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Differential miRNA expression in the mouse jejunum during garlic treatment of *Eimeria papillata* infections

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Abstract Accumulating evidence indicates a critical role of microRNAs (miRNAs) in the outcome of diseases. Here, we investigate the effect of garlic on the intestinal miRNA signature of male Balb/c mice during infections with *Eimeria papillata*. Garlic decreases the intracellular development as evidenced by a lowered fecal output of *E. papillata* oocysts from 3,150±410 to approximately 1,750±390 oocysts per gram feces on day 4 postinoculation. This anti-coccidial activity of garlic is associated with an inhibition of the *E. papillata*-induced increases of interferon gamma, inducible nitric oxide synthase, nitrite/ nitrate, and malondialdehyde and decrease in glutathione. Moreover, garlic downregulates the *E. papillata*-induced increases in the expression of the miRNAs miR-1959, miR-203, and miR-21, and it upregulates the expression of the 11 miRNA species miR-142-5P, miR-15A, miR-10A, miR-29B, miR-1902, miR-125A-5P, let-7E, miR-148A, miR-130A, miR-10B, and miR-93, respectively, as revealed by miRXplore microarray technology. Real-time PCR confirms these effects of garlic in the jejunum of *E. papillata*-infected mice. Our data indicate that the anti-coccidial activity of garlic is associated with specific changes in the miRNA signature of the mouse jejunum, the target site of *E. papillata*. These changes may reflect an involvement of miRNAs in garlic-activated pathways to reduce and/or to repair *E. papillata*-induced tissue injuries.

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Introduction

The unicellular eukaryote *Eimeria* is the infectious agent of coccidiosis worldwide affecting the digestive tract of vertebrates. *Eimeria tenella* is by far the most dangerous species among the approximately 800 different species which causes dramatic economic losses in poultry farms (Mehlhorn 2008) due to diarrhea, weight loss, desiccation, and fatal outcome of the disease. The *Eimerian* parasites are characterized by fast reproduction and by infecting especially young animals (Ryley and Robinson 1976; Licois et al. 1992; Gres et al. 2003; Pakandl 2005). Infection begins with oral uptake of *Eimerian* oocysts, which release infectious sporozoites in the intestine. These, in turn, invade mainly epithelial cells of the intestine, in which they asexually multiply before oocysts are finally discharged with the feces.

Numerous reports indicate the efficacy of garlic in the prevention and treatment of a variety of diseases. For instance, garlic exhibits antitumor, antithrombotic, antiarthritic, hypolipidemic, and hypoglycemic activities (Kiesewetter et al. 1993; Milner 1996; Karasaki et al. 2001; Duraka et al. 2002; Kumar et al. 2003). Moreover, garlic has been found to be effective against a wide variety of infectious diseases. Thus, garlic possesses antiviral (Fenwick and Hanley 1985), antibacterial (Sharma et al. 1977), and antifungal properties (Yamada and Azuma 1977). Moreover, garlic is even effective against diverse diseases caused by the much more complex parasites such as *Amoeba* (Peyghan et al. 2008), *Leishmania* (Ghazanfari et al. 2006), *Trypanosoma* (Nok et al. 1996) and *Cryptosporidium* (Wahba 2003), and *Giardia* (Calzada et al. 1999). Very recently, garlic has been also found to be effective against the intestinal parasite *Eimeria* (Dkhil et al. 2011).

During the last years, evidence has accumulated that microRNAs (miRNAs) play a critical role in the outcome of diseases (Tili et al. 2007). MicroRNAs are ~22 nts small non-coding RNAs which are ubiquitously distributed among all species. These miRNAs posttranscriptionally regulate gene expression by targeting 3′ ends of mRNA for degradation or for repression of translation. Meantime, the miRNAs are known to play important regulatory roles in many biological processes including proliferation, differentiation, apoptosis, development, and immune responses (Bartel 2004; Baltimore et al. 2008; Bala et al. 2009; Bi et al. 2009). Currently, however, the information about the role of miRNAs in the pathophysiology and diseases of the intestine is mainly confined to bowel disease and colorectal cancer (Slaby et al. 2009; Liu and Chen 2010). There is only one very recent report describing an induction by *Eimeria* of a few distinct miRNA species in the intestine of mice (Dkhil et al. 2010).

The present study investigates the effect of garlic on miRNA expression profiles in the intestine during *Eimerian* infection. *Eimeria papillata* has been chosen as a model, which is known to develop predominantly in the jejunal part of the intestine.

Material and methods

Animals

Balb/c mice were bred under specified pathogen-free conditions and fed a standard diet and water ad libitum. The experiments were performed only with male mice at an age of 8–9 weeks and were approved by state authorities and followed Saudi Arabian rules for animal protection.

E. papillata infections

E. papillata, kindly provided by Prof. Mehlhorn (University of Duesseldorf, Germany), was previously characterized (Danforth et al. 1992; Chobotar et al. 1993; Hnida and Duszynski 1999; Zhao and Duszynski 2001). Oral gavage of mice was done with 10^3 sporulated oocysts of *E. papillata* suspended in 100 μl sterile saline. Oocysts were collected from feces of naturally infected Balb/c mice, surface-sterilized with sodium hypochlorite and washed at least four times with sterile saline before oral inoculation as described by Schito et al. (1996). Once a day at 1 p.m., fresh fecal pellets were collected and weighed for each mouse, and the bedding in the cages was changed to eliminate re-infection. Fecal pellets were suspended in 2.5% (w/v) potassium dichromate before diluting in saturated NaCl, causing the oocysts float. The latter were counted in a McMaster chamber and expressed as number of oocysts per gram of wet feces (Schito et al. 1996).

Preparation of garlic extract

Garlic extract was prepared according to Balasenthil et al. (1999). In brief, cloves of garlic were homogenized in sterile saline at a final concentration of 20 mg/ml, centrifuged at 3,000×g for 10 min, and the supernatant was used for the experiments.

Histological analysis

Pieces of jejunum were freshly prepared, fixed in 10% neutral buffered formalin, and then embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin according to Drury and Wallington (1980). According to Dommels et al. (2007), tissue sections were scored for inflammatory lesions (infiltrations by mononuclear cells, neutrophils, eosinophils, and plasmacytes, for fibrin exsudation and lymphangiectasis, for tissue destruction (enterocyte loss, ballooning degeneration, edema, and mucosal atrophy), and for tissue repair (hyperplasia, angiogenesis, granulomas, and fibrosis). A rating score between 0 (no change from normal tissue) and 3 (lesions involved most areas and all the layers of the intestinal section including mucosa, muscle, and omental fat) was given for each aspect of inflammatory lesion, tissue destruction, and tissue repair. The sum of inflammatory lesions, tissue destruction, and tissue repair scores was used to represent the total histological injury score (HIS) for each intestinal section. The sum of the inflammatory lesions was multiplied by 2 to give more weight to this value since the tissue changes were mainly characterized by inflammatory lesions (Dommels et al. 2007).

Biochemical determinations

Jejunum tissue was aseptically removed, homogenized, and prepared for the various biochemical determinations as described by El Shenawy et al. (2008). Nitrite/nitrate and malondialdehyde (MDA) were assayed colorimetrically according to the method of Berkels et al. (2004) and Buege and Aust (1987), respectively. Glutathione (GSH) was determined by the method of Ellman (1959), modified by Nishikimi et al. (1972) and Aebi (1984).

Quantitative RT-PCR of mRNA

Pieces of jejunum were aseptically removed, rapidly frozen, and stored in liquid nitrogen until use. Total RNA was isolated using Trizol (Invitrogen). Contaminating genomic DNA was digested with the DNA-free™ kit (Applied Biosystem, Darmstadt, Germany), before cDNA was synthesized using QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). Real-time PCR (RT-PCR) was performed in a TaqMan7500 (Applied Biosystems) using the QuantiTect™ SYBR® Green PCR kit (Qiagen) and the gene-specific QuantiTect™ primer assay (Qiagen) according to the manufacturer's instructions. Qiagen (Hilden, Germany) delivered the primers for interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon gamma (IFN-γ), inducible nitric oxide synthase (iNOS), and 18S rRNA. Initial incubation was done at 50°C for 2 min, followed by Taq polymerase activated at 95°C for 10 min, 55 cycles followed at 95°C for 15 s, at 60°C for 35 s, and for 30 s at 72°C. All PCR reactions yielded only a single product of the expected size as detected by melting point analysis and gel electrophoresis. Quantitative evaluation was performed with Taqman7500 system software v.1.2.3f2 (Applied Biosystems) and the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Expression of genes was normalized to that of 18S rRNA (Delic et al. 2010).
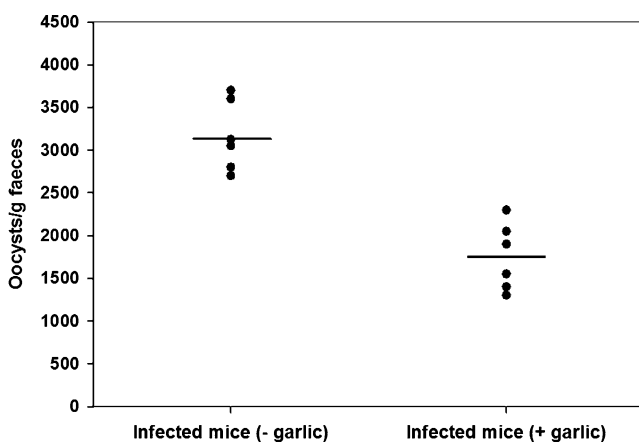

Fig. 1 Output of oocysts from mice infected with 10^3 sporulated E. papillata oocysts. Individual values for each infected mouse (− or + garlic) are presented

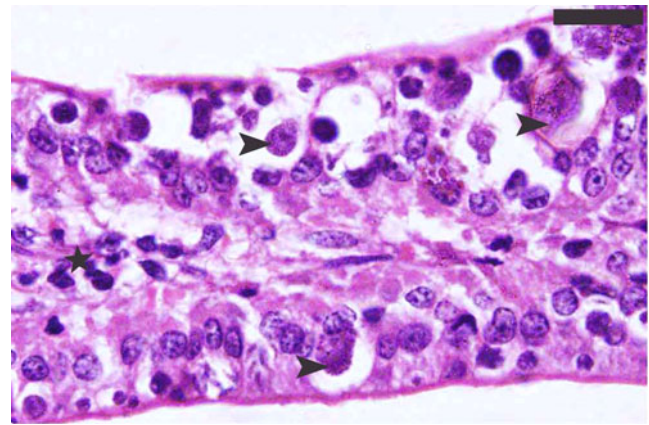Fig. 2 Sections of mouse jejunum infected with E. papillata on day 4 p.i.. Jejunum with only some inflammatory cellular infiltration (star). Arrow heads indicate different developmental stages of Eimeria. Sections are stained with hematoxylin and eosin. Bar=25 μm

RNA-labeling and microarrays

Quality and integrity of the isolated RNA were first determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was quantified at $A_{260 \text{ nm}}$ in a ND-1000 Spectrophotometer (NanoDrop Technologies). The miRCury Power Labelling Kit (Exiqon) was then used to label the RNA samples according to the manufacturers' instructions. Control RNA samples (2 μg) were labeled with the green fluorescent Hy3 and the experimental sample with the red fluorescent Hy5.
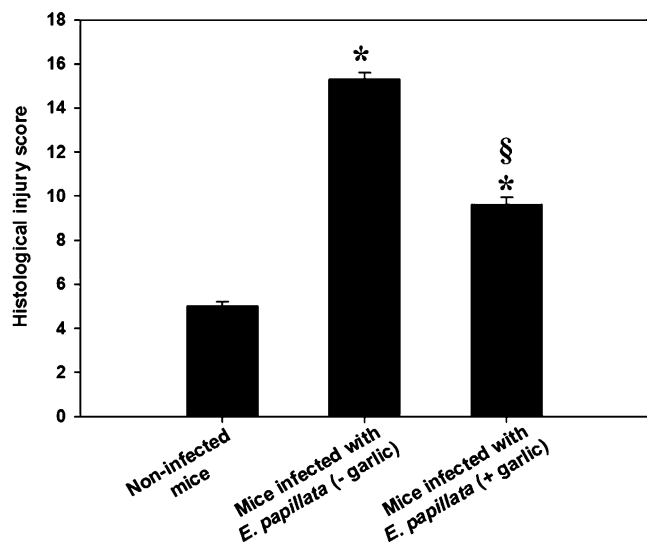

Fig. 3 Total HISs in jejunum of mice infected with E. papillata on day 4 p.i.. Values are means±SD. Asterisk significant difference vs. the control (p<0.01) and section sign significant difference between infected and infected garlic-treated mice (p<0.01)
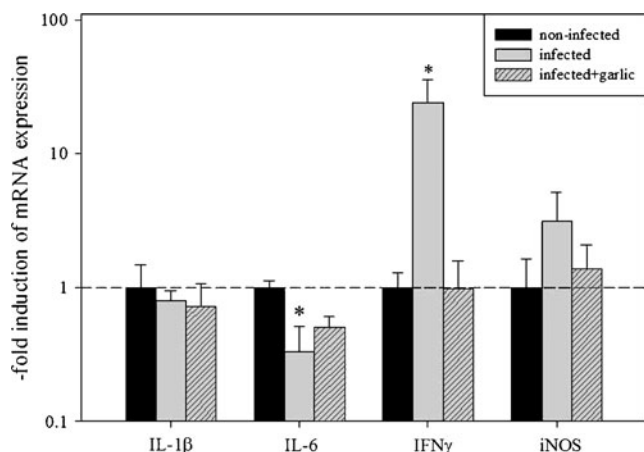
Fig. 4 RT-PCR analysis of IL-1β, IL-6, IFN-γ, and iNOS in the mouse jejunum. Expression of mRNA was analyzed in jejuna isolated from non-infected mice, mice infected with *E. papillata* on day 4 p.i., and infected mice treated with garlic on day 4 p.i. Signals were normalized to 18S rRNA signals, and relative expression is given as -fold increase compared to control mice. All values are means±SD. *Asterisk* significant data with respect to non-infected mice ($p<0.01$)

Hybridization of miRXplore™ microarrays

The a-Hyb™ hybridization station (Miltenyi Biotec) was used for hybridization according the manufacturer's instructions. Briefly, equal amounts of the corresponding Hy3 and Hy5 samples were combined, and the volume was adjusted to 100 μl with nuclease-free water, before adding 100 μl of 2x hybridization solution (Miltenyi) pre-warmed to 42°C. After mixing, the solution was pre-heated to 70°C for 2 min before applying to the a-Hyb station. Microarray processing on this station was performed by incubating in pre-hyb solution (Miltenyi) at 42°C for 5 min, hybridizing with the labeled RNAs at 42°C for 960 min, washing with wash buffer I (Miltenyi) at 10°C (two cycles) for 1 min and with wash buffer II (Miltenyi) at 10°C (two cycles) for 1 min.

Scanning and data analysis

Fluorescence signals of the hybridized miRXplore™ microarrays were read out with the Agilent's Microarray

Scanner System (Agilent Technologies). The ImaGene® software (Biodiscovery) was then used to determine mean signal and mean local background intensities for each spot of the miRXplore™ microarray images. Ratio calculation and normalization were done with the miRXplorer software. Here, two samples were compared directly to each other, and the signal intensities of both channels were normalized by using the lowest normalization. Only those values that were above the cut-off of 2.0 ($p<0.01$), in at least one of the two compared groups, were taken.

Quantification of miRNA

The isolated RNA was treated with DNase (Applied Biosystems, Darmstadt, Germany) as described above and then converted into cDNA following the manufacturer's protocol using the miScript™ Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with miScript™ SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primers for miR-1959, miR-203, miR-21, miR-15A, miR-10A, miR-29B, let-7E, and miR-148A used for qRT-PCR were commercially provided as miScript™ primer assays by Qiagen (Hilden, Germany). PCR reactions were conducted as follows: 94°C for 15 min as initial activation step to activate HotStarTaq DNA polymerase followed by 40 cycles at 94°C for 15 s, at 55°C for 35 s, and at 70°C for 30 s. Reaction specificity and quantitative evaluation of amplification data were analyzed as described above. For quantification, the U6 small nuclear RNA was used as an internal standard.

Statistical analysis

Values are presented as means±SD. Two-way ANOVA was used, and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS version 17.0).

Table 1 Effect of garlic on the level of nitrite/nitrate, malondialdehyde, and glutathione in jejunum of Balb/c mice infected with *E. papillata*

Parameter	Non-infected mice	Infected (− garlic)	Infected (+ garlic)
Nitrite/nitrate (μmol/g)	0.20±0.01	0.22±0.01[a]	0.21±0.01
MDA (μmol/g)	83±0.03	1.1±0.04[a]	0.81±0.04[b]
GSH (μmol/g)	1.75±0.40	1.57±0.50[a]	1.65±0.7

Values are means±SD ($n=6$)

[a] Significant change at $p<0.05$ with respect to non-infected mice

[b] Significant change at $p<0.05$ with respect to infected mice

Results

The effect of garlic on the outcome of *E. papillata* infections was investigated in male Balb/c mice. We compared three groups of mice, with six animals per group; all mice were individually caged. Mice of the first group served as a control and received only sterile saline. Mice of the second and third groups were infected with 10^3 sporulated oocysts of *E. papillata*, and only mice of the third group were treated with 100 μl garlic extract (20 mg/ml) daily for 4 days during infections. During the first 3 days of infection, there was no fecal output of oocysts. On day 4 postinoculation (p.i.), the output differed between garlic-treated and non-treated mice. In the latter, the number of excreted oocysts varied among the individual mice between 2,700 and 3,700 per gram feces (mean value, 3,150±410) (Fig. 1). However, the garlic treatment significantly lowered the shedding of oocysts, the number of

which varied in the range between 1,300 and 2,300 (mean, 1,750±390) per gram feces on day 4 p.i. (Fig. 1). Light microscopical inspection of hematoxylin-and-eosin-stained sections revealed that the epithelial cells of the jejunum were infected by *E. papillata* (Fig. 2). Concomitantly, there occurred some histological changes which were semi-quantified by applying the scoring according to Dommels et al. (2007).

Histological analysis revealed that mice infected with sporulated oocysts of *E. papillata* suffered a moderate inflammatory injury in jejunum (Fig. 3). This injury was diminished when mice were treated with garlic (Fig. 3).

In accordance, the mRNAs of the pro-inflammatory cytokines IL-1β and IL-6 were not affected or even downregulated, respectively, during infection with *E. papillata* (Fig. 4). Also, the iNOS mRNA was only slightly increased during infection. In accordance, total nitrite/nitrate in the jejunum increases slightly upon *E. papillata*

Table 2 miRNA expression in the jejunum of mice as revealed by miRXplore microarrays

miRNA name	Gene ID	Infected/non-infected mice	Infected+garlic/non-infected mice	Functions	PMID
miR-1959	40534	3.06	2.31	Unknown	
MCMV-miR-M23-1-5P	40249	2.8	2.77	Unknown	
miR-203	38328	2.31	1.22	Epigenetically silenced tumor-suppressive miRNA in hepatocellular carcinoma, elevated expression in pancreatic tumors is associated with poorer survival	19843643, 19551852
miR-21	35883	2.31	1.5	Is induced early in pancreatic ductal adenocarcinoma precursor lesions, androgen-receptor regulated, key regulator of oncogenic processes, negatively regulates Cdc25A and cell cycle progression in colon cancer cells	20093556, 19738047, 19614619, 19816956
miR-142-5P	39219	1.19	3.35	Tumor-suppressive expression in lung cancer	19618089
miR-15A	35864	1.39	3.02	Targets multiple oncogens, including Bcl-2, Mcl-1, Ccnd1 and Wnt3a	19498445
miR-10A	35824	1.55	2.81	Contributes to retinoic acid-induced smooth muscle cell differentiation	20118241
miR-29B	38569	n.d.	2.59	Tumor suppressor role in acute myeloid leukemia	19850741
miR-1902	40460	1.54	2.49	Unknown	
miR-125A-5P	39208	1.47	2.35	Metastatic inhibitor of lung cancer, regulated by EGFR	19702827
let-7E	39155	1.07	2.33	Role in drug-resistant ovarian cancer cells	18823650
miR-148A	35857	1.05	2.29	Overexpression leads to lowered PXR mRNA expression	18268015
miR-130A	35837	1.47	2.08	Involved in regulation of angiogenesis	17957028
miR-10B	39202	1.43	2.07	Overexpressed in malignant glioma and associated with tumor invasive factors	19536818
miR-93	35926	1.56	2.06	Regulates expression of tumor suppressor gene FUS1	19671678

infection just as malondialdehyde (MDA), an indicator of oxidative stress, whereas the content of glutathione (GSH) is significantly decreased (Table 1). Only IFN-γ mRNA was significantly upregulated on day 4 p.i. (Fig. 4). Garlic treatment, however, prevents this E. papillata-induced increase in the IFN-γ response (Fig. 3). Moreover, garlic hindered the infection-induced increase in nitrite/nitrate and MDA, respectively, whereas the decrease in GSH induced by E. papillata was apparently not affected under garlic treatment (Table 1).

In order to examine possible changes of the miRNA signature of the jejunum during infection with E. papillata under garlic treatment, RNA was isolated from jejuna of non-infected mice, those mice infected with E. papillata on day 4 p.i. and those mice infected but treated with garlic for 4 days. Then, the RNA was subjected first to a screening using miRExplore™ microarrays, which contain 634 mouse miRNAs (Fig. S1). Quantitative evaluation yields the miRNA species shown in Table 2. Surprisingly, only four miRNA species were upregulated during E. papillata infections. The miR-1959 was increased by 3.06, the MCMV-miR-M23-1-5P by 2.8, and the miR203 and the miR-21 were upregulated by 2.31, respectively. The upregulation of miR-1959, miR-203, and miR-21 was also verified using quantitative RT-PCR with commercially obtained primers (Fig. 5).

The miRNA expression is different after garlic treatment. Only the MCMV-miRNA was unaffected, whereas the infection-induced increase in miR-1959, miR-203, and miR-21 was lowered. In addition, the expression of 11 other different miRNA species, which were miR-142-5P, miR-15A, miR-10A, miR-29B, miR-1902, miR-125A-5P, let-7E, miR-148A, miR-130A, miR-10B, and miR-93, was increased during garlic treatment. This pattern of expression was confirmed for expression of several arbitrarily selected miRNAs by quantitative RT-PCR (Fig. 5).

Discussion

This study is the first report that describes an association of the anti-coccidial activity of garlic against E. papillata with specific changes of the miRNA signature in the mouse jejunum, the target site of E. papillata. Garlic treatment causes a significant lowering in the fecal output of E. papillata oocysts (Fig. 1). Obviously, garlic impairs the intracellular development of parasites in epithelia of host jejunum before the relatively inert oocysts are formed and finally released. These data confirm previous studies showing that garlic possesses anti-coccidial activity in rabbit coccidiosis caused by Eimeria stiedae (Toulah and Al-Rawi 2007). However, the effective components which mediate anti-coccidial activity of garlic are still unknown. The sulfur-containing allicin was thought for a long time to

be the major effective compound of garlic. However, garlic efficacy is presumably also due to smaller metabolic breakdown products of allicin, as, e. g., diallyl sulfide and diallyl disulfide found in the urine after uptake of garlic (Bartzatt et al. 1992) or allyl mercaptan found in the blood (Koch 1996; Cho and Xu 2000). Besides such allyl compounds, garlic also contains numerous other components, as, e. g., kaemferol and quercetin, which have been also demonstrated to be effective, for example, against the intestine parasite Giardia (Calzada et al. 1999). Currently, it cannot be excluded that the synergistic action of different components rather than a single compound is responsible for the anti-coccidial activity of garlic we describe here.

Remarkably, the jejunum of mice infected with E. papillata is characterized by low inflammation and garlic does apparently reduce this low inflammation. Indeed, garlic did not largely influence the mRNAs of the pro-inflammatory cytokines IL-1β and IL-6, the iNOS mRNA, total nitrite/nitrate, and MDA. The only exception is the IFN-γ mRNA, which is significantly upregulated by E. papillata, and this upregulation is prevented by garlic (Fig. 4). IFN-γ is considered as a major host defense mechanism against primary infections with E. papillata (Celada and Schreiber 1986). It has been suggested to be produced mainly by natural killer cells (Schito and Barta 1997). In this context, it is also noteworthy that garlic reduces the inflammatory response of the liver to infections with E. papillata, though the liver is not directly targeted by these parasites (Dkhil et al. 2011).
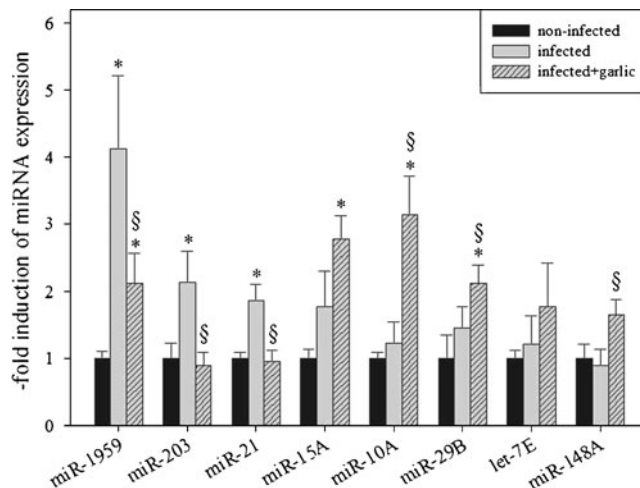

Fig. 5 Quantitative RT-PCR of miRNA expression in mouse jejuna. RNA was isolated from non-infected mice (n=3), mice (n=3) infected with E. papillata for 4 days, and infected and garlic-treated mice (n=3). Relative miRNA expression was normalized to the mean of the expression level of the corresponding non-infected control mice. Values are means±SD. Asterisk significant difference vs. the control (p<0.01) and section sign significant difference between infected and infected garlic-treated mice (p<0.01)

Garlic affects the miRNA expression in the jejunum (Fig. 5, Table 2). Thus, garlic apparently impairs the *E. papillata*-induced expression of the three miRNAs miR-1959, miR-203, and miR-21, but obviously increases expression of 11 miRNA species such as miR-142-5P, miR-15A, miR-10A, miR-29B, miR-1902, miR-125A-5P, let-7E, miR-148A, miR-130A, miR-10B, and miR-93. The functions of miR-1959, MCMV-miR-M23-1-5p, and miR-1902 are unknown to date, whereas some data exist for the other 12 miRNAs, but only in context with diverse cancer diseases (Table 2). Moreover, there is no information available about garlic effects on intestinal miRNA expression. Conspicuously, the miR-142-5p and miR-29B among the 11 upregulated miRNA species by garlic in *E. papillata*-infected jejunum have been very recently found to be required in NK cell activation, survival, and function (Bezman et al. 2010). However, there were no changes in the expression of miR-143-5p, miR-150, miR-16, miR-23a, miR-15b, miR-29a, miR-30b, and miR-26a, which have been also reported to be typical for NK cell functions (Bezman et al. 2010). Furthermore, we have not detected any changes in miR-21, miR-221, miR-222, and miR-146a which have been reported to be expressed in NK cells besides CD8$^+$ T cells (Landgraf et al. 2007; Wu et al. 2007). Also, neither garlic nor *E. papillata* has induced miR-152, the over-expression of which has been reported to increase NK cell-mediated cytolysis in host cells (Zhu et al. 2010). Thus, it is possible that the increased expression of miR-142-5p and miR-29b we have found here in jejuna of garlic-treated mice does not reflect any specific changes in NK cells but rather is due to other cells affected by garlic. Indeed, it is noteworthy that these two miRNA species have been also found to be critically involved in the transition of CD4$^-$CD8$^-$ T cells to CD4$^+$CD8$^+$ T cells (Sonkoly et al. 2008).

Future work is required to unravel the functions of the specific miRNA species detected here in the mouse jejunum to be associated with garlic treatment of *E. papillata* infections. In this context, it is worthwhile mentioning that it has been recently suggested that tissues damaged by pathogens send alarming signals which initiate and guide specific tissue-directed immune mechanisms (Matzinger 2007). It is therefore attractive to speculate that at least some of the miRNA species observed here are involved in pathways to activate such alarm signals and/or to repair tissue damages due to *E. papillata* infections.

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