

Video Article

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

Anna Lange¹, Andrea Schäfer¹, Julia-Stefanie Frick¹

¹Department for Medical Microbiology, Hygiene, Interfaculty Institute for Microbiology, Infection Medicine, University of Tübingen

Correspondence to: Julia-Stefanie Frick at Julia-Stefanie.Frick@med.uni-tuebingen.de

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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 expression⁵. 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 oxide 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^{6,8,9,10}. Generally, 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 response¹⁰. 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 properties⁹. 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* model¹². 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 mortality¹³. 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 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. 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.
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.
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

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. 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.
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 10⁹/mL.

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

1. Force-feed each larva with 10 µL of the adjusted bacterial suspension containing 10⁷ bacteria per dose. Use an insulin syringe with a blunted needle for oral application of the bacterial suspension.
 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.
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

1. Work under a hood and wear safety glasses. Clean the hood and spray reagent to prevent RNase contamination.
 1. Snap-freeze the living larvae after incubation in liquid nitrogen and homogenize them. Use a mortar and pestle for homogenization. Add liquid nitrogen to the mortar and grind each larval individual until powdered homogenates are produced.
 2. Pour the homogenate to a disposable weighing boat and wait for the liquid nitrogen to evaporate.
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.
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. 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.
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.
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.
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.

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**).
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.
 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.

1. Preparation of plasmid standards
 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.
 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).
 1. Use 16S *E. coli* primers (p_forward: GTTAATACCTTTGCTCATTGA, p_reverse: ACCAGGGTATCTAATCCTGTT¹⁷, 320 bp) or 16S *B. vulgatus* primers (p_forward: AACCTGCCGTCTACTCTT, p_reverse: CAACTGACTTAAACATCCAT¹⁸, 400 bp) for amplification.
 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.
 4. Prepare *E. coli* DH5α competent cells.
 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.
 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 CH₃COOK, 100 mM KCl, 10 mM CaCl₂, 50 mM MnCl₂, adjust pH 5.8 with glacial acid, sterile filtered). Fill each tube with additional 45 mL of RFI solution.
 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 RFI (10 mM MOPS, 15 mM CaCl₂, 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. 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.
 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.
 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.
 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.
 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).
 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.
 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.
 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.
 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.
 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.
 4. Discard the flowthrough and add 200 µL of endotoxin removal wash and centrifuge at maximum speed in a centrifuge for 15 s.
 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.
 6. Centrifuge at maximum speed in a centrifuge for 30 s (see **Table of Materials**).
 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**).

10. Prepare standard concentrations in 10-fold serial dilutions in a range of 10-100,000 copies: Calculate the mass of the single plasmid ($m = (n) \times (1.096 \times 10^{-21} \text{ g/bp})$, n = plasmid size, m = mass). Calculate the mass of plasmid DNA needed to contain the desired copy numbers of interest (copy number of interest $\times$ mass of single plasmid = mass of plasmid DNA needed).
2. Preparation of samples for quantification
 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.
 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.
 2. Quantify cDNA concentrations fluorometrically like described in step 5.1.9.
3. Measurement of bacterial load
 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).
 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

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 CCAGTCTGCTGCTGATAAACC¹⁹ (housekeeping)

Nox-4 159 bp: forward TGGCAGGCGCATCAGTTATCA, reverse ACAGCGACTGTCATGTGGAA⁸

Nos 76 bp: forward ATGAAGGTGCTGAAGTCACAA, reverse GCCATTTACAATCGCCACAA⁸

Gst 156 bp: forward GACAGAAGTCCTCCGGTCAG, reverse TCCGTCTTCAAGCAAAGGCA⁸

ApolI 265 bp: forward AGACTTGACGCCATCAAGA, reverse TGCATGCTGTTTGTCACTGC⁸

hemolin 267 bp: forward CTCCCTCACGGAGGACAAAC, reverse GCCACGCACATGTATTCACC⁸

gallerimycin 161 bp: forward GAAGTCTACAGAATCACACGA, reverse ATCGAAGACATTGACATCCA⁸

cecropin 158 bp: forward CTGTTCTGTGTTTCGCTTGTGT, reverse GTAGCTGCTTCGCCCTACCAC⁸

gloverin 101 bp: forward GTGTTGAGCCCGTATGGGAA, reverse CCGTGCATCTGCTTGCTAAC⁸

morcin 124 bp: forward GCTGTACTCGCTGCACTGAT, reverse TGGCGATCATTGCCCTCTT⁸)
2. Assess primer efficiency to be $E=2$.
 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).
 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.
 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.
 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).
 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.
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.
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 controls²⁰.

$$R = \frac{E_{\text{target}}^{\Delta \text{ct}_{\text{target}}(\text{control-sample})}}{E_{\text{housekeeping}}^{\Delta \text{ct}_{\text{housekeeping}}(\text{control-sample})}}$$

R: ratio

E_{target} : efficiency of S1-4 measured with target primer pair

$E_{\text{housekeeping}}$: efficiency of S1-4 measured with housekeeping primer pair

$\Delta \text{ct}_{\text{target}}(\text{control-sample})$: Δct of (ct of DPBS-fed sample)-(ct of bacteria-fed sample) measured with target primer pair

$\Delta \text{ct}_{\text{housekeeping}}(\text{control-sample})$: 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 is 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 dosage⁹. 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 (*Apoll*) and hemolin (**Figure 3A,B**) were shown to be generally higher expressed in *E. coli*-administered larvae compared to *B. vulgatus*-administered larvae⁸. 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.

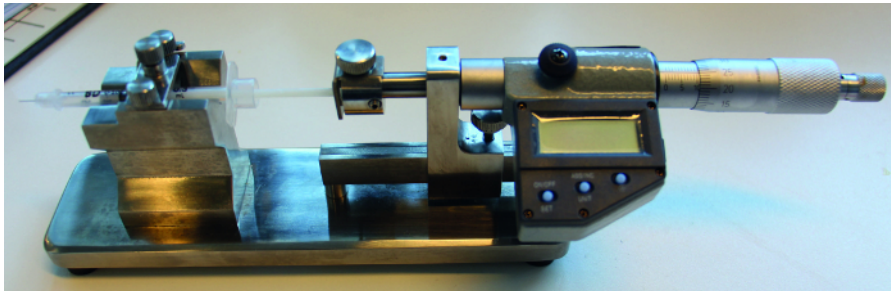


Figure 1: Force-feeding setup using a microsyringe pump. A blunt-ended needle is adjusted into microsyringe pump which allows precise injection of bacteria. [Please click here to view a larger version of this figure.](#)

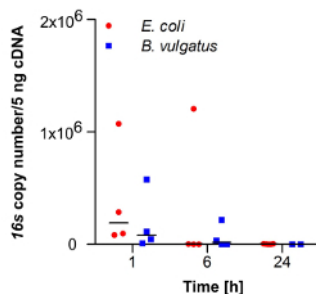


Figure 2: Persistence of bacterial load in *Galleria mellonella* larvae after force-feeding. Copy numbers of *B. vulgatus*- and *E. coli*-specific 16s rDNA genes were determined from 5 ng of cDNA at different time points using RT-PCR. Data points are shown with indication of the median. Modified from reference 8. [Please click here to view a larger version of this figure.](#)

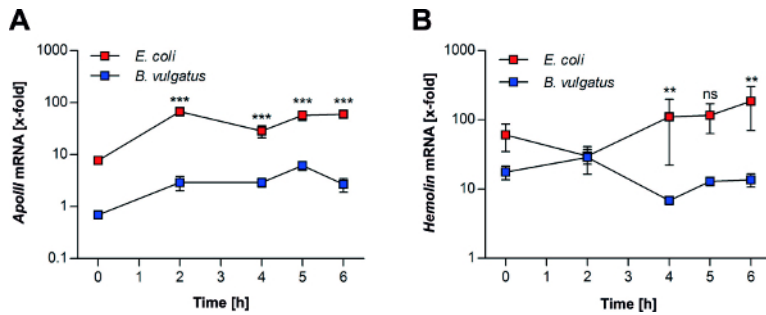


Figure 3: Differential pattern recognition of bacteria by *G. mellonella*. The larvae were administrated with two different intestinal commensals, RNA was isolated after 1-6 h, and mRNA expression of LPS recognition molecule apolipophorin (*ApolIII*) (A) and hemolin (B) was determined. Data points represent geometric means with standard error of the mean (SEM) ($p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).⁸ Please click here to view a larger version of this figure.

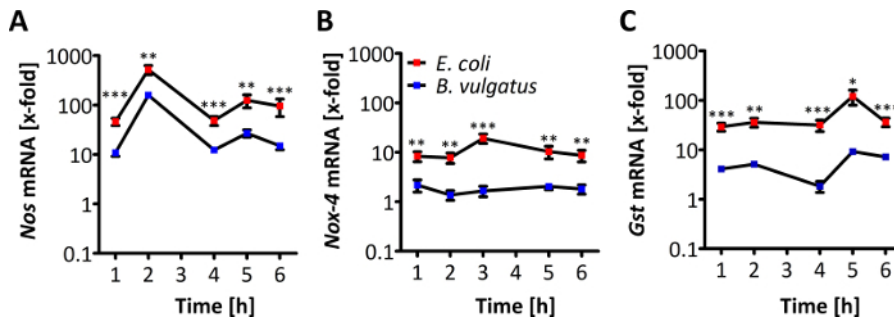


Figure 4: ROS marker gene expression after bacterial challenge. *E. coli* and *B. vulgatus* were force-fed and ROS defense marker gene expression was analyzed over time. *Nos* (A), *Nox-4* (B) and *Gst* (C) mRNA expression was measured in isolated larval RNA. Data points represent geometric means with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

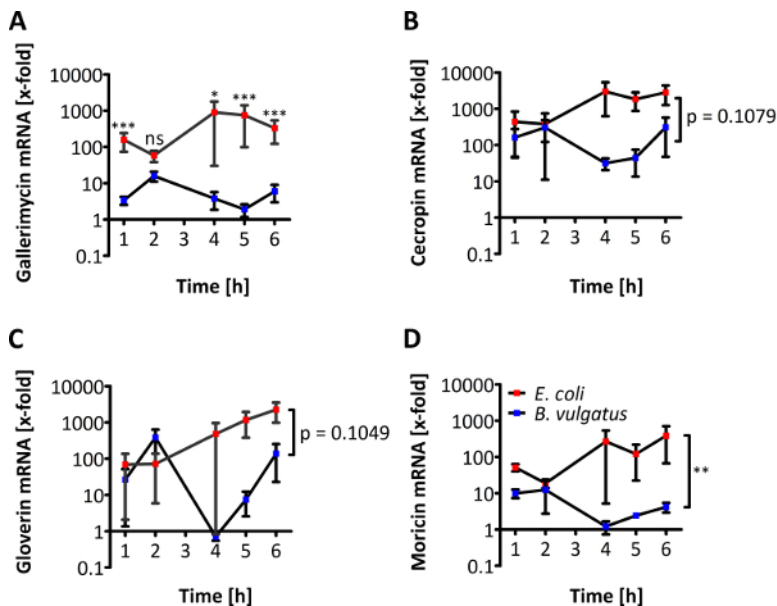


Figure 5: Commensal-induced defensin-like antimicrobial peptide expression in *G. mellonella* larvae and human epithelial cells. Larvae were orally administered with *B. vulgatus* or *E. coli*, immune responses were observed over time and RNA was isolated from larval individuals. *gallerimycin* (A), *cecropin* (B), *gloverin* (C), *morcin* (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. Please click here to view a larger version of this figure.

Discussion

The *G. mellonella* model is a frequently used model to assess bacterial virulence factors in a systemic infection approach²¹. 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 °C⁵. *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 gut²².

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 system⁹. 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 similarity⁸. *G. mellonella* harbors further a defensin-like antimicrobial peptide gallerimycin which shares structural similarities with mammalian β -defensin 2⁸.

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* administration⁸.

Disclosures

The authors have nothing to disclose.

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