Journal of Visualized Experiments

Galleria mellonella oral administration model to study commensal-induced innate immune responses

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59270R1
Full Title:	Galleria mellonella oral administration model to study commensal-induced innate immune responses
Keywords:	Galleria mellonella, oral infection, force-feeding, intestinal commensals, insect model, immunogenic, innate immunity
Corresponding Author:	Julia-Stefanie Frick Universitatsklinikum Tubingen Tübingen, Baden-Württemberg GERMANY
Corresponding Author's Institution:	Universitatsklinikum Tubingen
Corresponding Author E-Mail:	Julia-Stefanie.Frick@med.uni-tuebingen.de
Order of Authors:	Anna Lange
	Andrea Schäfer
	Julia-Stefanie Frick
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tübingen, Baden-Württemberg, Germany

Prof. Dr. Julia-Stefanie Frick
Institute of Medical Microbiology and Hygiene
University of Tübingen
72076 Tübingen
Germany
Telephone: 0049-7071-29 82352
Fax: 0049-7071-29 5440
E-mail: julia-stefanie.frick@med.uni-tuebingen.de

November, 28th 2018

Dear Sir or Madam,

we are pleased to resubmit the revised version of the manuscript **"*Galleria mellonella oral administration model to study commensal-induced innate immune responses*"** by Anna Lange, Andrea Schäfer and Julia-Stefanie Frick to be considered for publication in **"Journal of Visualized Experiments"** in the section **"Immunology & Infection"**.

We thank the editor and reviewers for their constructive suggestions, which we have all incorporated into our manuscript.

We have the impression that our manuscript has significantly improved, thanks to the work and suggestions of the reviewers and hope that our work is now suitable for publication in **"Journal of Visualized Experiments".**

Yours sincerely,

Julia-Stefanie Frick

TITLE:

A *Galleria mellonella* Oral Administration Model to Study Commensal-Induced Innate Immune Responses

AUTHORS AND AFFILIATIONS:
Anna Lange, Andrea Schäfer, Julia-Stefanie Frick

Department for Medical Microbiology, Hygiene, Interfacultary Institute for Microbiology, Infection Medicine, University of Tübingen, Germany

Corresponding author:
Julia-Stefanie Frick
julia-stefanie.frick@med.uni-tuebingen.de

Email addresses of co-authors:
Anna Lange: anna.lange@med.uni-tuebingen.de
Andrea Schäfer: andrea.schaefer@med.uni-tuebingen.de

KEYWORDS:
Galleria mellonella, oral infection, force-feeding, intestinal commensals, insect model, immunogenic, innate immunity

SUMMARY:
Here, we provide a detailed protocol for an oral administration model using *Galleria mellonella* larvae and how to characterize induced innate immune responses. Using this protocol, researchers without practical experience will be able to use the *G. mellonella* force-feeding method.

ABSTRACT:
The investigation of the immunogenic potential of commensal bacteria on the host immune system is one essential component when studying intestinal host-microbe interactions. It is well established that different commensals exhibit a different potential to stimulate the host intestinal immune system. Such investigations involve vertebrate animals, especially rodents. Since increasing ethical concerns are linked with experiments involving vertebrates, there is a high demand for invertebrate replacements models.

Here, we provide a *Galleria mellonella* oral administration model using commensal non-pathogenic bacteria and the possible assessment of the immunogenic potential of commensals on the *G. mellonella* immune system. We demonstrate that *G. mellonella* is a useful alternative invertebrate replacement model that allows the analysis of commensals with different immunogenic potential such as *Bacteroides vulgatus* and *Escherichia coli*. Interestingly, the bacteria exhibited no killing effect on the larvae, which is similar to mammals. The immune responses of *G. mellonella* were comparable with vertebrate innate immune responses and involve recognition of the bacteria and production of antimicrobial molecules. We propose that

G. mellonella was able to restore previous microbiota balance, which is well known from healthy mammalian individuals. Although providing comparable innate immune responses in both *G. mellonella* and vertebrates, *G. mellonella* does not harbor an adaptive immune system. Since the investigated components of the innate immune system are evolutionary conserved, the model allows a prescreening and first analysis of bacterial immunogenic properties.

INTRODUCTION

The intestinal microbiome is an essential component for maintenance of homeostasis, and involves both innate and adaptive immune responses[1,2]. The commensal microbiota community is characterized by different main commensal constituents: symbionts that confer beneficial effects by important immunomodulatory functions, and pathobionts that can have detrimental effects in genetically predisposed hosts and promote and trigger intestinal inflammation[3,4]. Many studies on symbionts and pathobionts and their influence on the host immune system have been published mainly studying adaptive immune responses.

Since these studies involve many animals for the investigations and the protection and replacement of animals used for experimentation is of increasing public interest, we seek to find a replacement model to allow for a screening of different bacterial immunogenic properties. Insects, especially *Galleria mellonella*, are a widely used replacement model in infection research. *G. mellonella* combines different advantages such as low costs and high throughput; it allows oral administration of bacteria, which is the natural exposure route, and it allows for systemic infection[5,6]. *G. mellonella* further enables incubation at 37 °C, which is the physiological body temperature of mammals and the optimum for bacterial virulence factor expression[5]. The main advantage of *G. mellonella* is the conserved innate immune system that enables the discrimination of self from non-self and encodes a variety of pattern recognition receptors like apolipophorin or the opsonin hemolin[6,7]. Upon microbe recognition, *G. mellonella* can trigger different downstream humoral immune responses. It can induce oxidative stress responses and secrete reactive oxygen species (ROS) which involves the activity of NOS (nitric oxidase synthase) and NOX (NADPH oxidase)[6,8]. In addition, *G. mellonella* activates a potent antimicrobial peptide (AMP) response, which results in the secretion of a mixture of different AMPs such as gloverin, moricin, cecropin or the defensin-like gallerimycin[6,8-10]. Generally, AMPs have quite broad host specificity against Gram-positive and Gram-negative bacteria and fungi and have to provide an potent response since insects are lacking any adaptive response[10]. Gloverin is an AMP active against bacteria and fungi and inhibits outer membrane formation[6,11]. Moricins exhibit their antimicrobial function against Gram-positive and Gram-negative bacteria by penetrating the membrane and forming a pore[9,11]. Cecropins provide activity against bacteria and fungi and permeabilize the membrane similarly like moricins[9,10]. Gallerimycin is a defensin-like peptide with anti-fungal properties[9]. Interestingly, it was found that the combination of cecropin and gallerimycin had a synergistic activity against *E. coli*[10].

Due to their easy-to-use character *G. mellonella* larvae are an often used infection model to assess bacterial pathogenicity. In particular, studies in which data obtained from *G. mellonella* correlate with data obtained from mice support the strength of this alternative host model. It was found that the most pathogenic serotypes of *Listeria monocytogenes* in a mouse infection

model lead also to higher mortality rates in *G. mellonella* after systemic infection. Further, less virulent serotypes turned out to be also less virulent in the *G. mellonella* model[12]. Similar observations have been made with the human pathogenic fungi *Candida albicans*. Virulence of different *C. albicans* strains has been assessed by systemic infection and subsequent monitoring of larval survival. Mouse avirulent strains were also avirulent or exhibited reduced virulence in *G. mellonella*, whereas the mouse virulent strains lead also to high larval mortality[13]. The *G. mellonella* model could further be used to identify type 3 secretion system pathogenicity factors of *Pseudomonas aeruginosa*[14].

Since most investigations involving *G. mellonella* were focused on virulence factors using the systemic infection approach we were especially interested in providing a method suitable for the analysis of intestinal commensals in an oral force-feeding model in which we can apply a distinct dosage of bacteria per larvae and not only observe the larval mortality rate but analyze different hallmarks of innate immune responses to maintain intestinal homeostasis.

Our method helps to increase the use of *G. mellonella* as a replacement model since we combine the application of bacteria and the analysis of RNA expression. It is not only useful to strengthen the meaning of bacterial pathogenesis studies when including the analysis of immune responses after oral administration and not only the observation of mortality rates after systemic infection. Our methods allows for the analysis of immunogenic properties of bacterial non-pathogenic commensals since it is provides more complex conditions than cell culture by offering an intestinal barrier in a living organism.

PROTOCOL

1. *G. mellonella* rearing and preparation of the larvae for the experiments

NOTE: The cycle from egg to last instar larva takes approximately 5-6 weeks.

1.1. Transfer the eggs laid by adult moths to 2 L boxes containing wax moth substrate (22% corn grits, 22% wheat meal, 17.5% beeswax, 11% skimmed milk powder, 11% honey, 11% glycerol, 5.5% dried yeast). Perform the whole breeding at 30 °C in the dark.

1.2. Transfer 25 g of substrate containing the larvae into fresh substrate after approximately 1-2 weeks when small and tiny larvae were visible. Synchronize the larvae after 2 weeks according to their size and keep groups of 30-40 larvae in 2 L containers on wax moth substrate for additional 2 weeks.

1.3. Select the larvae for experiments by weight. Use only pale and fast moving larvae with a mass of 180-200 mg.

2. Cultivation and preparation of *Bacteroides vulgatus* and *Escherichia coli* for oral administration

2.1. Grow the obligate anaerobic bacterium *Bacteroides vulgatus* mpk at 37 °C anaerobically using jars and sachets for creating an anaerobic environment (see **Table of Materials**)[15,16]. Cultivate *B. vulgatus* for 2 days and grow an overnight subculture in brain heart infusion (BHI) broth.

2.2. Grow the facultative anaerobic bacterium *Escherichia coli* mpk under aerobic conditions in Luria-Bertani (LB) broth at 37 °C. Cultivate *E. coli* overnight in LB broth and grow subculture for 2 h at 37 °C on the day of the experiment.

2.3. Harvest the cultures by centrifugation at 1,700 x *g* for 5 min. Resuspend the bacterial pellets in DPBS (Dulbecco's Phosphate-Buffered Saline). Determine the optical density (OD) of the bacterial cultures at OD 600 nm and calculate the bacterial concentrations. The bacterial concentrations were adjusted to 10^9/mL.

3. Force-feeding of *G. mellonella* larvae with bacterial suspensions

3.1. Force-feed each larva with 10 µL of the adjusted bacterial suspension containing 10^7 bacteria per dose. Use an insulin syringe with a blunt-ended needle for oral application of the bacterial suspension.

3.1.1. Fix the syringe was fixed into a microsyringe pump (**Figure 1**) to ensure the accuracy of the applied suspension volume to each larva (see **Table of Materials**). Insert the syringe carefully between their mandibles. Do not force the syringe between the mandibles. Wait for the larvae to open it mouthparts and insert then the syringe.

3.2. Incubate the force-fed larvae in the dark at 37 °C between 1-24 h. Use DPBS-administered larvae as mock background controls to exclude potential stress responses induced due to the handling of the larvae during force-feeding.

4. Processing of orally administered larvae and RNA isolation

4.1. Work under a hood and wear safety glasses. Clean the hood and spray reagent to prevent RNase contamination.

4.1.1. Snap-freeze the living larvae after incubation in liquid nitrogen and homogenize them. Use a mortar and pistil for homogenization. Add liquid nitrogen to the mortar and grid each larval individual until powdered homogenates are produced.

4.1.2. Pour the homogenate to a disposable weighing boat and wait for the liquid nitrogen to evaporate.

4.2. Mix the liquid nitrogen-free frozen powdered homogenates with 1 mL of Trizol in a 2 mL tube and incubate the mixture at room temperature for 1 h.

4.3. Centrifuge the mixture at 8,000 x *g* for 15 min at room temperature and transfer the supernatant into a fresh tube and discard the pellet. Mix the supernatant with 200 µL of 1-Bromo-3-Chloropropane (BCP). Incubate the mixture for 5 min at room temperature and for 10 min on ice.

4.4. Centrifuge the BCP-added reactions at 18,000 x *g* for 15 min at 4 °C. Transfer the upper transparent layer into a new 2 mL tube and discard the rest. Precipitate the RNA of the transferred upper layer with 500 µL isopropanol by mixing and inverting the tube for 5 min.

4.5. Centrifuge the tube at 18,000 x *g* for 15 min at 4 °C. Wash the precipitated RNA pellet with 500 µL of 75% ethanol.

4.6. Dry the RNA pellet for 5-10 min at RT. Take care to not over dry it as it will be hard to dissolve later.

4.7. Dilute ribonuclease inhibitor (1:100) in nuclease-free water and use 100 µL of the solution to resuspend the dried RNA pellet. Vortex the tube carefully until the pellet is completely dissolved.

4.8. Measure RNA quality and quantity. Ensure that the 260/280 ratio is approximately 2.0 and 260/230 ratio in the range of 2.0-2.2 (see **Table of Materials**).

4.9. Use 5 µg of the isolated RNA for DNase digestion. Mix 5 µL of 10x buffer, 1 µL of ribonuclease inhibitor enzyme, 2 µL of DNase enzyme, 5 µg of RNA, and fill up with nuclease-free water up to 50 µL. Incubate for 30 min at RT.

4.9.1. Add 6 µL of inactivation reagent and incubate for 2 min at RT and vortex reaction occasionally. Centrifuge reaction at 10,000 x *g* for 1 min. Transfer supernatant into fresh 1.5 mL tube.

NOTE: The RNA contains the larval RNA as well as the bacterial RNA of the respective strain used for oral administration.

5. Quantification of the bacterial 16S copy numbers after force-feeding

NOTE: The copy numbers of the expressed bacterial 16S was determined using cDNA synthesized from the RNA extracted in section 4. Final quantification is calculated with the help of a standard curve of plasmid in which the 16S PCR fragment of either *B. vulgatus* or *E. coli* was cloned.

5.1. Preparation of plasmid standards

5.1.1. Amplify 16S fragments from *E. coli* mpk or *B. vulgatus* mpk genomic DNA by PCR. Mix 10 µL of 5x buffer, 1 µL of 10 mM dNTP solution, 2.5 µL of 10 µM forward primer and 2.5 µL of

10 µM reverse primer dilution, 1 µL of DMSO, 1 µL of genomic DNA template, 31.5 µL of nuclease-free water and 0.5 µL of proof-reading enzyme.

5.1.2. Run the PCR (initial denaturation: 98 °C for 30 s, denaturation: 98 °C for 10 s, annealing: 60 °C for 30s, extension: 72 °C for 30 s, final extension: 72 °C for 5 min, repeat denaturation, annealing and extension for 30 cycles).

5.1.2.1. Use 16S *E. coli* primers (p_forward: GTTAATACCTTTGCTCATTGA, p_reverse: ACCAGGGTATCTAATCCTGTT[17], 320 bp) or 16S *B. vulgatus* primers (p_forward: AACCTGCCGTCTACTCTT, p_reverse: CAACTGACTTAAACATCCAT[18], 400 bp) for amplification.

5.1.3. Use the *E. coli* and *B. vulgatus* 16S PCR fragments for blunt-end cloning into a cloning vector. Set up ligation and mix 10 µL of 2x buffer, 1 µL of non-purified PCR product, 1 µL of blunt-end cloning plasmid, 7 µL of nuclease-free water and 1 µL of T4 DNA Ligase. Incubate ligation for 10 min at RT.

5.1.4. Prepare *E. coli* DH5α competent cells.

5.1.4.1. Inoculate 100 mL of LB medium in an Erlenmeyer flask with 1 mL of an overnight culture. Grow the culture until OD 600 nm is between 0.4-0.6. Split the resulting culture into two 50 mL tubes and incubate the cultures on ice for 10 min.

5.1.4.2. Centrifuge the cultures at 1,700 x *g* for 10 min at 4 °C. Discard the supernatant and resuspend each pellet carefully with 5 mL of RFI solution (30 mM CH_3COOK, 100 mM KCl, 10 mM CaCl, 50 mM $MnCl_2$, adjust pH 5.8 with glacial acid, sterile filtered). Fill each tube with additional 45 mL of RFI solution.

5.1.4.3. Centrifuge the cultures at 1,700 x *g* for 10 min at 4 °C. Discard the supernatant and resuspend each pellet carefully with 6 mL of RFII (10 mM MOPS, 15 mM $CaCl_2$, 10 mM KCl, 15% glycerol, autoclaved) solution. Pool both fractions and incubate the 12 mL suspension on ice for 15 min. Prepare cell suspension aliquots (200 µL). Store the aliquots at -80 °C.

5.1.5. Transfer the ligation reaction to one aliquot of competent *E. coli* DH5α cells and leave the reaction on ice for 15 min. Heat shock the cells for 45 s at 42 °C and add 1 mL of LB medium.

5.1.5.1. Incubate transformation for 45 min at 37 °C. Add 100 µL of the transformation to a LB agar plate containing ampicillin and incubate overnight at 37 °C.

5.1.6. Perform colony PCR of 8 resulting transformants from the LB agar plate of step 5.1.5. Pick each colony with a toothpick, dip it onto a fresh LB plate containing ampicillin (master plate) and then dip the same toothpick into a well containing 5.5 µL of nuclease-free water in a PCR stripe.

5.1.6.1. Add 7.5 µL of 2x PCR mix, 0.5 µL of 10 µM forward primer and 0.5 µL of 10 µM reverse primer dilution. Use the same primer pairs mentioned in section 5.1.1.

5.1.6.2. Run PCR (initial denaturation: 95 °C for 5 min, denaturation: 95 °C for 1 min, annealing: 60 °C for 30s, extension: 72 °C for 1 min, final extension: 72 °C for 7 min, repeat denaturation, annealing and extension for 35 cycles).

5.1.7. Verify the size of the 16S fragments on a 1% agarose gel. Use 0.5x Tris-Borate-EDTA (TBE) buffer to dissolve 1 g of agarose and boil it in a microwave. Add 1:50,000 dye to gel and pour it. Add the colony PCR reactions and a 100 bp DNA ladder to the gel, and run the gel for 45 min at 110 V.

5.1.8. Inoculate a 5 mL LB overnight culture containing ampicillin with one clone from the master plate (section 5.1.6) for each *E. coli* and *B. vulgatus* 16S plasmid that contains the right insert size.

5.1.8.1. Centrifuge the bacterial overnight cultures in a 2 mL tube at 1,700 x *g*. Discard the supernatant and resuspend the pellet in 600 µL sterile water.

5.1.8.2. Add 100 µL of lysis buffer and mix by inverting the tube 6 times. Add 350 µL of cold (4°C) neutralization solution and mix thoroughly by inverting the tube.

5.1.8.3. Centrifuge at maximum speed in a centrifuge for 3 min. Transfer the supernatant (~900 µL) to a spin column and centrifuge at maximum speed in a centrifuge for 15 s.

5.1.8.4. Discard the flowthrough and add 200 µL of endotoxin removal wash and centrifuge at maximum speed in a centrifuge for 15 s.

5.1.8.5. Add 400 µL of wash solution to the column and centrifuge at maximum speed in a centrifuge for 30 s. Transfer the column to a clean 1.5 mL tube, add 30 µL of elution buffer to the column incubate it for 1 min at room temperature.

5.1.8.6. Centrifuge at maximum speed in a centrifuge for 30 s (see **Table of Materials**).

5.1.9. Determine the plasmid DNA concentration by mixing 1 µL of plasmid DNA with 199 µL of working solution (1 µL of fluorescent dye per 199 µL of buffer for each reaction). Prepare two standards by mixing 10 µL of standard 1 or 10 µL of standard 2 with 190 µL. Vortex the sample and standard tubes and incubate reaction for 2 min. Measure the concentration (see **Table of Materials**).

5.1.10. Prepare standard concentrations in 10-fold serial dilutions in a range of 10-100,000 copies: Calculation the mass of the single plasmid (m = (n) x (1.096x10-21 g/bp), n = plasmid size, m = mass). Calculate the mass of plasmid DNA needed to contain the desired copy

numbers of interest (copy number of interest x mass of single plasmid = mass of plasmid DNA needed).

5.2. Preparation of samples for quantification

5.2.1. Synthesize cDNA. Mix 2 µL of 7x buffer, 1 µL of DNase-digested RNA from section 4 and 11 µL of nuclease-free water. Incubate for 2 min at 42 °C.

5.2.1.1. Place reaction immediately on ice. Mix 4 µL of 5x RT Buffer, 1 µL of RT (Reverse Transcriptase) primer mix, 1 µL of RT enzyme and the reaction of step 5.2.1. Incubate for 15 min at 42 °C. Incubate for 3 min at 95°C to inactivate RT enzyme.

5.2.2. Quantify cDNA concentrations fluorometrically like described in step 5.1.9.

5.3. Measurement of bacterial load

5.3.1. Adjust cDNA concentrations to 5 ng per 12 µL reaction for quantitative PCR. Mix 2x RT-PCR mix, 0.25 µL of 100 µM forward primer, 0.25 µL of 100 µM reverse primer (5.1.1) and 12 µL of adjusted cDNA. Run qPCR (initial denaturation: 95 °C for 5 min, denaturation: 95 °C for 10 s, annealing: 60 °C for 30 s, repeat denaturation and annealing for 35 cycles, melting: 95 °C, cool down to 4 °C).

5.3.2. Plot $\log_{10}$ concentrations of plasmid standard curve (10-100,000 copies), i.e. 1-5 (x-axis), against the corresponding ct-values (y-axis). Perform linear regression to obtain the regression equation. Solve the equation for x (concentration). Use the formula to calculate the $\log_{10}$ of the copy numbers by inserting ct-value into the formula. Calculate the antilogarithm to obtain copy numbers.

6. Determination of innate immune marker gene using quantitative RT-PCR

6.1. Check primers for gene-specificity by PCR and subsequent agarose gel electrophoresis to verify the correct fragment size. Perform PCR like described in section 5.1.5.
Ubiquitin 130 bp: forward TCAATGCAAGTAGTCCGGTTC, reverse CCAGTCTGCTGCTGATAAACC[19] (housekeeping)
***Nox-4* 159 bp:** forward TGGCACGGCATCAGTTATCA, reverse ACAGCGACTGTCATGTGGAA[8]
***Nos* 76 bp:** forward ATGAAGGTGCTGAAGTCACAA, reverse GCCATTTTACAATCGCCACAA[8]
***Gst* 156 bp:** forward GACAGAAGTCCTCCGGTCAG, reverse TCCGTCTTCAAGCAAAGGCA[8]
***ApoIII* 265 bp:** forward AGACTTGCACGCCATCAAGA, reverse TGCATGCTGTTTGTCACTGC[8]
hemolin 267 bp: forward CTCCCTCACGGAGGACAAAC, reverse GCCACGCACATGTATTCACC[8]
gallerimycin 161 bp: forward GAAGTCTACAGAATCACACGA, reverse ATCGAAGACATTGACATCCA[8]
cecropin 158 bp: forward CTGTTCGTGTTCGCTTGTGT, reverse GTAGCTGCTTCGCCTACCAC[8]
gloverin 101 bp: forward GTGTTGAGCCCGTATGGGAA, reverse CCGTGCATCTGCTTGCTAAC[8]
moricin 124 bp: forward GCTGTACTCGCTGCACTGAT, reverse TGGCGATCATTGCCCTCTTT[8])

6.2. Assess primer efficiency to be E=2.

6.2.1. Pool 2 µL of 5-10 different positive samples (i.e., samples that are expressing the gene for which the primer pair needs to be investigated).

6.2.2. Prepare a 1:5 dilution series of the sample pool: standard 1 (S1): undiluted pool; S2: 2 µL of S1 + 8 µL nuclease-free water; S3: 2 µL of S2 + 8 µL nuclease-free water; S4: 2 µL of S3 + 8 µL nuclease-free water.

6.2.3. Apply 1 µL of S1-S4 and a non-template control (nuclease-free water) to a 96-well qPCR plate. Add 5 µL of RT master mix, 0.1 µL of each 100 µM forward and reverse primer, 3.7 µL of nuclease-free water and 0.1 µL of RT mix per well.

6.2.4. Run quantitative RT-PCR (reverse transcription: 50 °C for 10 min, initial denaturation: 95 °C for 5 min, denaturation: 95 °C for 10 s, annealing: 60 °C for 30 s, repeat denaturation and annealing for 40 cycles, melting: 95 °C, cool down to 4 °C).

6.2.5. Plot $\log_{10}$ of relative units for S1-S4 (1, 0.2, 0.04, 0.008) (x-axis) against the corresponding ct-values (y-axis). Perform linear regression and determine the slope of the standard curve. Calculate the efficiency E: $E = 10^{-(1/\text{slope})}$.[20]

NOTE: A slope of -3.32 indicates ideal reaction conditions and primer efficiency of E=2.00. This means: the amount of PCR product doubles during each cycle.

6.3. Use 100 ng of digested RNA (100 ng/µL) as a template for RT-PCR. Mix RT-PCR reagents and run RT-PCR like mentioned in section 6.2. Measure all bacteria- and DPBS-administered samples with both housekeeping primer pair and target primer pairs. Always run the S1-S4 dilutions with the housekeeping primer pair and S1-S4 with the target primer pair on the same plate for efficiency determination.

6.4. Calculate ratio (R) of RNA gene expression according to the following formula using the experimentally determined primer efficiency of both the housekeeping and the target primer pair. Normalize bacteria stimulated samples to mock controls[20].

$$\boldsymbol{R} = \frac{\boldsymbol{E_{target}}^{\boldsymbol{\Delta ct_{target}(control-sample)}}}{\boldsymbol{E_{housekeeping}}^{\boldsymbol{\Delta ct_{housekeeping}(control-sample)}}}$$

R: ratio
E_{target}: efficiency of S1-4 measured with target primer pair
$E_{housekeeping}$: efficiency of S1-4 measured with housekeeping primer pair
$\Delta ct_{target}^{(control\text{-}sample)}$: Δct of (ct of DPBS-fed sample)-(ct of bacteria-fed sample) measured with target primer pair
$\Delta ct_{housekeeping}^{(control\text{-}sample)}$: Δct of (ct of DPBS-fed sample)-(ct of bacteria-fed sample) measured

with housekeeping primer pair

REPRESENTATIVE RESULTS

The *G. mellonella* hemolymph infection model in widely used to analyze the virulence factors of a huge variety of pathogens. Most measurements include the analysis of larvae mortality, which is a quite easy method. Nevertheless, this method does not allow conclusions about immune responses in general and link the results of *G. mellonella* immune responses with vertebrate immune mechanisms. The *G. mellonella* oral administration model on the other hand is only rarely used for oral infection or colonization of the larvae due to the difficulties to obtain exact infection dosage[9]. Further, only little is known about *G. mellonella* innate immune responses towards non-pathogenic bacteria especially mammalian intestinal commensals.

In contrast to pathogens, commensals challenge the host and trigger immune responses but the host immune system is able to maintain immune homeostasis. *G. mellonella* is able to clear the initial force-fed bacterial load until finally no bacteria were detectable anymore (**Figure 2**)[8]. The 16s gene copy numbers of both *B. vulgatus* and *E. coli* substantially decreased within 24 h.

We demonstrated that commensal-administered *G. mellonella* larvae induce RNA gene expression of different innate immunity marker genes: LPS-recognition molecules – apolipophorin (*ApoIII*) and hemolin (**Figure 3A,B)** were shown to be generally higher expressed in *E. coli*-administered larvae compared to *B. vulgatus*-administered larvae[8]. Further, marker gene expression of two kinds of antimicrobial molecules can be monitored. The production of reactive oxygen and nitrogen species (ROS/RNS) can be estimated by the measurement of *Nos* and *Nox-4* gene expression which were demonstrated to be strongly upregulated upon *E. coli* force-feeding compared to *B. vulgatus* (**Figure 4A,B**)[8]. Furthermore, gene expression of antioxidative *Gst* could be observed (**Figure 4C**).[8]

In addition we showed that different antimicrobial peptide expression was induced stronger after *E. coli* administration than in response to *B. vulgatus* force-feeding. We observed upregulation of defensin-like gallerimycin peptide, LPS-interacting gloverin peptide, cecropin and moricin (**Figure 5A,B,C,D**)[8].

FIGURES AND TABLES

Figure 1: Force-feeding setup using a microsyringe pump. A blunt-ended needle is adjusted into microsyringe pump which allows precise injection of bacteria.

Figure 2: Persistence of bacterial load in *Galleria mellonella* larvae after force-feeding. Copy numbers of *B. vulgatus*- and *E. coli*-specific 16s rDNA genes were determined from 5 ng of cDNA at different time points using RT-PCR. Data points are shown with indication of the median. Modified from reference 8.

Figure 3: Differential pattern recognition of bacteria by *G. mellonella*. The larvae were administrated with two different intestinal commensals, RNA was isolated after 1-6 h, and mRNA expression of LPS recognition molecule apolipophorin (*ApoIII*) (**A**) and hemolin (**B**) was

determined. Data points represent geometric means with standard error of the mean (SEM) ($p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).[8]

Figure 4: ROS marker gene expression after bacterial challenge. *E. coli* and *B. vulgatus* were force-fed and ROS defense marker gene expression was analyzed over time. *Nos* (**A**), *Nox-4* (**B**) and *Gst* (**C**) mRNA expression was measured in isolated larval RNA. Data points represent geometric means with standard error of the mean (SEM) ($p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Modified from reference 8.

Figure 5: Commensal-induced defensin-like antimicrobial peptide expression in *G. mellonella* larvae and human epithelial cells. Larvae were orally administered with *B. vulgatus* or *E. coli*, immune responses were observed over time and RNA was isolated from larval individuals. gallerimycin (**A**), cecropin (**B**), gloverin (**C**), moricin (**D**) mRNA expression was determined. Data points represent geometric means with standard error of the mean (SEM) ($p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Modified from reference 8.

DISCUSSION

The *G. mellonella* model is a frequently used model to assess bacterial virulence factors in a systemic infection approach[21]. Since many pathogens and bacteria enter the host via the oral colonization or infection route, new insights need to be found to evaluate *G. mellonella* as a model for oral colonization and infection.

The possibility to rear *G. mellonella* between 15-37 °C is a great advantage since most mammalian models maintain body temperatures of 37 °C[5]. *G. mellonella* larvae can be purchased from different suppliers but the establishment of an own breeding population provides many advantages such as the absence of antibiotics that interfere with the assays, better estimation when to start experiments since the suppliers do not always provide larvae in a ready-to-use stage and stress responses are avoided due to transportation or temperature changes. Due to the temperature tolerance of *G. mellonella* the temperature range at which breeding can be performed is high. Higher temperatures lead to faster development of the larvae and according to the breeding temperature, we can estimate the lifecycle from egg to last instar larva. When larvae were selected for experiments, only pale and fast-moving individuals were chosen to avoid any stress and immune reactions to interfere with the experiments.

In order to establish the force-feeding model, it needs to be assured that the oral application was successful. Therefore, it was helpful to set up several trials for which a strong bromophenol dye was added to the solution intended for force-feeding. This helps to exclude any injured larvae and select for the larvae that have the blue dye only within their gut[22].

Using this model, we found that *G. mellonella* larvae are useful to investigate innate immune response kinetics of certain marker genes. During the establishment of the oral administration model and the study of immune response kinetics we found gene expression to be not locally expressed in the midgut. First experimental trials to extract midgut RNA after oral

administration of commensal bacteria did not provide conclusive results. Therefore, the immune responses were determined "globally" in whole individuals. These findings support the hypothesis of global recognition via intestinal receptors, transmission of the signal and triggering extraintestinal gene expression. Generally, *G. mellonella* is able to induce AMPs mainly in the fat body, but further in hemocytes and the intestinal system[9]. Since there is no precise information available about tissue-specific production of antimicrobial molecules in *G. mellonella* larvae after infection, the whole larval RNA was extracted from complete individuals and used for assaying RNA gene expression. A further advantage of whole larval RNA extraction is the complete containment of the living bacteria inside the gut and the possibility to quantify the bacterial load. The dissection of the gut could lead to the loss of bacteria due to preparation.

Since most *G. mellonella* research is performed on bacterial virulence traits we were especially interested if and how the larvae trigger immune responses towards non-pathogenic bacteria which are part of the mammalian microbiota. Recently, we showed that both *G. mellonella* and mammals share similar components of the innate immune response, which are homologous and evolutionary conserved. The nitric oxid synthase (*Nos*) and NADPH oxidase (*Nox*) genes share a high degree of similarity[8]. *G. mellonella* harbors further a defensin-like antimicrobial peptide gallerimycin which shares structural similarities with mammalian β-defensin 2[8].

Using the oral administration model it was possible to demonstrate differential bacterial recognition of either anti-inflammatory symbiotic *B. vulgatus* or pro-inflammatory pathobiotic *E. coli*. In addition downstream oxidative stress responses and antimicrobial peptide production were higher induced after *E. coli* administration compared to *B. vulgatus* administration[8].

ACKNOWLEDGMENTS:

This work was funded by the DFG (SPP1656), the DFG research training group 1708, the Bundesministerium für Bildung und Forschung (BMBF), and the German Center for Infection Research (DZIF).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Nell, S., Suerbaum, S., Josenhans, C. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nature Reviews Microbiology.* **8** (8), 564-577 (2010).

2 Muniz, L. R., Knosp, C., Yeretssian, G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Frontiers in Immunology.* **3,** 310 (2012).

3 Ivanov, II, Honda, K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe.* **12** (4), 496-508 (2012).

4 Ayres, J. S. Inflammasome-microbiota interplay in host physiologies. *Cell Host Microbe.* **14** (5), 491-497 (2013).

5 Champion, O. L., Titball, R. W., Bates, S. Standardization of G. mellonella Larvae to

Provide Reliable and Reproducible Results in the Study of Fungal Pathogens. *Journal of Fungi (Basel).* **4** (3) (2018).
6 Wojda, I. Immunity of the greater wax moth Galleria mellonella. *Insect Science.* 10.1111/1744-7917.12325 (2016).
7 Buchmann, K. Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals. *Frontiers in Immunology.* **5** 459 (2014).
8 Lange, A. et al. Galleria mellonella: A Novel Invertebrate Model to Distinguish Intestinal Symbionts From Pathobionts. *Frontiers in Immunology.* **9** (2114) (2018).
9 Tsai, C. J., Loh, J. M., Proft, T. Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence.* 10.1080/21505594.2015.1135289 1-16 (2016).
10 Bolouri Moghaddam, M. R. et al. The potential of the Galleria mellonella innate immune system is maximized by the co-presentation of diverse antimicrobial peptides. *Biological Chemistry.* **397** (9), 939-945 (2016).
11 Casanova-Torres, A. M., Goodrich-Blair, H. Immune Signaling and Antimicrobial Peptide Expression in Lepidoptera. *Insects.* **4** (3), 320-338 (2013).
12 Mukherjee, K. et al. Galleria mellonella as a model system for studying Listeria pathogenesis. *Applied and Environmental Microbiology.* **76** (1), 310-317 (2010).
13 Brennan, M., Thomas, D. Y., Whiteway, M., Kavanagh, K. Correlation between virulence of Candida albicans mutants in mice and Galleria mellonella larvae. *FEMS Immunological and Medical Microbiology.* **34** (2), 153-157 (2002).
14 Miyata, S., Casey, M., Frank, D. W., Ausubel, F. M., Drenkard, E. Use of the Galleria mellonella caterpillar as a model host to study the role of the type III secretion system in Pseudomonas aeruginosa pathogenesis. *Infection and Immunity.* **71** (5), 2404-2413 (2003).
15 Waidmann, M. et al. *Bacteroides vulgatus* protects against *Escherichia coli*-induced colitis in gnotobiotic interleukin-2-deficient mice. *Gastroenterology.* **125** (1), 162-177 (2003).
16 Lange, A. et al. Extensive Mobilome-Driven Genome Diversification in Mouse Gut-Associated Bacteroides vulgatus mpk. *Genome Biology and Evolution.* **8** (4), 1197-1207 (2016).
17 Hermann-Bank, M. L., Skovgaard, K., Stockmarr, A., Larsen, N., Molbak, L. The Gut Microbiotassay: a high-throughput qPCR approach combinable with next generation sequencing to study gut microbial diversity. *BMC Genomics.* **14** 788 (2013).
18 Sato, K. et al. OmpA variants affecting the adherence of ulcerative colitis-derived Bacteroides vulgatus. *Journal of Medical and Dental Science.* **57** (1), 55-64 (2010).
19 Freitak, D. et al. The maternal transfer of bacteria can mediate trans-generational immune priming in insects. *Virulence.* **5** (4), 547-554 (2014).
20 Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research.* **29** (9), e45 (2001).
21 Ramarao, N., Nielsen-Leroux, C., Lereclus, D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. *Journal of Visualized Experiments.* 10.3791/4392 (70), e4392 (2012).
22 Ramarao, N., Nielsen-Leroux, C., Lereclus, D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. *Journal of Visualized Experiments.* 10.3791/4392 (70), e4392 (2012).

Figure 1

Click here to access/download;Figure;Figure 1.jpg

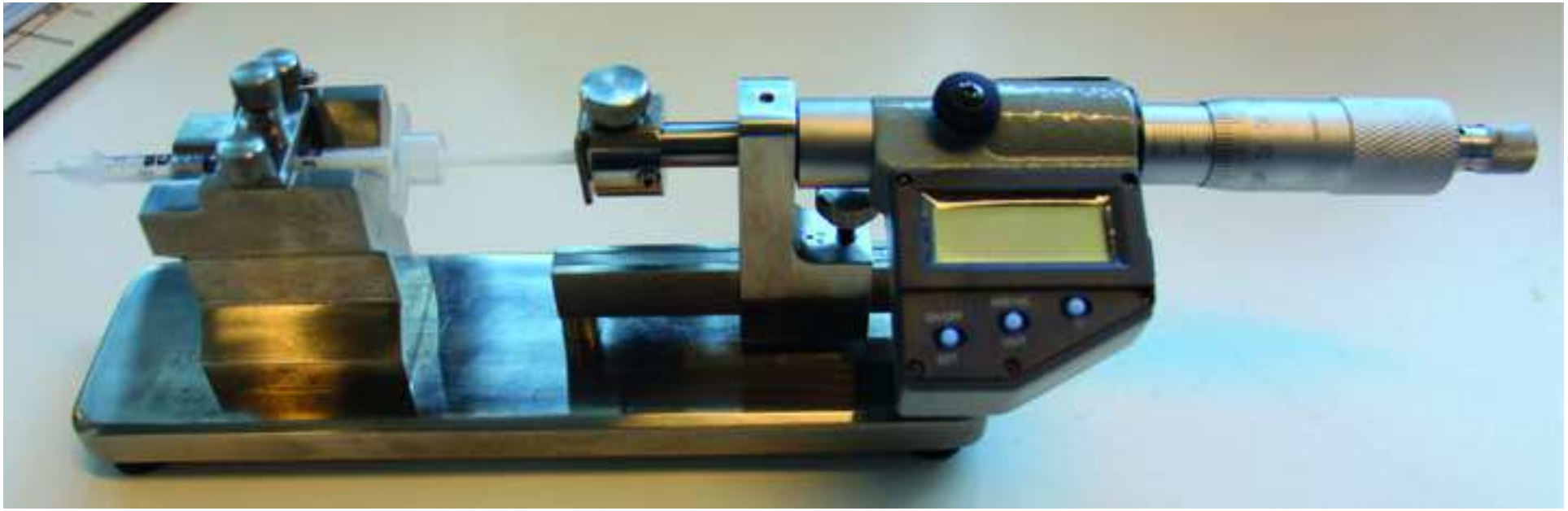


Figure 2

Click here to access/download;Figure;Figure 2.jpg

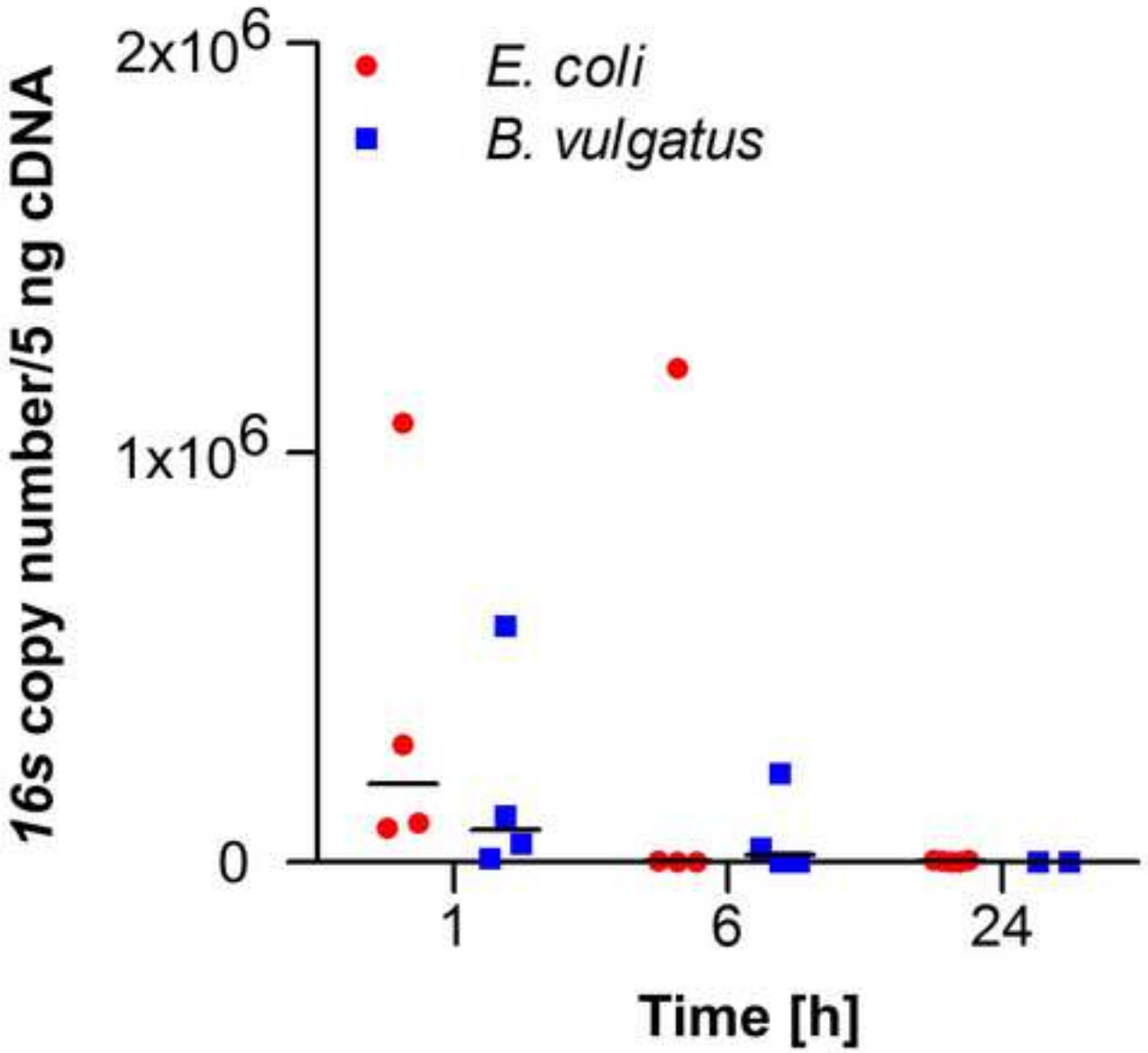


Figure 3

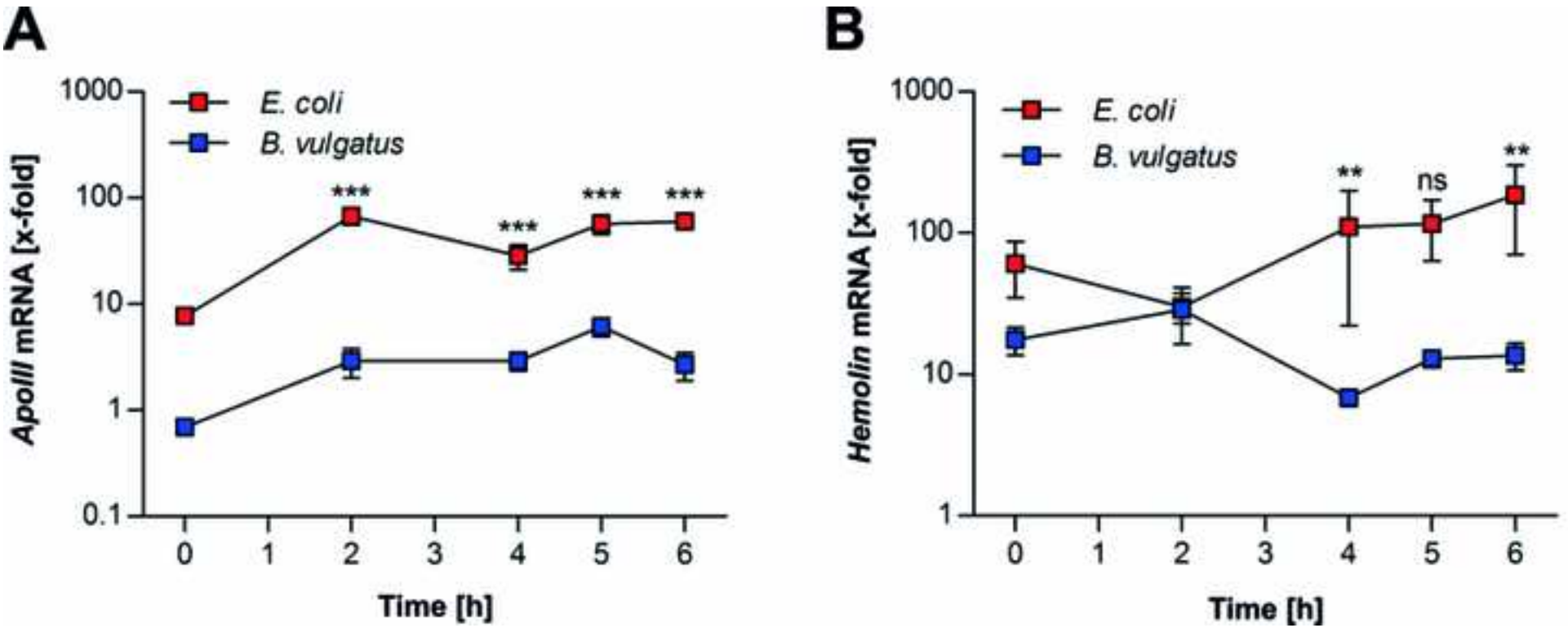


Figure 4

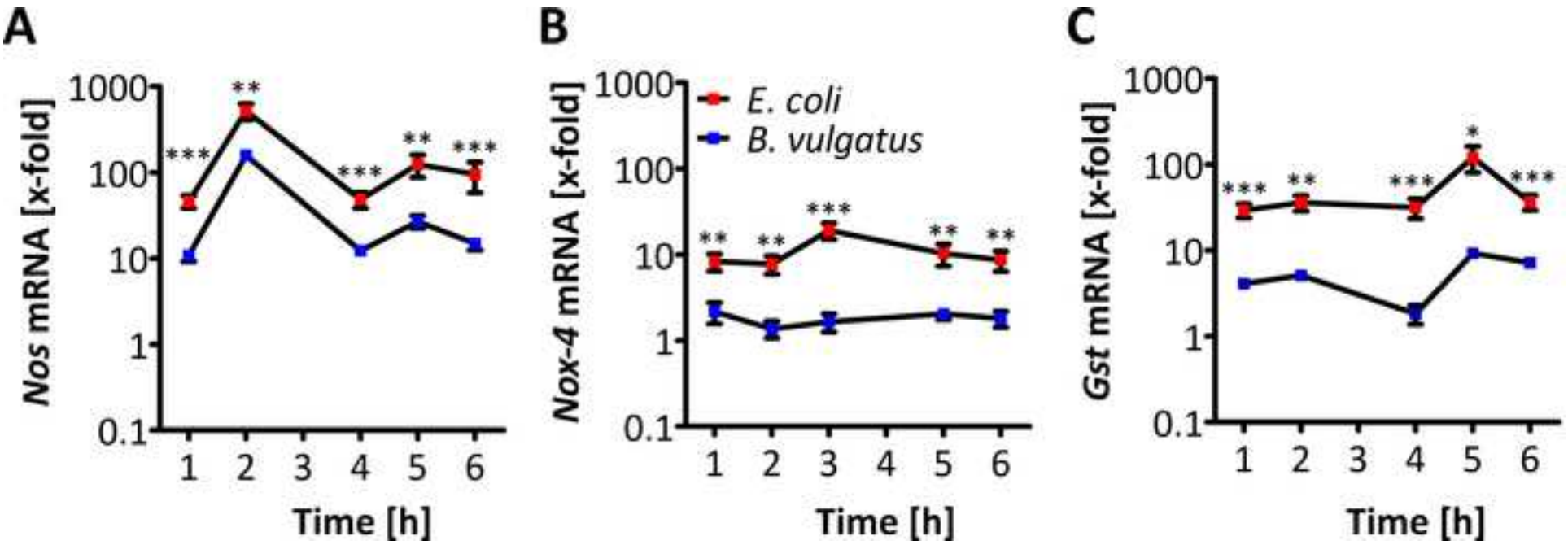
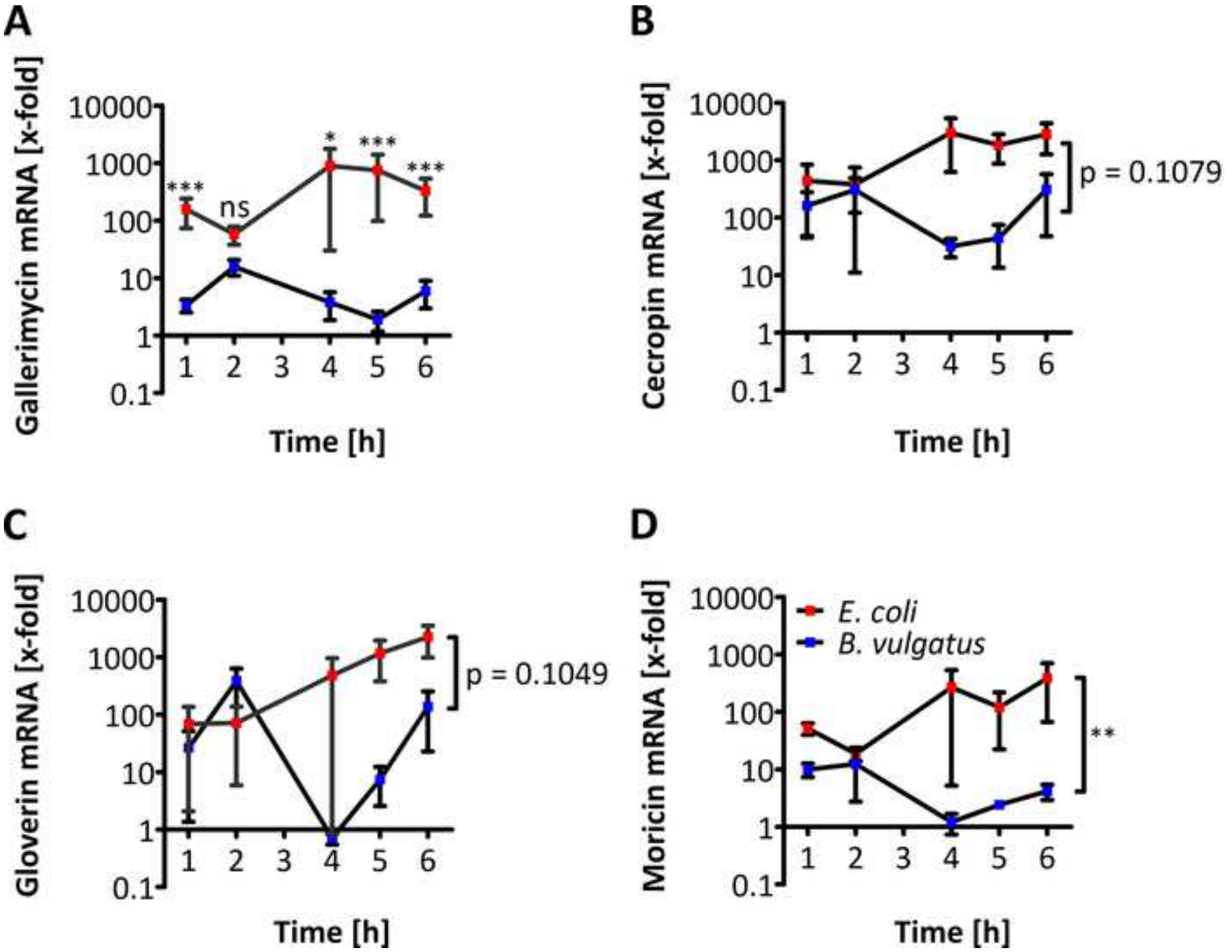


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL tubes	Eppendorf	0030120086	
100 bp DNA ladder	Thermo Fisher Scientific	15628019	
1-Bromo-3-Chloropropane (BCP)	Sigma-Aldrich	B9673	
2 mL tubes	Eppendorf	0030120094	
2x Mangomix	Bioline	BIO-25033	Colony PCR
50 mL tubes	Greiner Bio-One	210 261	
Agarose	Biozym	840004	
Beeswax	Mixed-Store.de	-	
Brain heart infusion broth	Thermo Fisher Scientific	CM1135	
CloneJET PCR Cloning Kit	Thermo Fisher Scientific	K1232	Cloning vector for 16S fragments
Corn grits	Ostermühle Naturkost GmbH	306	Organic cultivation
Difco LB Agar, Miller (Luria-Bertani)	Becton Dickinson	BD	
Difoco LB Broth, Miller (Luria-Berta	Becton Dickinson	244610	
DNA-free DNA Removal Kit	Thermo Fisher Scientific	244510	Dnase digestion
Dried yeast	Rapunzel	-	Organic cultivation
Dulbecco's Phosphate-Buffered Sal	Thermo Fisher Scientific	14040	
Ethanol	VWR	20821.330	
Glycerol	Sigma-Aldrich	W252506	
Honey	Ostermühle Naturkost GmbH	487	
Isopropanol	VWR	20842.330	
Lightcycler 480 Instrument II	Roche Molecular Systems	5015278001	
LightCycler 480 Multiwell Plate 96, white	Roche Molecular Systems	4729692001	
Manual Microsyringe Pump with Di	World Precision Instruments	DMP	
Micro-Fine+ U-100 insulin syringe 0	Becton Dickinson	324826	Oral administration
Mortar, unglazed	VWR	410-9327	
Nanodrop	Thermo Fisher Scientific	13-400-518	
Nuclease-free water	Thermo Fisher Scientific	10977035	
Oxoid AnaeroGen sachets	Thermo Fisher Scientific	AN0025A	Quality and quantity of RNA
PCR stripes	Biozym	710970	

Pestle, unglazed grinding surface	VWR	410-9324	
Phusion proof-reading enzyme	Thermo Fisher Scientific	F553S	
Primers	Biomers	-	
PureYield Plasmid Miniprep System	Promega	A1222	
QuantiFast SYBR Green PCR kit	Qiagen	204056	qPCR for bacterial copy number measurment
QuantiFast SYBR Green RT-PCR Kit	Qiagen	204156	qRT-PCR for gene expression measurements
QuantiTect Reverse Transcription K	Qiagen	205311	cDNA synthesis
Qubit Assay Tubes	Thermo Fisher Scientific	Q32856	
Qubit dsHS DNA kit	Thermo Fisher Scientific	Q32851	Quantification of plasmid and cDNA samples
Qubit fluorometer	Thermo Fisher Scientific	Q33226	Quantification of plasmid and cDNA samples
RNase-ExitusPlus	AppliChem	A7153	
Rnasin Ribonuclease Inhibitor	Promega	N2511	
Skimmed milk powder	Sucofin	-	
SYBR safe DNA Gel Stain	Thermo Fisher Scientific	S33102	
TRI reagent	Sigma-Aldrich	T9424	
Weighing boat	VWR	10803-148	
Wheat meal	Ostermühle Naturkost GmbH	6462	Organic cultivation

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Galleria mellonella oral administration model to study commensal-induced innate immune responses.

Author(s): Anna Lange, Andrea Schäfer, Julia-Stefanie Frick

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Julia-Stefanie Frick
Department:	University Hospital Tübingen
Institution:	Institute for Medical Microbiology & Hygiene
Title:	Prof. Dr.
Signature:	Julia Frick
Date:	19.10.2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

JoVE59270

Author's response Letter to the editor and reviewers

We want to thank you sincerely for your detailed comments and suggestion which we included in our work. We feel that it significantly improved our manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Comment: Thank you for the advice to thoroughly proofread our manuscript to avoid spelling or grammar problems.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Comment: Thank you for this suggestion we rephrased the Summary to describe the protocol and the method applications.

3. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Comment: Thanks for the comment. We exchanged centrifuge speeds from rpm to xg.

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Comment: Thank you for this suggestion we rephrased the protocol to avoid any personal pronouns.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Comment: Thank you for this advice. We revised the protocol and used the imperative tense throughout the protocol. In rare cases we included a "note" when the imperative tense could not be used. Further we moved discussion into the discussion section.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Oxoid AnaeroGen, TRI reagent, RNasin, Nanodrop, Phusion, Qubit, QuantiTect, QuantiFast SYBR, etc.

Comment: Thank you for this comment since we have not been aware of the commercial language. We revised the protocol and used generic terms instead. Sometimes we mentioned as advised "see table of materials".

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Comment: We agree to add more details to the protocol steps. (see point 8-13.)

8. 4.1: Please describe how to homogenize the larvae and specify the incubation temperature.
Comment: We added more details to this essential step of the protocol. We provided more details for how the homogenization needs to be performed and mentioned the incubation time.

9. 4.3: Is the pellet discarded? Is 500 µL isopropanol added to the new tube containing the upper layer? Please specify.

Comment: Yes, the pellet is discarded and the isopropanol is added to the upper layer. We modified the step to contain more details.

10. 5.1: Please specify PCR conditions.

Comment: We specified the PCR recipe and the program. Further we changed 5.1 into 5.1.1since there were more details and we still want the protocol to be clearly structured.

11. 5.2, 5.3, 5.5, 5.7, 6.2, 6.3: Please add more details to these steps. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

Comment: We added more details to these steps and changed 5.2 into 5.1.2-5.1.5, 5.3 into 5.1.6-5.1.7, 5.5 into 5.2.1, 5.7 into 5.3 (5.3.1 and 5.3.2), and6.2 into 4.9, 6.3 was left without renaming it. The renaming of the points should help to keep the clearly structure of the protocol.

12. Please ensure that conditions and primers are listed all PCR procedures.

Comment: We added more details and described the PCR recipe and the program.

13. 6.1: Please describe how to assess the primer efficiency.

Comment: We added more details to the primer efficiency assessment and devided 6.1 into 6.1 and 6.2. We provided essential information to calculate the primer efficiency.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Comment: After having introduced all the suggested and advised changes to the protocol we highlighted the essential steps of the protocol for the video. The highlights include almost 2 pages.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Comment: We assured to highlight the complete sentences.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Comment: We followed this advice and highlighted the details to perform the highlighted steps.

17. Figure 2: Please change "100 6" to "106" (i.e., delete 0 and the space between).

Comment: We followed this advice and performed the suggested changes.

18. Figure 3: Please describe the asterisk symbols in the figure legend.

Comment: We agree to explain the asterix symbol. Therefore we added the descriptions in the figure legends.

19. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Comment: Thank you for this comment we discussed critical steps like rearing in the discussion section. Further we discussed limitation like the lack of gut-specific RNA expression analysis.

20. References: Please do not abbreviate journal titles.

Comment: We followed this advice and performed the suggested changes

21. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Comment: Thanks for the comment. We sorted the items in alphabetical order and included more necessary material/equipment since we noticed that they were missing.

Reviewer 1:

Abstract: Improve and revise. Firstly, I advise to describe the focus of paper and the relation between invertebrate and vertebrate, link the importance of reduce of the number of vertebrate as the authors have already done. Finally, describe briefly the importance of intestinal microbiota and explain the validity of G. mellonella model in this contest and a final consideration about the obtained data.

Comment: Thank you for the advice to revise the abstract.

Protocol: line 119 : replace rpm with xg

Comment: Thanks for the comment. We changed rpm for xg.

Why the authors did not extract only the gut of the larvae? the procedure is quite simple.

Comment: We agree that the procedure of extracting the gut from the larvae is not difficult, and we performed this in a previous project but the expression analysis of midgut tissue was not conclusive. Since AMPs can be produced in several tissue types we chose to analyze "global" expression. That is why we use the RNA of whole larval individuals for RNA expression analysis. In our manuscript we mentioned this point already in the discussion section: "During the establishment of the oral administration model and the study of immune response kinetics we found gene expression to be not locally expressed in the midgut."

Protocol/Representative results: line 132: Authors described that larvae were incubated between 1-6 hours; in line 229, authors suggested that 16s gene copy of both bacteria decreased within 24 hours. Is not clear if the incubation is between 1-6 hours or 1-24 hours? and if authors incubated larvae within 24 hours, are they sure that the administration of 10^7 bacteria not killed larvae in this interval?

Comment: We agree that these statements lead to confusion since we missed to mention the 24 h incubation in the protocol. That is why we corrected the incubation times in the protocol into "1-24 h".
Further, we are sure that the larvae were still alive after 24 h since the RNA was only extracted from living *G. mellonella*. We added this information "living" in the protocol (4.1). After 24 h the oral-administered larvae were still light without any melanization symptoms. The administered bacteria are not pathogenic and lack any virulence factors.

Fig. 4 and 5: I advice to write one time the legend for these figures, the bacteria are only two. Define only one axis for "y", authors used different scale for the four graphs.

Comment: We followed this advice and included the legend of Figure 4 and 5 only once to each of the figures. Further, we changed the axis to have the same scale.

Caption of figure : "*" explain

Comment: We agree to explain the asterix. Therefore we added the explanations in the figure legends.

Reviewer 2:

I suggest to cite more pioneering original papers in which Galleria mellonella has been established as a powerful model for bacterial human pathogens to illustrate its importance as an alternative model host.

Comment: We followed this advice and cited more papers in the introduction to highlight the comparative character of *G. mellonella* and mice to support its alternative host model capabilities.

I would add more information on the selected antimicrobial peptides of Galleria mellonella (properties, activities). Gallerimycin is an antifungal peptide whereas cecropin has been demonstrated to display activity against human pathogen bacteria.

Comment: We thank for the advice to include more detailed information about the AMPs we selected, since this provide more helpful details for the reader. Therefore we introduced the following section into the introduction: **"**Generally, the AMPs have quite broad host specificity against Gram-positive and Gram-negative bacteria and fungi and have to provide a potent response since insects are lacking any adaptive response. Gloverins is an AMP which is active against bacteria and fungi and inhibits outer membrane formation. Moricins exhibit their antimicrobial function against Gram-positive and Gram-negative bacteria by penetrating the membrane and forming a pore. Cecropins provide activity against bacteria and fungi and permeabilize the membrane similarly like moricins. Gallerimycin is a defensin-like peptide with anti-fungal properties. Interestingly, it was found that the combination of cecropin and gallerimycin had a synergistic activity against E. coli."

Lane 27: replace homologous by similar because this term implicates a common ancestral origin.

Comment: We followed this advice and changed "homologous" to "similar".

Permissions

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Copyright Statement.pdf

Frontiers Copyright Statement

All content included on Frontiers websites (including Loop), such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of Frontiers if created by Frontiers, or of the person or entity who or which owned it prior to submission to Frontiers. If not owned by Frontiers, it is licensed to Frontiers Media SA (Frontiers) or its licensees and/or subcontractors.

The copyright in the text of individual articles (including research articles, opinion articles, book reviews, conference proceedings and abstracts) is not the property of Frontiers, and its ownership is not affected by its submission to or publication by Frontiers. Frontiers benefits from a general licence over all content submitted to it, and both Frontiers and its users benefit from a **Creative Commons CC-BY licence** over all content, as specified below.

Images and graphics not forming part of user-contributed materials are the property of or are licensed to Frontiers may not be downloaded or copied without Frontiers' explicit and specific permission or in accordance with any specific copyright notice attached to that material.

The combination of all content on Frontiers websites, as well as the design and the look and feel of the Frontiers websites, and the copyright and all other rights in such content and combination, are the sole property of Frontiers.

As an author or contributor you grant permission to others to reproduce your articles, **including any graphics and third-party materials supplied by you**, in accordance with the **Frontiers Terms and Conditions**. The licence granted to third parties over all contents of each article, **including third-party elements**, is a Creative Commons Attribution ("CC BY") licence. The current version is **CC-BY, version 4.0**, and the licence will automatically be updated as and when updated by the Creative Commons organisation.

You may include a requirement to reproduce copyright notices in materials contributed by you, but you may not restrict the right to reproduce the entire article, including third-party graphics. This means that you must obtain any necessary third-party consents and permissions to reproduce third-party materials in your articles submitted to Frontiers.

E-books are subject to the same licensing conditions as the articles within them.

Articles published prior to the effective date of this notice: Please note that reproduction of third-party graphics and other third-party materials contained in articles published prior to the effective date of this notice may be subject to third-party notices prohibiting their reproduction without permission. You must comply with those notices.

Articles published prior to July 2012: The licence granted for these articles may be different and you should check the pdf version of any article to establish what licence was granted. If an article, dating from before July 2012, carries only a non-commercial licence and you wish to obtain a commercial licence, please contact Frontiers at **editorial.office@frontiersin.org**.

All software used on this website, and the copyright in the code constituting such software, is the property of or is licensed to Frontiers and its use is restricted in accordance with the **Frontiers Terms and Conditions**. All copyright, and all rights therein, are protected by national and international copyright laws.

This Copyright Statement comes into effect on **25th May, 2018**.