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Kiel

Arbeitsgruppe Prof. Dr. Bosch

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Contents

Personal information

My name is Timon Utecht and I live in Bonn. I just graduated from school and I am currently looking forward to start my studies on "Molecular Biomedicine" in October 2019. I participated in the BiologieOlympiade last year; being able to reach the third round of the competition, the IBO-Verein provided me with an internship in Kiel at the Zoological Institute of the Christian-Albrecht-University of Kiel (CAU).

For four weeks I worked in the research group of Mr. Prof. Dr. Bosch, who is the head of the team. I was directly supervised by Tim Lachnit, who works at the institute as a scientist. The research group is part of the Collaborative Research Center 1182 "Origin and Function of Metaorganism" (Prof. Dr. Bosch is the spokeperson of the CRC), thus focussing on the interaction of the microbiome and its host. As a modell organism they are using Hydra, which is – seen form an evolutionary point of view – a very simple organism that belongs to the phylum of *Cnidaria*. It is widely known for its nearly endless capacity of regeneration. Prof. Dr. Bosch's research group currently counts 18 members, containing not only PhD- but also master- and bachelor-students as well as scientists and postdocs.

During my time at the institute I participated in wide range of experiments and was even allowed to make my own little experiment. I decided to deal with a typical disease phenotype derived by alterations in Hydra's microbiome in this report. Therefore I will first describe the disease phenotype. Secondly, I am going to focus on a quantitative analysis of the increasing apoptosis rate in sick Hydra, using techniques to quantify gene expression.

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Alterations in Microbiome Make Hydra Sick and Stimulate Host Apoptosis

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Abstract

The interaction of a host and its attached microbiome has been of special interest for the last decades. It is not yet fully understood which mechanisms underlie the complex interactions, but there is growing evidence that a specific microbiome is essential for the host's health. Not only pathogens that would initiate an immune response may be a threat for the host but also alterations in the host-specific microbiome. In this report, I analyse the consequences of alterations in the host-specific microbiome using the model organism *Hydra vulgaris*. Alterations in *Hydra*s microbiome do not only lead to a certain phenotype that is characterized by shortened tentacles and body but also to a different expression pattern of genes involved in both apoptotical as well as inflammatory signalling, such as AP-1, c-Jun and NLR2.

Introduction

The freshwater polyp *Hydra* (*Hydra vulgaris*) is well known for its seemingly endless capacity of regeneration for at least 200 years[1]. In spite of their key role as a model organism to investigate the molecular mechanisms of cellular regeneration, *Hydra* is known as a very common object of research throughout the entire field of biology. In the last few years the microbiome of *Hydra* has become a topic of special interest: in modern biology life has been redefined as a community of metaorganisms. The term metaorganism refers to the fundamental observation that life cannot be analysed detached from interspecific relations, thus the term metaorganism is defined as a host and the unique microbiome attached to it[2]. The microbiome may not only contain bacteria but also archaea, fungi as well as protists and viruses. All of them combined are thought to be of a high importance for maintaining the host's homeostasis.

Further research on the concept of metaorganisms revealed a key role of altered bacteria host interaction in diseases[3]. That leads to the development of a new viewpoint on diseases as well as therapies. *Hydra* is affected by alterations in the bacterial population as well. If *Hydra* is confronted with a nutrient-rich environment, growth rates of host specific bacteria are promoted which leads to shortened body and tentacle length in the polyp; further investigations revealed that the bacterial-host-interaction is essential for the development of this disease-phenotype as germ free animals do not show any alteration in their phenotype when exposed to nutrient-rich conditions[4].

In order to find out about the actual reason for the shortened tentacle- as well as body length two hypotheses should be taken into account. Firstly, the disease-phenotype might be a result of continuing contraction of body and tentacles. A second possibility is programmed cell death – also known as apoptosis – which in this case might work as an inflammatory or even defensive reaction of the polyps to get rid of bacteria-rich parts of their bodies[4]. To proof the second hypotheses, I analysed both the number of bacteria on the polyps during a nutrient treatment and the length of the body and tentacles of the freshwater polyp to describe the disease-phenotype. A qRT-PCR combined with the measurement of body- and tentacle length was supposed to link the decrease in size to the phenomenon of apoptosis. Therefore, the expression of seven genes, including genes such as c-Jun, AP-1 or NLR2, shall be analysed within this report.

Activating protein 1 (AP/AP-1) is a transcription factor that is composed of different proteins either belonging to the Jun protein family (including c-Jun, JunB and JunD) or to the Fos protein family (c-Fos, FosB, etc.) that form homo- or heterodimers (Jun-Jun, Fos-Jun); in fact AP-1 may also refer to other dimer-variants, as Jun is able to interact and form heterodimers with members of the activation transcription factor (ATF) family as well as the Jun dimerization protein (JDP) family, too[5]. All of these proteins have been shown to contain a basic leucine zipper (bZIP) domain that is crucial for the

dimerization of the proteins[5,6]. Another feature of the AP-1 family is the specific TPA (12-O-tetradecanoylphorbol-13-acetate) responsive element (TRE) and an associated consensus-sequence that can be found in every target gene[5]. Even though the structure of AP-1 as well as many functional properties of the transcription factor are known, its actual role in apoptosis remains unclear; AP-1 has not only been shown to be of high relevance for cell death (it is often called a "decision maker") but it has also been shown to keep cells alive, thus preventing them from programmed cell death, which reveals the dependency of AP-1 function on environmental (extracellular) circumstances[7]. There is evidence that c-Jun, which is the most popular part of the AP-1 family, is highly relevant for progressing through the G1-phase of the cell cycle as well as it does interact with NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) to prevent cells from UV-light-induced apoptosis; therefore c-Jun needs to be activated by Jun N-terminal kinases (JNKs, a mitogen activated protein-kinase)[8]. In contrast to that c-Jun has been shown to be a crucial factor when it comes to programmed cell death, which reveals opposing functions of c-Jun resulting from different environmental impacts[9].

The nucleotide-binding oligomerisation domain-like receptor 2 (NLR2/NOD2) is part of a group of receptors that is termed pattern recognition receptors (PRRs) that have been shown to be evolutionarily conserved, as there is evidence that PRRs exist in very simple organisms like *Hydra*[10,11]. *Hydra* is able to detect MAMPs and PAMPs (microbe/pathogen associated molecular patterns) as well as to initiate an innate immune response that relies on Toll-like receptor (TLR) signalling and the resulting secretion of AMPs[12]. The working mechanism of NLRs is similar to TLRs even though there is growing evidence that NLRs are situated inside the cell (not on the outer cell membrane); NLR1 and NLR2 are thought to play a crucial role in inflammatory signalling, as they indirectly activate MKKs, which phosphorylate MAPKs like JNK, thus making it possible for the MAPKs to phosphorylate transcription factors of the AP-1 family in the nucleus (like c-Jun)[13,14]. NLR1 and NLR2 also activate INF-signalling via TRAF3 (TNF receptor associated factor 3), which may play an important role in the initiation of programmed cell death[14,15].

First, the disease-phenotype of *Hydra* should be described, including the amount of bacterial colonisation as well as the decrease in size of tentacle- (TL) and body length (BL). Finally, this observation is linked to an increasing apoptosis rate in the polyps.

Results and Discussion

Disease-phenotype of *Hydra*

To show the impact of the amount of nutrients available for the bacteria situated on *Hydra*s epidermis control animals were kept in S-medium (s. Methods), whereas a group of other animals was exposed to R2A-rich medium, which provides optimal growing conditions for bacteria. Whenever *Hydra* is exposed to nutrient-rich conditions (R2A, s. Methods), bacteria that are situated on the epidermis of the freshwater polyp (which includes the body as well as the tentacles and the foot) are confronted with a change in the availability of nutrients. It is not well understood which groups of bacteria profit by the nutrient-rich environment and which groups might suffer from it but nonetheless the amount of bacteria that can be found on *Hydra*s epidermis increased (Fig. 1 A). The amount of colonisable bacteria attached to *Hydra* after 24 h was 6.9-fold higher on animals exposed to R2A-medium than on control animals; after 48 h the amount of colonisable bacteria on treated animals was even 7.5-fold higher than on the control animals. Besides, there was a slight increase in the amount of colonisable bacteria for the two different control groups, too (Fig. 1 C). That might arise due to individual differences between the animals, which also includes the individual capability to deal with stress. Another possible reason is that even in the S-medium bacteria are able to grow in very little dimension compared to the R2A-treatment.

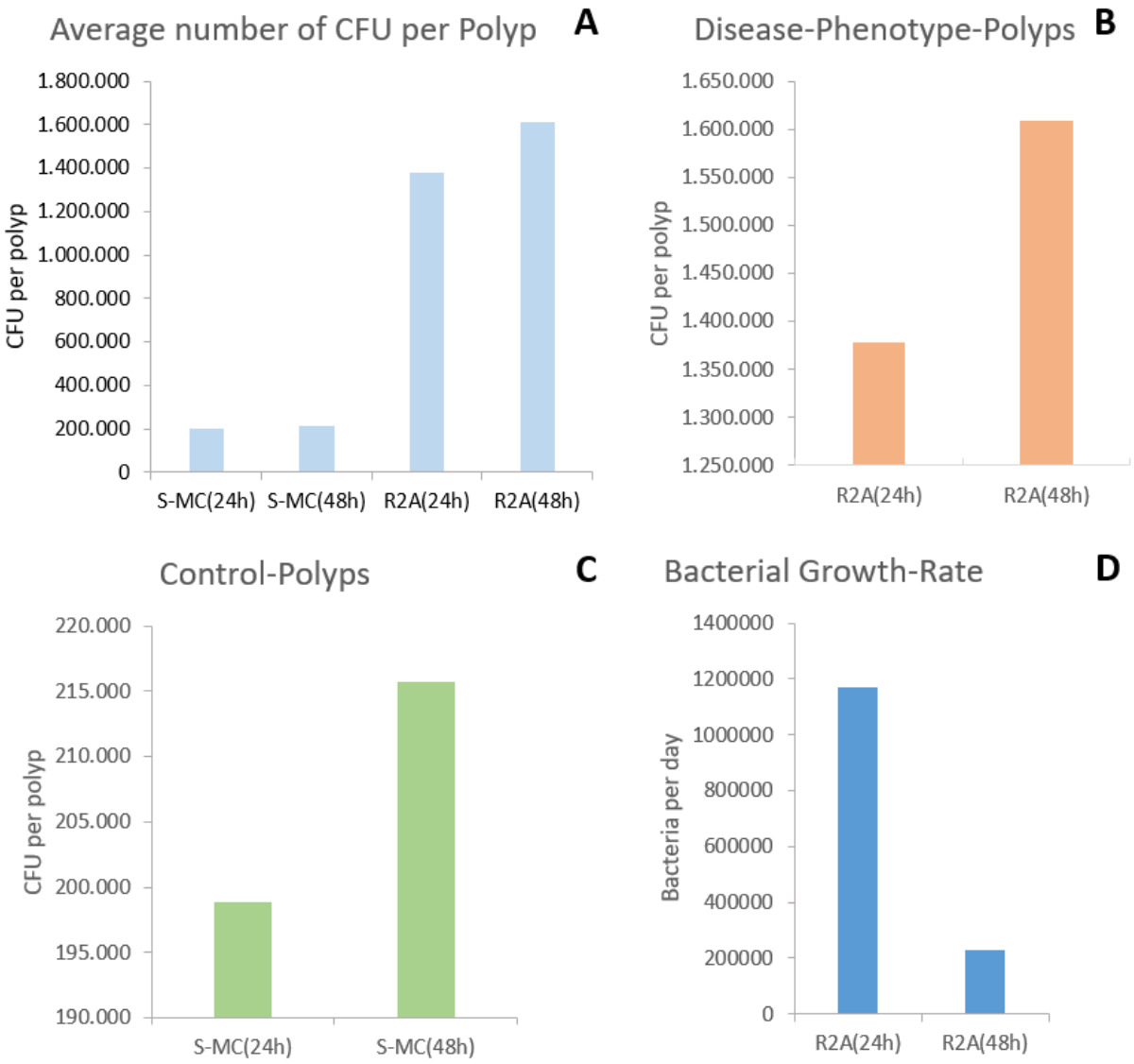

Fig. 1: (A) Two control-groups were exposed to S-Medium whereas two other groups were exposed to R2A-medium; polyps (n = 5) were plated out; CFU per polyp were counted (s. Methods), then averaged (B) Comparison of CFU per polyp for 24 h-control and 48 h-control (for each time step n = 5) (C) Comparison of CFU per polyp for R2A-treatment for 24 h/48 h-time step (n = 5 for each step).

A significant difference could be detected for the R2A-treatment as well when comparing the amount of colonisable bacteria on the 24 h group and the 48 h group (differing in approx. 200.000 CFU per polyp; Fig. 1 B). This further increase probably was a result of consecutive bacterial growth on the polyps. Nonetheless, the growth rate of bacteria attached to the animals dramatically decreased on the second day; a growth rate of approx. 1.2 million colonisable bacteria per day was measured for the first day, followed by a growth rate for the next day that only made up for one sixth of the original growth rate (approx. 200.000 colonisable bacteria per day; Fig. 1 D).

Due to that enormous growth of the bacterial community attached to the host the usual microbiome could not be maintained, which leads to a certain phenotype that is termed disease-phenotype. Wildtype *Hydras* that lived in a natural environment (S-medium) showed a long and relaxed body as well as long and relaxed tentacles (Fig. 2 A). Characteristic for the disease-phenotype were a shortened body as well as a decrease in tentacle length (Fig. 2 B). The increase of bacterial colonization on the animals epidermis was again verified by staining the bacteria with SYBR-GOLD (DNA-binding dye). *Hydras* exposed to R2A-medium showed an increase in the intensity of fluorescence on their epidermis (Fig. 2 C), which is correlated to a higher amount of bacteria attached to the host. Besides, it was observed that the mucus layer, which the animal gets rid of by contracting body and tentacles, is densely populated by

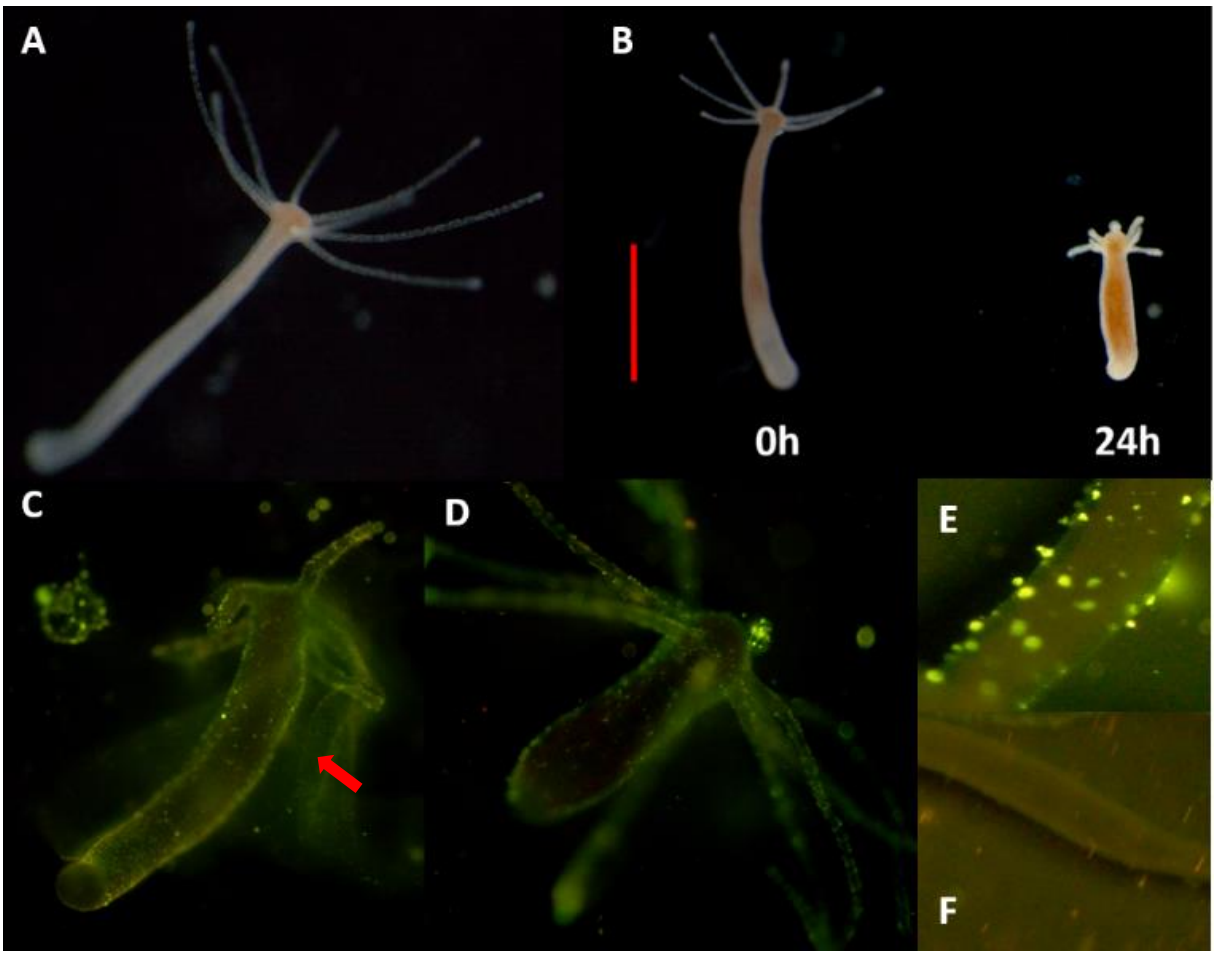

Fig.2: (A) Polyp was kept in S-Medium and shows normal phenotype (B) One polyp was pictured when put into nutrient-rich medium (0h) and after 24h; the polyp shows typical disease-phenotype (decrease in BL as well as a decrease in TL); red line is 2 mm long (C) Density of bacteria on polyp's epidermis is higher when the animal was confronted with nutrient-rich conditions (Bacteria were visualized by using fluorescent DNA-binding dye SYBR-GOLD) (D) Density of bacteria on a control-polyp kept in S-Medium (E) Density of bacteria on the lower part of the body (Treatment) (F) Density of bacteria on lower part of the body (Control)

bacteria as well. In fact this might be a defensive reaction of *Hydra* to reduce the big amount of attached bacteria (Fig. 2 C, red arrow). Another possibility would be that *Hydra* regularly gets rid of its mucus layer to ensure a proper contact to the environment (e.g. to have access to nutrients as well as oxygen). There was bacterial growth on control animals as well (Fig. 2 D) but it turned out to be much lower. Interestingly, the difference between bacterial growth on treated animals and control animals seemed to be highest for the lower part of *Hydra*s body (Fig. 2 E, Fig. 2 F). Here, the relative absence of bacteria on control animals compared to animals exposed to R2A-medium was clearly visible.

In order to further determine the disease-phenotype of *Hydra*, the body length as well as the tentacle length were measured (s. Methods). Even though there were some fluctuations, a certain trend was clearly visible: The control animals showed a constant body length (averaged) of 6.4 to 7.0 mm (Fig. 3 E), but the tentacle length slightly increases over a period of 48 h. Polyps that had just been exposed to S-medium showed an average tentacle length (d-TL) of 4.9 mm. After 24 h a d-TL of 8.4 mm was measured, then the d-TL slightly decreased (1 mm) during the second day (Fig. 3 E). It is possible that the increase in d-TL over time in S-medium was a result of a decreasing stress level of the polyps, as they gradually adapted to the new environment. In comparison to the control animals the polyps that were exposed to R2A-medium showed a decrease in both tentacle and body length (Fig. 3 F). At the beginning an average body length (d-BL) of 4.7 mm was observed. After the 48 h the body length only made up 71% of the original d-BL (appx. 3.4 mm). Analysing the d-TL, there was a similar trend detectable: Firstly (0 h), the animals exposed to R2A showed a d-TL of 2.4 mm. The d-TL only made up 49% of the original d-TL after 24 h, then further decreasing to one third of the d-TL measured at the

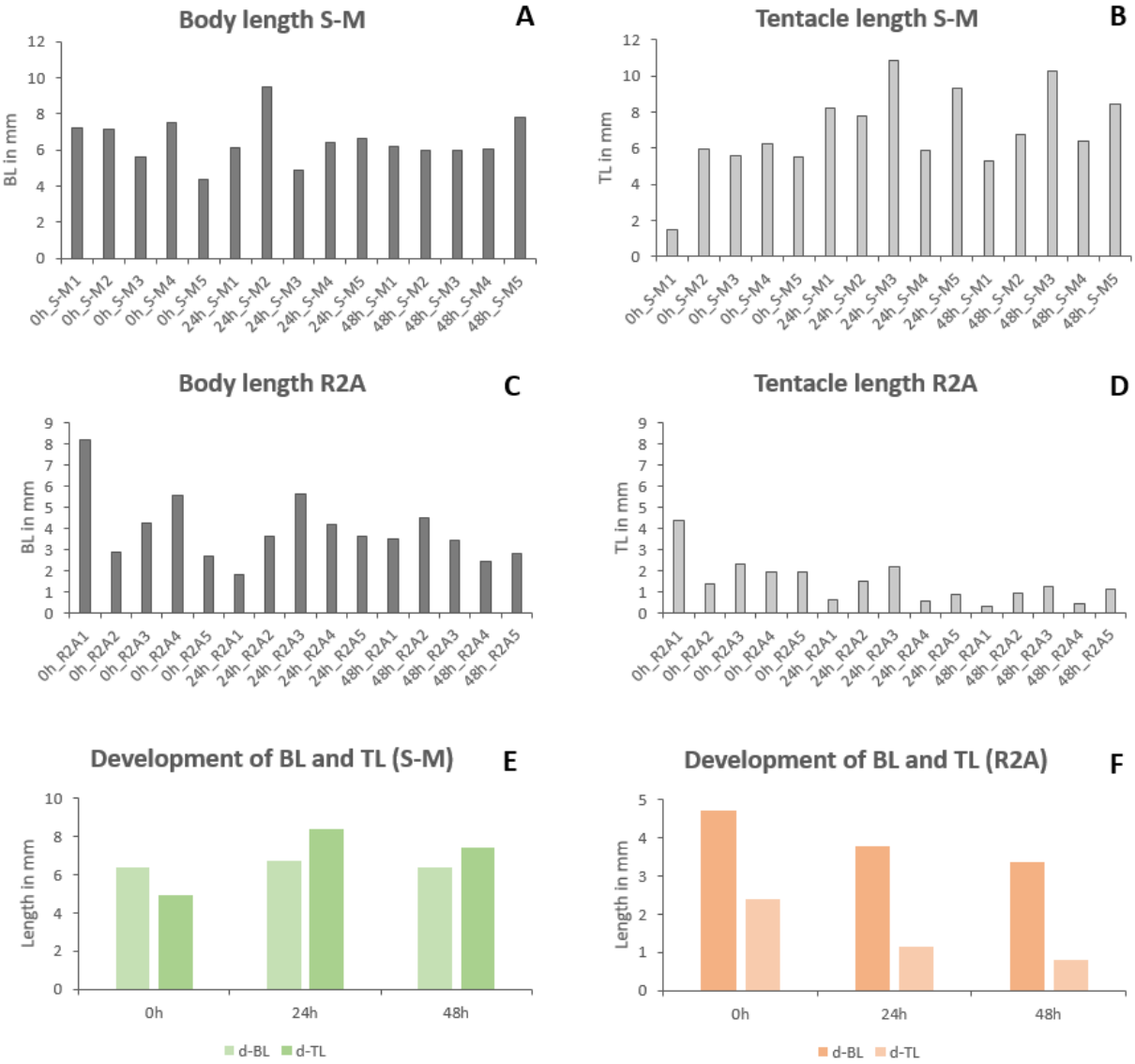

Fig.3: (A) Body length (BL) in mm of Hydra kept in S-Medium (S-M) for variable durations, data was collected at 3 time-steps with n = 5 (B) Tentacle length (TL) in mm of Hydra kept in S-M for variable durations, n = 5 for each time-step (C) BL in mm of Hydra kept in R2A-medium for variable durations, n = 5 for each time step (D) TL in mm of Hydra kept in R2A for variable durations, n = 5 for each time step (E) Development of BL and TL in S-M over a period of 48 h, values shown in this graph represent the arithmetic means of all data measured at one time-step with n = 5 (F) Development of BL and TL in R2A over a period of 48 h, values shown in this graph represent the arithmetic means of all data measured at one time-step with n = 5.

beginning. Comparing the decrease rates for both d-TL and d-BL it turned out that the decrease in TL is even higher than the decrease in BL.

Apoptosis in *Hydra* as a response to increasing bacterial colonization

To finally determine whether the disease-phenotype of *Hydra vulgaris* is just a result of continuing contraction of body and tentacles or a reduction of size via programmed cell death the expression of 7 genes (all relevant for inflammatory or apoptotical signalling) was analysed relative to a housekeeping gene (Elongation Factor (EF), s. Methods). Firstly, what could be observed when analysing all the expression patterns together is that most genes underlay a certain regulation, thus the gene expression of most of the tested genes differed from the gene expression in the control groups, which suggests the presence of the phenomenon of apoptosis in *Hydra*, when the polyp is exposed to nutrient-rich conditions. The expression of AP/AP-1 was a little higher in both R2A group as well as PEP group (approx. 10% higher) relative to the control group after 24 h of exposure to nutrient-rich conditions

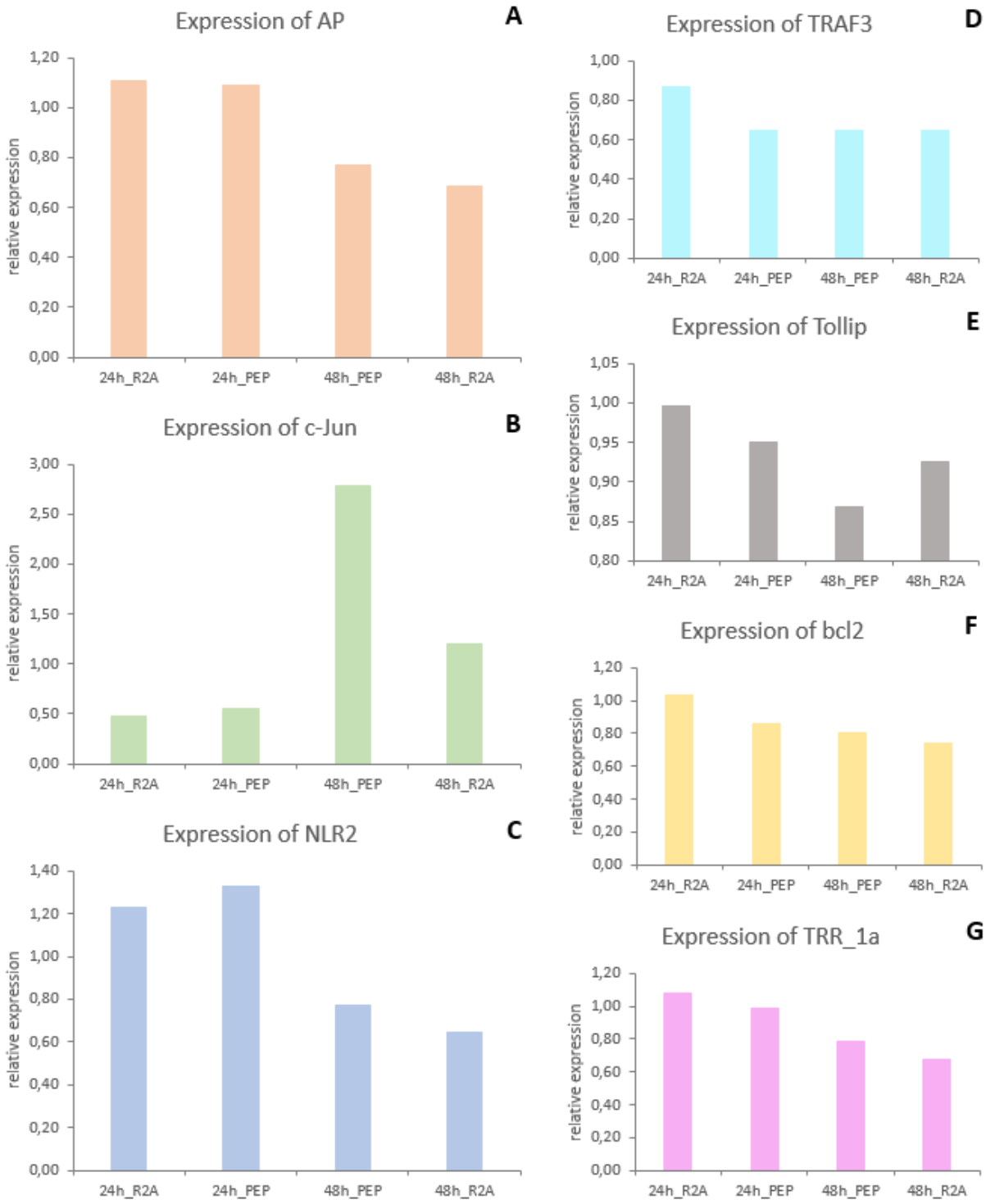

Fig.4: (A) Expression of AP; relative to Elongation Factor (EF) and 24 h-Control when labelled with 24 h, relative to EF and 48 h-Control when labelled with 48 h (calculated with ΔΔCt-Method, s. Methods); n = 4 for every column (averaged value) (B) Relative expression of c-Jun; n = 4 for very column (C) Relative expression of NLR2 (NOD2); n = 4 for every column (D) Relative expression of Toll interacting protein; n = 4 for every column (E) Relative expression of bcl2; n = 4 for every column (F) Relative expression of TRAF3; n = 4 for every column (G) Relative expression of TRR_1a; n = 4 for every column

(Fig. 4 A). That ratio changes during the second 24 h of the treatment as the expression of AP in the R2A group only makes up 77% (69% in the PEP group) of the expression in the control group. Therefore, one can assume that the expression of AP is slightly upregulated (or may even stay constant) first and starts getting downregulated after 24 h. In contrast to that, c-Jun behaves oppositely: The expression of c-Jun is downregulated during the first 24 h of the nutrient treatment (approx. one half of the c-Jun expression in the control group) and then strongly increases during the next 24 h of the

treatment (Fig. 4 B). Interestingly, the expression of c-Jun in the PEP group is almost three times higher than in the control group, whereas in the R2A group c-Jun expression is only 20% higher. Keeping in mind opposing functions of AP and c-Jun when it comes to the regulation of apoptosis, both them may promote apoptosis when up- or downregulated, as it is not clear when apoptosis rates are the highest during the treatment. It may be that the apoptosis rate decreases with time which would suggest that a slight upregulation of AP and a downregulation of c-Jun are apoptosis-promoting conditions but it is also possible that apoptosis rates increase with time which would confirm the opposite regulation mechanism of apoptosis for AP and c-Jun. The expression of NLR2 (NOD2) shows a pattern similar to the expression of AP. After a period of 24 h NLR2 expression is upregulated (relative to the control) in both PEP- and R2A-exposed animals (23% higher in R2A group, one third higher in PEP group); then the expression of NLR2 decreases (relative to control), thus only making up appx. 70% of the expression level of NLR2 in the control group (Fig. 4 C). The similar expression pattern of AP-1 and NLR2 might be a result of their interaction. As NLR2 has been shown to indirectly activate transcription factors of the AP family by activating MAPKs like JNK, their upregulation may also be induced via NLR2 pathways, thus resulting in an enhancement of the NLR2/AP-1 signal in the nucleus. Interestingly, the expression of TRAF3 is downregulated relative to the control for both treatment and both time points (Fig. 4 D). TRAF3 expression in PEP animals made up 65% of the expression in the control group and remains constantly downregulated over a period of 48 h. In R2A animals the expression of TRAF3 made up 87% of the expression of TRAF3 in the control group after 24 h; 24 h later the expression of TRAF3 in the treated animals only made up 65%. As NLR2 and TRAF3 work together when it comes to the regulation of apoptosis via INF-signalling a constant downregulation of TRAF3 is surprising, as it should hinder their working efficiency.

Toll-interacting protein (Tollip) has been shown to have an inhibitory impact on NF-κB-activation[16]. As NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) is known for its promoting impact on cell survival an upregulation of Tollip would lead to a lower expression of NF-κB, resulting in a higher apoptosis rate[17]. It turned out that Tollip is downregulated in the R2A as well as PEP group relative to the expression of Tollip in the control group (Fig. 4 E), which suggests an upregulation of NF-κB and therefore a lower apoptosis rate. As the phenomenon of apoptosis is clearly visible in the R2A and PEP group, it can be imagined that the downregulation of Tollip may not be of high efficiency concerning an upregulation of NF-κB in this scenario. In fact it may be sufficient to have a little lower amount of Tollip to inhibit NF-κB-activation. Besides, Bcl2 (B-cell lymphoma 2) is known to be a critical regulator of apoptosis; there is evidence that an overexpression of bcl2 promotes cell survival in multiple animals such as humans but also in organisms like Hydra[18]. In the PEP- and R2A group the expression of bcl2 is significantly downregulated after 48 h of treatment: There is a difference of 20% between the expression levels of bcl2 in control and PEP treatment and a difference of 26% between control and R2A treatment (Fig. 4 F). The downregulation of bcl2 enhances apoptosis in this case, as it cannot maintain its cell survival-promoting function. Finally, the expression of TRR_1a (Toll-receptor-related 1a) seemed to be downregulated after a period of 48 h as well. In fact the expression of TRR_1a in PEP animals only made up 79% of the expression of TRR_1a in the control group whereas the expression of TRR_1a in the R2A group made up 68% of the expression of the gene in the control group (Fig. 4 G). TLRs have been shown to exist in *Hydra vulgaris*; they play an important role in *Hydras* immune response because of their capability to recognize PAMPs[19]. The downregulation of TRR_1a seemed inappropriate here, as though the animal has to deal with a growing number of bacteria to be detected and fought against. There might be a link between the downregulation of TRR_1a and an upregulation of the overall apoptosis rate.

Conclusions

The introduction of the concept of the metaorganism has widened our eyes when it comes to the analysis of diseases of any form as it is necessary to keep in mind the interactions of host and microbiome when developing a new therapy to cure them. In this report I showed the relevance of an intact microbiome for the basal metazoan *Hydra vulgaris* that showed a certain disease-phenotype when confronted with nutrient-rich conditions that promote bacterial growth rates. Even though *Hydra* is a very simple organism the most basic result of this piece of work – that an intact microbiome is highly essential for maintaining the host's health – can easily be transferred on different species. Even in human there is growing evidence that some diseases might be a result of an altered microbiome; one example is inflammatory bowel disease (IBD) that has been linked to an altered immune response in the gut, leading to inflammatory reactions. Rather new approaches revealed a key role of an intact microbiome to maintain the regular function of the gut which nowadays replaced the old hypothesis[20].

Besides, the disease-phenotype of *Hydra vulgaris* was linked to higher apoptosis rate. Here, I provided evidence that the expression of a certain number of genes, involved in both apoptotical and inflammatory signalling, in treated animals differed from the gene expression in the control animals. In fact, that suggests an important role of bacterial signals as key factors for inducing the increasing apoptosis rates of the host, as it has been shown that the upregulation of the apoptosis rate was not induced by just adding the nutrients[4]. Even though the expression of genes like NLR2, bcl2 or TRR_1a that have been linked to the overall field of the immune system was regulated in a certain manner it can be hypothesized that the apoptosis rate is upregulated via inflammatory signalling induced by secreted molecules of the bacteria. Another possibility is the detection of LPS (lipopolysaccharides) via immune receptors like TLRs that induces a response of the immune system. In contrast to that the upregulated apoptosis rate might arise due to an apoptotical response to bacterial signals, thus activating the immune system as a result of a higher apoptosis rate in epithelial cells. To further determine the actual reasons of the upregulated apoptosis rate it will be necessary to verify one of the proposed hypotheses in order to identify the key factor in this defensive reaction of the polyp.

Methods

Preparation of the polyps

The polyps were derived in 6-well plates. Each of them was kept in a single well exposed to 5 ml of a certain medium. Polyps that were part of the control group were confronted with normal S-medium (S-M; S-M contains 0.1% $CaCl_2$-solution (42.18 g $CaCl_2 \times 2H_2O$ per liter) and 1% of carbonate buffered $MgSO_4$-solution (8.116 g $MgSO_4 \times 7\ H_2O$, 4.238 g $NaHCO_3$, 1.0958 g K_2CO_3 per liter of distilled water) as well as milli Q water). Next to the control group two different groups of polyps were either exposed to 5 ml of R2A (Reasoner's 2A agar)- or PEP (pepton/tryptone)-rich solutions. Therefore stock solutions were prepared containing either 10% R2A-medium (0.3% of R2A both in milli Q (mQ) water) or 0.2% PEP. The polyps were kept in these media for up to 72 h.

Quantification of bacteria

In order to quantify the amount of bacteria situated on the polyps epidermis a series of 10 polyps was either exposed to 5 ml of R2A- or S-medium (approx. 20 °C). Then, 5 polyps were collected after 24 h

and the remaining polyps were collected after 48 h of treatment. To measure the amount of colonisable bacteria situated on the polyps they were each put into 100 µl of mQ, then shredded by a small piston. The piston was washed by slowly adding 900 µl of mQ. To further process the probes all of them were diluted two times (100 µl of the original sample were transferred into a new tube already containing 900 µl of mQ) to minimalize the amount of bacteria in the sample. Finally, 200 µl of the final samples were plated out on R2A-agar (hence the amount of colonies that can be seen on the plates made up for 0.2% of the original amount of bacteria attached to the polyp). The R2A-agar contained 1.81% R2A as well as mQ. After 3 days the CFU (colony forming unit) per polyp were counted using the ImageJ-software of the NIH. Afterwards, the CFU per polyp were averaged for the five replicates per each time-step and treatment.

To visualize the bacterial growth on *Hydras* epidermis the fluorescence DNA-binding dye SYBR-GOLD was used. As SYBR-GOLD binds to the bacterial DNA easily, it was possible to detect bacteria attached to Hydra. Images were taken for both, control and R2A polyps.

Description of the disease-phenotype

To further describe the disease-phenotype of *Hydra vulgaris*, the length of tentacles and the body was measured. Again, a group of 5 polyps per treatment was either exposed to R2A or S-medium. The polyps of every group were imaged once at the beginning (0 h), after 24 h and after 48 h. Next, the body length and tentacle length of every polyp was measured using the ImageJ-software of NIH. To describe the overall development of tentacle- as well as body length the associated values were averaged for every time-step.

Analysis of *Hydra*s gene expression

To quantify the gene expression of seven genes (c-Jun, AP-1, NLR2, Tollip, bcl2, TRAF3, TRR_1a) linked to apoptotical or inflammatory signalling a qRT-PCR (quantitative real time-polymerase chain reaction) was performed. The expression of these genes was analysed in a PEP, a R2A as well as in a control group over a period of 48 h (4 replicates per treatment and per time-step). The isolated RNA as well as the matching cDNA were provided by Eva Herbst. In a second step the specific primers were diluted to 10pmol/µl (concentration was measured using NanoVue technology and their molecular weight was calculated, s. Table 1). Next, forward and reverse primers of every primer-pair were mixed (30 µl per forward primer and 30 µl per reverse primer). Afterwards the primer mixes were distributed on the 64-well plate, adding 2 µl per tube and the master mix containing 110 µl of the GoTaq®-master mix (Promega), 8.8 µl of a specific cDNA and 83.6 µl of mQ was prepared. Finally, 23 µl of the master mix were distributed along one lane of the plate (s. Table 2, plate setup).

In order to amplify the cDNA and to detect the emitted fluorescence the plate was first heated to a final temperature of 95 °C for 2 min. In the following the machine was programmed to run a normal PCR-program (denaturation: 95 °C, 15 s; annealing: 57 °C, 30 s; amplification: 60 °C, 30 s). This procedure was repeated 40 times. The fluorescence signal was only measured during the amplification period.

The quantification was done relative to the expression of the elongation factor (housekeeping gene) using the ΔΔCt-method. The gene expression measured in the control-animals was used as a reference.

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Appendix

Table 1: Molecular weight and concentration of the primers

Primer (forward and reverse)		ng/µl	MW (g/mol)	pmol/µl	add water to 10 µl primer
SF_cJun_RT	SF_cJun_RT_F	972	7107	137	127
	SF_cJun_RT_R	768	7017	109	99
SF_bcl2_RT	SF_bcl2_RT_F	776	7170	108	98
	SF_bcl2_RT_R	636	6601	96	86
SF_Tollip_qRT	SF_Tollip_qRT_F	458	6691	68	58
	SF_Tollip_qRT_R	525	7393	71	61
SF_TRAF3_RT	SF_TRAF3_RT_F	647	7318	88	78
	SF_TRAF3_RT_R	565	7243	78	68
SF_AP_qRT	SF_AP_qRT_F	479	6342	76	66
	SF_AP_qRT_R	463	6613	70	60
SF_TRR_1a	SF_TRR_1a_F	465	7074	66	56
	SF_TRR_1a_R	518	6957	74	64
SF_NLR2_qRT	SF_NLR2_qRT_F	547	7679	71	61
	SF_NLR2_qRT_R	537	7670	70	60
Ak_EF1a	Ak_EF1aF	693	6534	106	96
	Ak_EF1aR	643	6453	100	90

Table 2: Plate setup

	c-Jun	bcl2	Tollip	TRAF3	AP	TRR_1a	NLR2	EF
24h_C1/ 24h_PEP1/ 48h_PEP1								
24h_C2/ 24h_PEP2/ 48h_PEP2								
24h_C3/ 24h_PEP3/ 48h_PEP3								

24h_C4/ 24h_PEP4/ 48h_PEP4								
24h_R2A1/ 48h_C1/ 48h_R2A1								
24h_R2A2/ 48h_C2/ 48h_R2A1								
24h_R2A3/ 48h_C3/ 48h_R2A1								
24h_R2A4/ 48h_C4/ 48h_R2A1								

Further Data:

I was provided with additional data on the change of the gene expression pattern of the polyps during the nutrient treatment that shall be included here as the two approaches turned out to show different results.

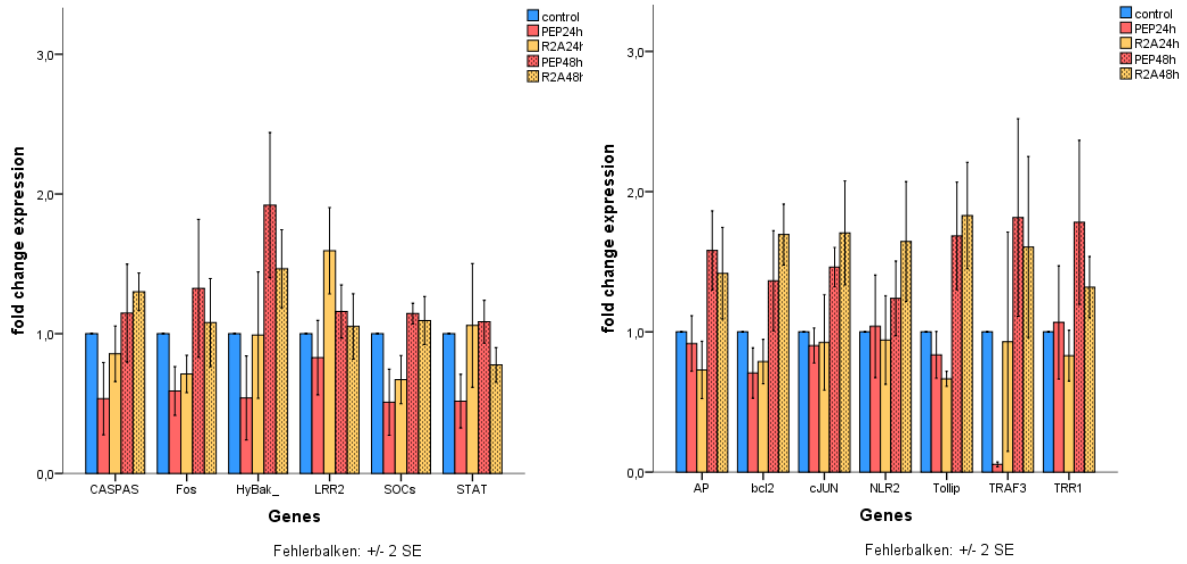

Fig. 5: Relative gene expression of Caspase3, Fos, Hybak, LRR2, SOC, STAT, AP, bcl2, cJUN, NLR2, Tollip, TRAF3, TRR1; gene expression relative to control at 24 h for 24 h samples and relative to control at 48 h for all 48 h samples as well as relative to the expression of EF (24 h and 48 h).

The left part of Fig. 5 shows a few other genes that were tested during further analysis (qPCR performed by E. Herbst). All of them have been shown to regulate inflammatory signalling or apoptosis. The right part shows the change in the expression pattern of the genes I analysed during my internship. When comparing my results and Fig. 5 some differences can be observed: To start with, here the expression of AP increased at 48h (relative to control at 48h; s. Fig. 5), whereas my data (s. Fig. 4) showed a decrease relative to control. Another example is the expression pattern of Tollip: According to Fig. 4 the expression of Tollip decreased after 48h (relative to control) but according to Fig. 5 the expression of Tollip after 48h is higher in the treated animals compared to the control animals.