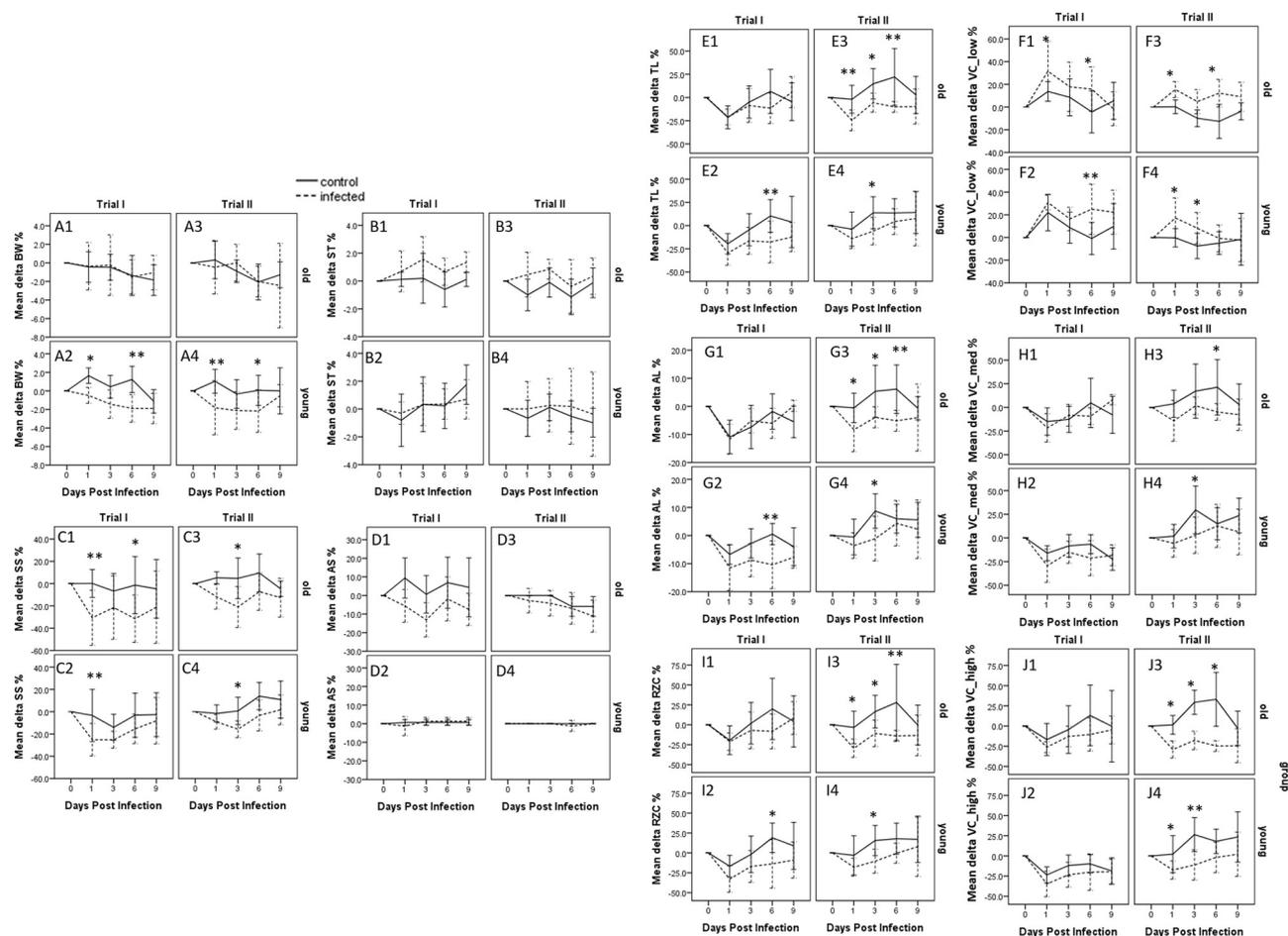


Fig. 3. Changes of phenotype parameters after infection.

Relative changes (delta) % of phenotype parameters (referred to baseline) collected in old (panels 1 and 3) and young (panels 2 and 4) mice during trial I (panels 1 and 2) and trial II (panels 3 and 4). A1–A4: changes % of Body Weight (BW); B1–B4: changes % of ventral Surface Temperature (ST); C1–C4 = changes % of Strength Score; D1–D4: changes % of Aspect score (AS); E1–E4: changes % of Track Length (TL); F1–F4: changes % time spent at speed of 0–6 cm/s (VC_low); G1–G4 = changes % of Activity (AL); H1–H4: changes % of time spent at speed of 6–20 cm/s (VC_med); I1–I4: changes % of Rated zone Counts (RZC); J1–J4: change % of time spent at speed of 20–40 cm/s (VC_high). Means \pm 95%CI are displayed; Controls = continuous line; Infected mice = dotted line; * $p < 0.05$ compared to controls and ** $p < 0.01$ compared to controls by mixed models analysis for longitudinal data (SPSS).

against our *E. coli* strain. The results of this trial are displayed in Fig. 5. The computed scores confirm their sensitivity to quantify the sickness behavior associated with infection ($p < 0.05$ at least between infected and control mice at days 1, 3 and 6 post infection) and, most importantly, they are able to quantify the effectiveness of the antibiotic in reducing the time and the burden associated with infection. Significant differences between antibiotic treated mice and infected mice can be detected at days 3, 6 and 9 post infection in young mice, as well as at day 3 post infection in old mice. The overall estimated mean response of the three groups for the period comprised from the 1st to the 6th day post infection (computed by mixed models for longitudinal analysis) clearly represents the differences among the three groups (supplementary data 1). However, the difference between the antibiotic and infected group appears to be significant only in young mice. Results using other scores described in Table 3 were similar to those shown in Fig. 5 (data not shown). Near the end of the experiments (day 9) we had to suppress an old mouse in the antibiotic group due to evident signs of suffering. Post mortem examination revealed that death was caused by a liver cancer thus being unrelated to the treatment or infection.

4. Discussion

A non-invasive mouse model of mild oral *E. coli* O157:H7 infection as well as scores to monitor the response to infection were developed

for young and aged C57BL6 mice. The model is based on the oral administration of 10^7 – 10^8 CFU of *E. coli* O157:H7 in C57BL6 mice not previously treated with streptomycin, thus avoiding permanent colonization of the pathogen and the associated lethal outcome. The scores used to monitor infection are mainly based on fecal shedding and on changes to baseline of physical performance variables. The scores display that the sickness behavior of infected mice lasts approximately one week (or little more) post infection before returning to baseline levels. The contribution of fecal shedding to the score is higher during the first days post infection, but alterations in the physical parameters were prominent after 3 days when viable *E. coli* in the feces disappeared.

This is in agreement with the outcome of the *E. coli* O157:H7 infection in humans where usually the illness resolves in 6–14 days [6]. Our model is based on previous results on the susceptibility of laboratory mice to intestinal colonization by *E. coli* [4,7,8], where fecal shedding of the pathogen was observed in a range of 8–14 days. Since we used a dose of pathogen lower of 1–2 order of magnitude it was expected to observe a faster fecal shedding. Indeed, in our model the shedding of the pathogen was completed in 6 days with some exceptions in old mice.

While the mice did not display evident signs of infection, we found significant changes in the battery of tests related to physical performances. These changes could be related to a transient post-infection weakness as well as to altered motivation associated with the symptom

Table 3
Discriminant analysis models and coefficients of Recovery Scores.

Score (Model)	1		2		3		4		5	
Subgroups	All mice		Young mice		Old mice		Young mice		Old mice	
variables	all		all		all		Physical performance		Physical performance	
Parameter	Correlations	Coefficients								
Log(CFU)	-0.706	-0.456	-0.604	-0.360	-0.730	-0.502	-0.817	-0.477	-0.776	-0.510
%ΔBW	0.248	0.188	0.559	0.342	-0.164					
%ΔSS	0.466	0.019	0.400	0.022	0.479	0.021	0.542	0.023	0.510	0.026
%ΔST	-0.205		0.008		-0.318	-0.217				
%ΔAS	0.250	0.142	0.005		0.145					
%ΔTL	0.317		0.366		0.331		0.543		0.360	
%ΔAL	0.278		0.277		0.282		0.455		0.278	
%ΔRZC	0.309		0.396	0.014	0.275		0.536	0.014	0.313	
%ΔVC_low	-0.321		-0.338		-0.373	-0.018	-0.537		-0.396	-0.018
%ΔVC_med	0.178		0.233		0.176		0.451		0.188	
%ΔVC_high	0.394	0.012	0.304		0.340		0.479		0.349	
Constant		0.864		0.768		0.888		0.724		0.910
Computation	Score at group centroid									
Control	0.981		1.042		1.034		0.770		0.972	
Infected	-0.981		-1.042		-1.034		-0.770		-0.972	

^aWithin-groups correlations between discriminating variables and the respective score; Wilks' Lambda $p < 0.001$ for each score; Scores were computed by discriminant analysis (stepwise method) of mice infected versus controls using the absolute change (%Δ) of each parameter with respect to the day before infection as discriminating variables. Absolute values of correlations > 0.3 are highlighted in bold. The coefficients of the minimum set of variables included (stepwise method) in the discriminant function are shown in the next column on the right to the correlation column for each model. Abbreviations: Body weight (g) (BW), Water intake (ml) (WI), Food intake (g) (FI), Strength Score (SS), Ventral surface temperature (°C) (ST), Aspect Score (AS), Track Length (cm) (AL), Activity (%) (AL), Rated Zone Counts (n) (RZC), Time (s) spent at speed of 0–6 cm/s (VC_low), Time (s) spent at speed of 6–20 cm/s (VC_med), Time (s) spent at speed of 20–40 cm/s (VC_high).

of the infection [10]. Taking into account that an immune response to oral challenge with *E. coli* has been already shown in C57BL/6 mice [7], it is reasonable to assume that the pathogen challenge may produce an immunologically induced fatigue similar to the one reported in models of challenge with cytokines or lipopolysaccharide [14,15].

As alternative or contributing factor, our results may be related to a “loss of interest” behavior and not to a real muscle weakness. Our observation related to changes in muscle strength measurement is in contrast with the data recently reported on an *E. coli* (O18:K1) septic peritonitis mouse model where no change in grip strength was observed up to 48 h post infection both in young and aged mice [16]. Although these differences could be related “*in primis*” to the *E. coli* strain used in the experiments, there are at least two additional important differences. The first one relates to the method used to test hand grip strength. Indeed, we did not use a grip strength meter but rather, a sequential weight lifting method, which might involve a component of resistance in the measurements. Moreover, our measurement might be related to an increased loss of interest in holding the grip of infected mice compared to controls. Indeed, frequent testing, as performed in our case, has been reported to lead to a loss of interest in holding the grip [17], which might be exacerbated in infected mice due to physical impairment. Hence, our recorded behavioral changes may not be the direct consequence of a general debilitating effects of the infection, but rather the results of changes in the motivational state. This change was hypothesized to be a functional response useful to potentiate the host response and facilitate recovery to infection [18].

As we know aging is a complex process that has dramatic impacts on most systems in the body [19]. Our results confirm the previous observation that age is one of the critical factors to be carefully considered when designing behavioral and physiological tests [20] as well as physical performance measures [21]. Indeed, with the exclusion of changes in grip test, both baseline levels of several physical parameters and the minimum set of variables that discriminate infected from control mice appears to be different in old and young mice. The additional observation that the recovery to baseline levels of the scores is delayed in old male mice treated with the highest dose of pathogen

(Trial II in experiments series I) suggests that physical performance in old individuals may be more affected by the infection than the younger counterpart. Our study shows that the recovery post infection is therefore delayed in old mice compared to young mice even with a mild *E. coli* infection supporting the fact that the most prominent manifestations of aging is an increase in susceptibility to infections. In the validation trial, however, we detected an apparent slower recovery in young mice, due to less significant changes after the 1st day post infection. The reason could be checked in the highest variability observed in the old mice groups. In this case, undiagnosed diseases, such as our case of liver cancer in the antibiotic group, and other sources of heterogeneity caused by advanced aging may play a role.

All the scores developed in this study discriminated the response of infected from control mice up to the 9th day post infection, where we recorded the normalization of most parameters. The developed scores were also enough sensitive to detect inter-group changes in the validation trial (experiment series II). The developed scores were able to detect differences between the infected and control group as well as to confirm the beneficial effects of an antibiotic on the recovery post infection. This is likely the consequence of the extremely fast fecal shedding of *E. coli* observed in response to the antibiotic, but also to a minimization of the impact of the infection on physical performances. With the exclusion of the mice who died for liver cancer, all the other mice were still living several days post infection without any particular sign of alteration or disease. Last, but not least, there are several strain apparatuses commercially available that can provide graded data of muscle strength and locomotor activity than those that we propose in this study, but their cost may put them beyond the reach of many laboratories which do not specialize in testing physical performance. Hence, the model here developed can be used to refine animal experiments of infection with *E. coli* with a significant reduction in costs as well as in suffering of mice used in the experiments.

Limits of the study: although we used oxytetracycline to provide evidence that our scores can be used to develop treatments against *E. coli*, it is important to remark that the effects of the antibiotic used in this study may be limited to the particular strain of *E. coli* used in the

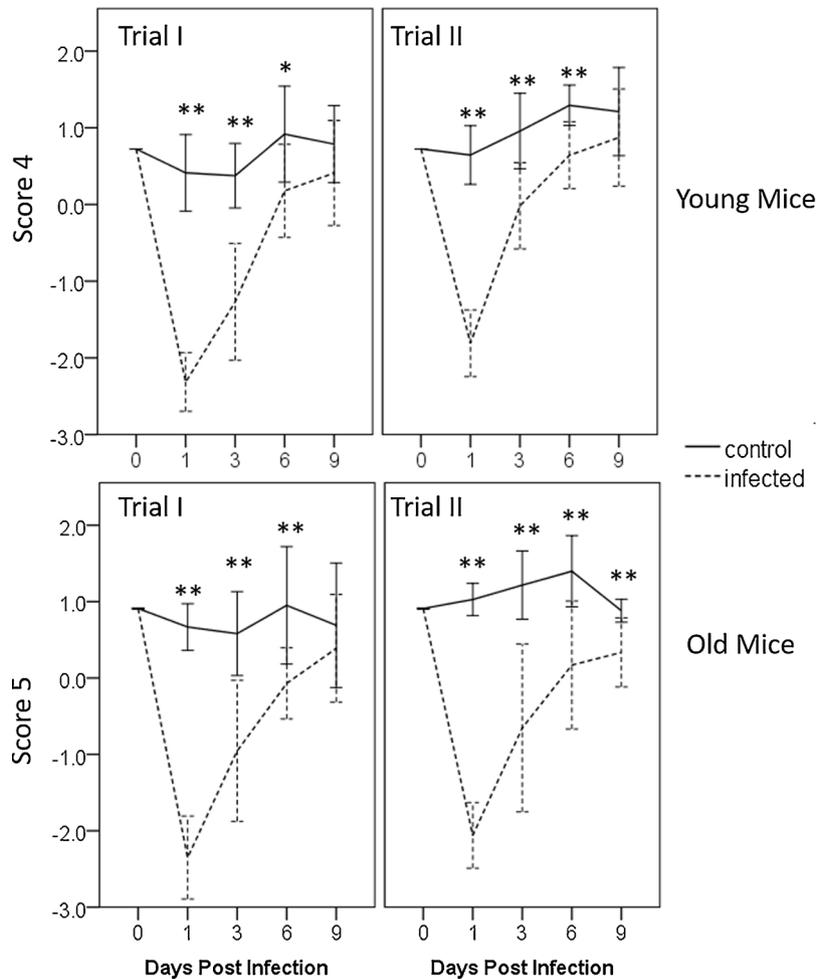


Fig. 4. Change of computed scores after infection. Time course of two scores computed by discriminant analysis to quantify the longitudinal response to infection in young (score 4) and old mice (score 5). Means \pm 95%CI are displayed; Controls = continuous line; Infected mice = dotted line; * $P < 0.05$ and ** $p < 0.01$ based on overall test results from mixed models analysis for longitudinal data (SPSS).

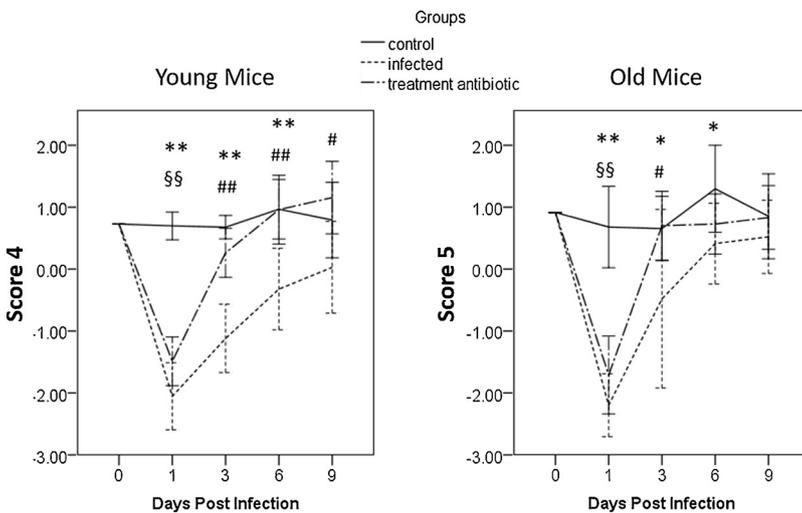


Fig. 5. Use of computed scores to study the efficacy of an intervention.

Quantification of the response to infection in mice infected with *E. coli* (5×10^7 CFU) in presence or absence of treatment with oxytetracycline and versus untreated/uninfected mice. The response to infection is evaluated with the scores (score 4 for young mice and score 5 for old mice) developed during experiment series II. Means \pm 95%CI are displayed; Controls = continuous line; Infected mice = dotted line; infected mice treated with oxytetracycline = composite continuous and dot line; * $P < 0.05$ and ** $p < 0.01$ for controls vs infected mice; §§ $p < 0.01$ for controls vs infected mice treated with oxytetracycline; # $p < 0.05$ and ## $p < 0.01$ for infected mice vs infected mice treated with oxytetracycline. Significance is based on results from mixed models analysis for longitudinal data (SPSS).

experiments and that these experiments may need to be reproduced in the context of different bacteria strain and species. Given the differences between the human and mouse species, the increasing phenomenon of antibiotic resistance and the relative high mutation rate of most bacteria species, caution is recommended in the interpretation of the results as a demonstration of the efficacy of oxytetracycline against *E.*

coli O157:H7.

5. Conclusions

Resistance to antibiotics has been growing fast and this includes most *E. coli* strains [22]. Growing antibiotics resistance to multiple

strains of *E. coli* and to other bacteria has triggered search for alternative therapy. For example, probiotics have shown promise [23]. We have been able to develop a model of transient and non-lethal mild *E. coli* infection that due to the sensitivity of several integrated non-invasive measurements could be particularly suited for the development of new therapies. The model could be useful to identify potential disease prevention strategies and therapeutics aiming to ameliorate or reduce the sickness behavior including physical impairment associated with the subclinical bacterial infection. Our model also contributes to decreasing experimental costs and to refine the procedures for testing treatments against infection in laboratory animals.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.cimid.2018.12.003>.

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