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Studies on pathogen-host interaction: Impact of the emerging zoonotic pathogen *Arcobacter butzleri* on human macrophages

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Für Lennart

A. List of Abbreviation

A	Adenine
A.	*Arcobacter*
ACTB	β-Aktin
ATR	Ataxia telangiectasia and Rad3-related protein
B2M	β-2-microglobulin
BSA	Bovine serum albumine
C.	*Campylobacter*
CaMKII	Calcium/calmodulin-dependent protein kinase II
CD	Cluster of differentiation
CDK4/6	Cyclin-dependent kinase 4/6
cDNA	Complementary deoxyribonucleic acid
CLuc	Cypridinia Luciferase
CO_2	Carbon dioxide
CT	Cycle threshold
CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1
DAPI	4',6-Diamidin-2-phenylindol
DAVID	Database for Annotation, Visualization and Integrated Discovery
DGCR8	DiGeorge syndrome chromosomal region 8
DNA	Deoxiribonucleic acid
dNTP	Deoxynucleotide
DR 5	Death Receptor 5
ds	Double strand
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food safety Authority
e.g.	*exempli gratia*
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain
Fc	Fragment crystallizable
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
fw	forward
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GLuc	Gaussia Luciferase
h	hour
H.	*Helicobacter*
H_2O	Water
HCV	Hepatitis C Virus
Hk	heat killed
hsa	Homo sapiens
Ig	Immune globulin
IL	Interleukin
iNOS	Nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
LB	Lysogeny Broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
M.	*Mycobacterium*
MAPK	Mitogen-activated protein kinase
MHB	Mueller-Hinton blood agar
MHC II	Major histocompatibility complex II
min	minute
miR	microRNA
miRNA	microRNA
ml	mililiter
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response protein
Nc	negative control
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NT	Non target
OD	Optical density
P-bodies	Processing bodies
p.i.	*post infectionem*
PAMP	Pathogen-associated-molecular pattern
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PDCD4	Programmed cell death protein 4
PMA	Phorbol-12-myristate-13-acetate
Pre-miRNA	Precurser miRNA
Pri-miRNA	Primary miRNA
PRR	Pattern-recognition receptor
PSMB10	Proteasome (prosome, macropain) subunit, beta type, 10
rev	reverse
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAseq	RNA sequencing
rRNA	ribosomal RNA
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard deviation
SDS	Natriumdodecylsulfate
sec	second
SFM	Serum free medium

siRNA	small interfering RNA
spp.	Species pluralis
TAE	Tris-acetate-EDTA
TBP	TATA box binding protein
TLR	Toll-like-receptor
TNFα	Tumor necrosis factor-alpha
TNFRSF10b	Tumor necrosis factor receptor superfamily, member 10b
TRAF	TNF receptor associated factors
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF related adapter molecule
TRBP	transactivating response RNA-binding protein
TRIS	Tris(hydroxymethyl)aminomethane
U	Uracil
3' UTR	3 prime untranslated region
vs	versus
WHO	World Health Organization

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1. Introduction

1.1. Zoonotic diseases

Zoonoses are referred to as infectious diseases, which can be transmitted between vertebrate animals and humans and vice versa. They are caused by all types of pathogenic organisms, including parasites, viruses, fungi and bacteria (WHO, 2015).

Most infectious diseases caused by emerging pathogens over the last years are zoonoses (> 60%) and 72% of these zoonotic infections originate from wildlife (Jones et al., 2008; Palmer et al., 2013).

Direct or indirect exposure to animals, animal products (food) and their environment bare the greatest risks for disease transmission. Bites from infected animals and insects constitute another source of infection. In many cases zoonotic pathogens colonize their animal hosts without obvious effects on their health but are able to cause severe disease in humans, mainly via consumption of contaminated food (e.g. *Campylobacter*-enteritis, Salmonellosis). Therefore, food safety control is of crucial relevance to prevent foodborne diseases. Successful control requires joint veterinary and medical efforts, which includes development of global surveillance systems (Palmer et al., 2013). Nevertheless, the fact that animals are often silent carriers of severe pathogens without exhibiting symptoms makes the successful identification of contaminated food products very difficult. In addition, contamination often occurs during the slaughtering processes, where formerly healthy animals get cross-contaminated and are subsequently passed onto the next step of the food chain.

Factors favoring the development of zoonotic diseases are manifold. They include climate change, which promotes the occurrence and spread of vectors, movement of infected animals, changes in land use and livestock management practices as well as lack of public health services. Most important are the consequences resulting from the growth in world population and the increasing need for animal protein, which leads to intensive animal husbandry. Additionally, numbers of immunocompromised patients being more susceptible to zoonotic agents have increased over the last years (Palmer et al., 2013).

Intensive animal husbandry often involves large-scale use of antibiotics for growth promotion and mass prophylaxis (Graham et al., 2007). This overuse of antibiotics is a major contributor for the development of antibiotic resistance and favors the emergence and spread of resistant bacteria.

Antimicrobial resistance refers to the ability of microorganisms to withstand antimicrobial treatments (EFSA). It is a natural process of adaptation in bacterial evolution (WHO, 2012).

Mutation in DNA sequences and horizontal gene transfer give rise to resistant bacterial genotypes (Thomas and Nielsen, 2005; Enright, 2003).

A diverse range of antimicrobial resistance has been proven in foodborne pathogens such as *Campylobacter* spp. and *Salmonella* spp. – the two leading causes for bacterial gastroenteritis worldwide. The European Food safety Authority (EFSA) points out that further transfer of antimicrobial resistance will affect human health (EFSA Panel on Biological Hazards, 2008) and the WHO alerts in its report about "The Evolving Threat of Antimicrobial Resistance" that a crisis has been building up over decades, so that today many common and life-threatening infections are becoming difficult or even impossible to treat, sometimes turning a common infection into a life-threatening one (WHO, 2012).

Identifying new targets to combat bacterial pathogens is necessary to treat infectious diseases appropriately in the near future. The identification of such targets depends on a broad and widespread understanding of the molecular pathogenicity of the disease. Molecular interactions between the pathogen and the host that occur upon infection underlie many signaling events, which favor the process of disease. In turn, they are potential targets for alternative combatting strategies.

The contribution to a broader knowledge of the molecular pathogenesis and the pathogen-host interaction of the new emerging zoonotic organism *Arcobacter butzleri* (*A. butzleri),* causing foodborne diseases are the fundament of this work.

1.2. Arcobacter spp., A. butzleri

Arcobacter spp. are gram negative, spiral shaped, motile bacteria with one unsheathed single polar flagellum at one or both ends of the cell. They were first isolated from aborted bovine fetuses in 1977 (Ellis et al., 1977) and assigned to the genus *Campylobacter* (Neill et al., 1979). A new genus *Arcobacter* was proposed by Vandamme et al. in 1991 containing aerotolerant *Campylobacter*-like-organisms that can grow microaerobically or aerobically, which is one feature that distincts *Arcobacte*r spp. from *Campylobacter* spp. (Vandamme et al., 1991). Another difference regarding cultivation conditions is the ability to grow at low temperatures (15°C).

To date, the genus *Arcobacter* spp. contains 17 species isolated from various origins and belongs to the family of *Campylobacteraceae* within the class of *Epsilonbacteria* (Figueras et al., 2014). Certain members of this family such as *C. jejuni* are known to be a major cause of infectious diarrhea worldwide (Young et al., 2007). Within the genus *Arcobacter* spp., *A. butzleri* is reported to be the predominant one associated with disease. Contaminated and

undercooked meat products, mainly poultry and pork, as well as polluted water constitute the main source of infection (Collado and Figueras, 2011; Rivas et al., 2004; Vandenberg et al., 2004). In farm animals such as cattle and pigs, cases of mastitis, abortion and gastrointestinal diseases were reported. Nevertheless, *Arcobacter* spp. have also frequently been isolated from asymptomatic animals (Collado and Figueras, 2011; Van Driessche et al. 2004). In poultry, different *Arcobacter* species (mainly *A. butzleri*, *A. cryoaerophilus* and *A. skirrowii*) have been isolated from chicken, ducks, turkeys and geese carcasses and intestines (Atabay et al., 2006; Aydin et al., 2007, Atabay et al.; 2008) but without any association to clinical symptoms.

Watery and persistent diarrhea as well as recurrent abdominal cramps have been described as typical characteristics of human infection (Vandenberg et al., 2004, Vandamme et al., 1992). Additionally, there are single reports of peritonitis and bacteremia (Lau et al., 2002; On et al., 1995). However, the role of *A. butzleri* in the pathogenesis of bacterial gastroenteritis is still not clear and due to the lack of routine diagnostic and standardized isolation and identification methods, the incidence of *A. butzleri* associated diseases cannot be properly evaluated (Vandenberg et al., 2004; Collado and Figueras, 2011). The prevalence of *Arcobacter* spp. among diarrheic patients was investigated in different studies throughout the world and was reviewed by Figueras et al. It ranged from 0.1-13%, with *A. butzleri* being the most prevalent one, and highly depended on the identification method (0.1-1.25% with culturing methods, 0.4-13% with PCR techniques) (Figueras et al., 2014). Houf and Stephan examined the presence and characteristics of *Arcobacter* spp. in feces of asymptomatic humans in Switzerland and found *A. cryoaerophilus* to be the only *Arcobacter* species present (1.4% of the samples), *A. butzleri* was not isolated (Houf and Stephan, 2007). Similarly, Vandenberg et al. reported that *A. butzleri* was isolated more often from diarrheic stool samples than from non-diarrheic patients (Vandenberg et al., 2004). These prevalence studies support the classification of *A. butzleri* as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

In 2007, Miller et al. published the whole genome sequence of an *A. butzleri* human clinical strain and revealed the presence of putative virulence factors such as invasion or fibronectin binding proteins as well as a surprisingly high level of antibiotic resistance genes (Miller et al., 2007). But if and to what extent these virulence factors enhance *A. butzleris* pathogenicity still remains unknown. To unravel underlying mechanisms of host colonization, previous *in vitro* studies focused on the interaction of *A. butzleri* with intestinal epithelial cells and its ability to adhere to and invade host cells has been demonstrated by several authors (Karadas et al., 2013; Ho et al., 2007; Levican et al., 2013). Another study demonstrated that

A. butzleri induces epithelial barrier dysfunction by changes in tight junction proteins and induction of epithelial cell apoptosis, which are mechanisms that are consistent with a leak flux type of diarrhea in *A. butzleri* infection (Bücker et al., 2009) and indicate a high pathogenic potential. Further evidence of pathogenicity has been given by proving cytotoxicity and induction of inflammation in epithelial cells mediated by IL-8 (Villarruel-López et al., 2003; Ho et al., 2007) even though *A. butzleri* does not possess any known gene for the cytolethal-distending toxin which is found in *C. jejuni* and is known to produce cytotoxicity on host cells by directly damaging eukaryotic DNA (Miller et al., 2007; Lee et al., 2003; Jinadasa et al., 2011). As indicated above, the pathogenic potential of *A. butzleri* has been mainly studied using epithelial cells. However, little is known about the reaction of innate immune cells towards *A. butzleri* infection.

1.3. Innate immunity/early immune response to bacterial infection

Invasive microorganisms, which are able to overcome anatomic host barriers such as epithelial surfaces to enter the organism are immediately recognized by phagocytic and antigen-presenting cells of the innate immune system such as macrophages or dendritic cells. Upon infection, monocytes, which derive from hematopoetic stem cells are rapidly recruited to the tissue, where they differentiate into tissue macrophages. They are able to phagocyte and thereby eradicate pathogens but also play a role in connecting innate and adaptive immune response via antigen presentation. Their presence in almost all tissues (Murphy et al., 2012) and their different abilities make them crucial for successful elimination of invading pathogens. Therefore, macrophages have a fundamental role in the first line of defense during bacterial infection.

The process of pathogen recognition is mediated via pathogen-associated-molecular patterns (PAMPS) on the outer side of the microorganism and respective pattern-recognition receptors (PRRs) such as Toll-like-receptors (TLRs) found on the membranes and in the cytoplasm of host cells (Kawai and Akira, 2006). Human TLRs consist of 10 different members. TLR2 recognizes microbial lipopeptides supported by TLR1 and 6. TLR4 recognizes LPS and LOS and TLR5 is specific for microbial flagellin. The intracellular TLRs 3,7,8 and 9 sense microbial nucleic acids and are usually localized in intracellular compartments such as endosomes (Takeda and Akira, 2005).

The activation of a PRR is followed by downstream activation of different signaling events such as the MyD88 (myeloid differentiation primary response protein) pathway resulting in release of NFκB, a nuclear transcription factor for many cytokines. These cytokines have different functions in immune response related to cellular and humoral immunity. PRRs also

mediate phagocytosis by either recognizing PAMPs (e.g. Mannose receptor) or by detecting humoral components that opsonize pathogens before being recognized by host cell receptors. Opsonization may be unspecific such as opsonization with complement, which is recognized by complement receptors or it may be pathogen-specific with antibodies recognized by the Fc receptor (Aderem and Underhill, 1999). Activation of phagocytosis induces the rearrangement of the actin cytoskeleton of the host cell, which is followed by internalization of the pathogen. The intracellular bacteria-containing vacuole (phagosome) subsequently fuses with early and late endosomes and finally lysosomes to form the phagolysosome (Aderem and Underhill, 1999). This enclosed space enables the host cell to generate an acidic milieu, where additionally high levels of reactive oxygen species, reactive nitrogen intermediates and antimicrobial peptides are used to eliminate the residing bacteria (Diacovich and Gorvel, 2010).

Additionally, after phagocytosis of the microorganisms and digestion in the phagolysosome, macrophages present the antigen to CD4+ T-cells via Major Histocompatibility Complex II (MHCII) and thereby orchestrate the collaboration of innate and adaptive immune response (Murphy, 2012).

Hence, the undisturbed function of macrophages is of fundamental need for a successful elimination of developing infectious diseases. The sensitive signaling process involved in these first but mandatory defense processes is therefore a potential target for invading bacteria. Modulating macrophage signaling allows pathogens to evade the host's immune response and to establish an environment allowing further replication, which finally leads to manifestation of disease (Rosenberger and Finley, 2003).

1.4. Immunmodulation as survival mechanism of pathogens

Bacterial pathogens have coevolved with their hosts and therefore developed mechanisms of escaping host immune defense. Although infected cells apply several mechanisms to kill bacteria, pathogens have developed strategies to manipulate host defense mechanisms to survive and eventually replicate (Diacovich and Gorvel, 2010). Survival mechanisms are diverging and often depend on the specific evolutionary acquired lifestyle of the pathogen. For example, some pathogens are able to invade eukaryotic cells and replicate inside, whereas others prevent their own uptake by phagocytes (Diacovich and Gorvel, 2010; Moine and Abraham, 2004). Pathogens can also modulate innate immune responses by expressing protein effectors, which directly interfere with Toll-like-receptor signaling pathways (Diacovich and Gorvel, 2010), whereas other pathogens benefit from inflammation favoring bacterial dissemination from the site of infection towards manifesting systemic disease. Many

pathogens are also able to change the structure of their PAMPs to exhibit recognition of immune cells and thereby prevent eradication by the host. Best example for modulation of innate immunity as a survival mechanism constitutes *Mycobacterium tuberculosis,* which prevents the fusion of the phagosome with the lysosome after being phagocytized by macrophages (Hart et al., 1972). As a consequence, the increase of reactive oxygen is hampered and unables the macrophage to kill and digest the pathogen. The pathogen, in turn, established a niche to survive and replicate inside the host cell without being accessible for further eradication mechanisms of the immune system.

Another efficient way to attenuate host defense is modulation of the apoptotic machinery of mononuclear phagocytic cells. It is commonly known that different bacteria act in both ways and are either able to inhibit or induce apoptosis to enhance their virulence potential. Intracellular bacteria such as *M. tuberculosis* benefit from decreased host cell death (Häcker et al., 2006) whereas bacteria such as *Salmonella* spp. induce apoptosis of macrophages to impair phagocytosis and eradication by immune cells (Navarre and Zychlinsky, 2000).

Apoptosis, phagocytosis and TLR signaling in immune cells during bacterial infection are efficiently regulated by several effector molecules including microRNAs (miRNAs) (Sharbati et al., 2011; Moon et al., 2014; Naqvi et al., 2015). Identification of miRNAs expressed in response to infection contributes to a better understanding of underlying regulatory mechanisms and provides the basis for the development of alternative strategies to treat infectious diseases.

1.5. miRNAs

MiRNAs are small non-coding RNAs with a length of approximately 21 nucleotides. They are key regulators of post transcriptional gene expression by binding to a complementary sequence within a 3' untranslated region (UTR) of respective mRNAs leading either to mRNA degradation or inhibition of protein translation (Filipowicz et al., 2008). This process is commonly referred to as RNA silencing. Since the first identification of miRNAs in 1993 (Lee et al., 1993) hundreds to thousands of miRNAs have been discovered and it is currently estimated that miRNA regulate 60% of all protein coding genes in humans. MiRNAs are therefore involved in most biological processes (Jovanovic and Hengartner, 2006). Thus, dysregulation in many cases leads to diseases such as inflammatory and metabolic disorders or cancer. As a consequence the use of miRNAs as a diagnostic tool for diseases but also the therapeutic application has become a major focus of investigation.

The first step in miRNA biogenesis is generally performed by the RNA polymerase II which produces the primary miRNA (pri-miRNA), a stemloop containing imperfectly base-paired

parts. One pri-miRNA stem loop can contain sequences for various different miRNAs. Subsequent to transcription pri-miRNAs are processed into mature miRNAs through canonical or non-canonical biogenesis pathways (Li and Rana, 2014). During canonical biogenesis, the pri-miRNA is processed by the endonuclease Drosha to become a hairpin structured precursor miRNA (pre-miRNA), which generally consists of around 70 nucleotides with a 2 nucleotide 3' overhang. Drosha is part of a processing complex containing several proteins of which a protein with double-strand (ds) RNA binding capacities (DGCR8) plays another important role for the processing step. During non-canonical biogenesis pre-miRNAs are generated by mRNA splicing machinery and are not cleaved by Drosha. In both pathways, the transfer from the nucleus to the cytoplasm is mediated by Exportin 5 - a shuttle protein- which recognizes the 2 nucleotide overhang. In the cytoplasm the pre-miRNA is processed by another endonuclease called Dicer, which cleaves the precursor to produce a mature miRNA duplex with around 22 base pairs. The endonuclease Dicer again works in complex with a protein posessing dsRNA binding capacities called TRBP. TRBP is responsible for recruiting Argonaute proteins to the mature miRNA duplex and together with Dicer the RNA-induced silencing complex (RISC) is formed. Only one strand of the miRNA duplex is loaded into RISC while the other one degrades (Filipowicz et al., 2008; Bushati and Cohen, 2007).

MiRNAs ususally function via base-pairing with the 3' UTR of their target mRNA. One mRNA can be targeted by several miRNAs and one miRNA can target several different mRNAs. When the full range Watson-Crick base pairing occurs, miRNA-mRNA interaction results in mRNA degradation by Argonaute protein-mediated endonucleolytical cleavage right in the middle of the miRNA-mRNA duplex (Filipowicz et al., 2008). This occurs most often in plants. However, according to the current understanding, most mammalian miRNAs do not bind their target mRNA perfectly which results in repression of protein translation and deadenylation often leading to subsequent decapping and decay of the targeted mRNA. The fact that one mRNA can be targeted by several miRNAs and one miRNA can target several different mRNAs, would give rise to unlimited possibilities of interaction. Fortunately, miRNA binding to its target usually follows a set of canonical rules (Filipowicz et al., 2008; Bartel, 2009) facilitating prediction of binding partners with bioinformatic tools.

Most important for miRNA binding to its binding site is perfect base pairing of miRNA nucleotides 2 to 8. This region is commonly referred to as the "seed" region (Filipowicz et al., 2008). The middle region of the miRNA-mRNA duplex consists of mismatches forming a bulge region. The 3' end of a miRNA usually binds with a good complementary across nucleotides 13-16 to ensure binding integrity of the duplex although cases of mismatches do occur. Binding integrity can be improved if the binding region is close to the poly(A) tail or the termination codon and through AU-enriched sequences. These factors are known to make

the mRNA binding site more accessible to recognition by the RNA-induced-silencing-complex. However, there are several exceptions to these canonical rules. It was recently shown that the binding of most miRNAs includes the 5′ seed region, but seed interactions are often non-canonical, containing bulged or mismatched nucleotides. Furthermore, some miRNA-mRNA interactions were shown to involve the miRNA 3′ end, with little evidence for 5′ contacts (Helwak, Kudla et al., 2013).

Usually, there is more than one binding site in the mRNA 3' UTR, which can function as binding partners for the same or different miRNAs.

In which step protein translation is inhibited by miRNAs still remains to be unraveled. There are controversial reports if the event occurs during the translation initiation or at a post-initiation step. MiRNA repressed mRNAs, Argonaute proteins and miRNAs accumulate in processing bodies (p-bodies), which occur as dynamic structures found in the cytoplasm. Although p-bodies are not necessarily required for mRNA repression, several proteins necessary for inhibition of translation and mRNA decapping are also found in these cytoplasmic formations. Since disruption of p-bodies has no influence on the degree of inhibition of translation it has been argued that the p-body formation may be a consequence of repression rather than its cause (Chu and Rana, 2006) and therefore might also serve as a storage site for translationally repressed mRNAs since miRNA mediated repression and p-body formation are reversibel (Bushati and Cohen, 2007).

MiRNA expression patterns are time-, stimulus- and tissue-specific. Similar to mRNAs, miRNA expression is controlled by transcription factors but can additionally be regulated post-transcriptionally, usually at the pre-miRNA stage or via RNA binding proteins (Staedel and Darfeuille, 2013). Concomitant to mRNA transcription factors, miRNAs control signaling pathways and cell function in most biological processes and in response to different stimuli. Dysregulation of miRNA expression and function can therefore have severe consequences and lead to infectious diseases, inflammatory and metabolic disorders, cardiovascular diseases or cancer.

1.5.1. miRNAs in infectious diseases

The role of miRNAs in infectious diseases has become more and more apparent and there are numerous reports about their involvement in viral and parasite infection. The contribution of miRNAs in bacterial diseases has been less explored. However, over the last years subsequent studies reported an increasing evidence of miRNA host response to bacterial infection (Eulalio et al., 2012; Staedel and Darfeuille, 2013).

First report about the role of miRNAs in bacterial infection was published in 2006 by Navarro et al. who observed an upregulation of the plants miR-393a induced by a flagellin derived peptide of *Pseudomonas syringae*. This miRNA represses auxin signaling which is a negative regulator of innate immunity thereby leading to resistance against the pathogen (Navarro et al., 2006).

Several studies ascribe miRNAs to play a major role in TLR signaling (Nahid et al., 2011; Taganov et al., 2006; O'Neill et al., 2011). A subset of miRNAs are described to be induced via TLR signaling in cells of innate immunity. However, there are subtle differences in miRNA expression profiles depending on the TLR stimulus, the treatment time and the cell type used for investigation (O'Neill et al., 2011). In turn, miRNAs are known to target TLR signaling pathways by regulating different components of the signaling cascades including TLRs themselves as well as downstream signaling proteins such as MyD88, transcription factors such as NFκB as well as cytokines. MiRNAs can thereby control strength, location and timing of TLR response (O'Neill et al., 2011).

Investigation of miRNA expression of human monocytes in response to stimulation with bacterial LPS indicated some miRNAs as common regulators of TLR4 mediated innate immune response (Taganov et al., 2006). Especially miR-155 and miR-146a/b were found to be upregulated in immune cells induced by bacterial infection. These miRNAs are known to be essential for fine-tuning the inflammatory cytokine production in NFκB signaling pathways (Staedel and Darfeuille, 2013; Eulalio et al., 2012) usually leading to a decline in inflammatory reaction.

C. jejuni was demonstrated to activate TLRs 2, 4 and 9 (Haag et al., 2012, Bereswill et al., 2011). Since *A. butzleri* also belongs to the family of *Campylobacteraceae,* a miRNA mediated regulation of downstream signaling of these TLRs during *Arcobacter* infection could be hypothesized. TLR5 activation by *A. butzleri* needs to be the matter of further investigations since *Epsilonproteobacteria* such as *Campylobacteraceae* were shown to escape TLR5 recognition although many of them possess flagellins, a TLR5-ligand in many bacterial species (de Zoete et al., 2010).

Besides cytokine production, phagocytosis is another macrophage function of particular importance in terms of pathogen clearance which is known to be controlled in a miRNA dependent manner during bacterial infection. Moon et al. demonstrated that miR-15a/16 altered phagocytosis and bacterial clearance by targeting, at least partially, TLR4-associated pathways, subsequently affecting the survival of septic mice. Bacterial infection and/or bacterial-derived LPS enhanced the level of miR-15a/16 in bone marrow-derived macrophages. Overexpression of miR-15a/16 using miRNA mimics led to decreased phagocytosis and decreased generation of mitochondrial reactive oxygen species whereas

deletion of miR-15a/16 in myeloid cells significantly decreased the bacterial infection-associated mortality in sepsis mouse models. (Moon et al., 2014). In addition, miR-142-3p was shown to directly regulate protein kinase Cα, a key gene involved in phagocytosis (Naqvi et al., 2015).

Another example of the involvement of miRNAs during infectious diseases is the modulation of macrophage apoptosis. This has been shown by Sharbati and colleagues. They demonstrated that miR-29a and let-7e were specifically induced after mycobacterial infection of human macrophages. Validated targets of these miRNAs have been shown to be caspase 3 and 7. MiRNA expression therefore resulted in inhibition of apoptosis. Thus, this is an example where miRNA induction favours mycobacterial virulence since inhibition of cell death allows this intracellular pathogen to replicate and evade immune defense mechanisms (Sharbati et al., 2011).

Because of their mode of action miRNAs have become an interesting tool to modulate dysregulated mRNA expression to treat diseases (Li and Rana, 2014; van Rooij et al., 2012; Davidson and McCray, 2011) and first miRNA-based therapeutics are currently under investigation in clinical trials (Li and Rana, 2014). MiRNA therapeutics are based on supplementation (miRNA mimics) or the inhibition of a certain miRNA (antisense oligonucleotides, antagomirs) (Krutzfeldt et al., 2005; Krutzfeldt et al., 2007). The latter is the most widely used therapeutic approach (Broderick and Zamore, 2011; van Rooij et al., 2012, Li and Rana, 2014). The development of suitable RNA molecules as possible therapeutics faces some great challenges. Resistance to nucleases, high binding affinity to its anti-miR and efficient delivery to the tissue or cell of interest have to be assured. Many efforts have been made to address these needs and first promising results in pre-clinical and clinical trials have already been achieved (Li and Rana, 2014). As an example, sufficient therapeutics are still desperately needed in viral infections. Viruses are known to express miRNAs to alter the immune response of their host cells (Stern-Ginossar et al., 2007), employ miRNAs for maintaining latency (Umbach et al., 2008) or use cellular miRNAs to enhance their pathogenicity (Ellis-Connell et al., 2010). In case of Hepatitis C virus, the cellular and liver specific miR-122 is needed to facilitate replication of the viral RNA and constitutes therefore a potential target for antiviral intervention (Jopling et al., 2005). Hence, intravenous administration of a therapeutic inhibitor of miR-122 led to suppression of HCV viremia. Two weeks after application, viral titer of HCV was 400 and 200 times lower in serum and liver, respectively without any apparent liver toxicity and even improved liver pathology (Lanford et al., 2010).

In case of bacterial infections, miRNA based therapy could constitute a promising alternative to conventional medication and therefore a new possibility to counteract the rising antibiotic

resistance. However, the complex network of miRNA function also bares risks such as off targets effects e.g. due to modulation of miRNA function in non target tissue.

Fundament for the development of a safe therapeutic is therefore an extensive understanding of the multiple miRNA functions under different cellular conditions. As described for bacterial infections, miRNA expression often changes alongside immune reaction and is needed to protect the host against overwhelming inflammation. On the other hand, several pathogens such as *Mycobacteria* spp. are known to specifically modulate miRNA levels to enhance their virulent potential (Staedel and Darfeuille, 2013; Sharbati et al., 2011). Increasing the knowledge about miRNA expression in response to a specific pathogen contributes to a better understanding of regulative processes that occur upon infection but also provide a potential basis to target bacterial infections without antibiotics in the near future.

1.6. Aim of the study

Recent publications reported cases of severe gastroenteritis caused by *A. butzleri* but its relevance associated with disease still remains to be evaluated. The aim of this study was to broaden the knowledge about the impact of *A. butzleri* on human host cells. Apart from this work there is hardly any data investigating the influence of *Arcobacter* on innate immunity. Since phagocytic immune cells have a fundamental role in the first line of host defense during bacterial infection this study focused on *A. butzleris* interaction with human macrophages. The thesis therefore concentrated on two major aims:

The first investigations considered the inflammatory response, intracellular survival and modulation of host cell apoptosis as potential virulence mechanisms employed by different *A. butzleri* isolates. THP-1 cell line derived macrophages were used as an *in vitro* infection model. Inflammatory response was analyzed by quantification of mRNA of different cytokines typically produced during bacterial infections. Gentamycin protection assays were used to study the ability of 5 different *A. butzleri* strains to survive and resist the intracellular hostile environment of macrophages. Luminescence based caspase assays as well as immunostaining of active caspase 3 and TUNEL assays were employed to investigate the influence of *A. butzleri* on apoptotic processes in infected macrophages. Furthermore, a potential role of a certain host cell miRNA interacting with the initiator caspase 8 was analyzed by *in silico* prediction followed by reporter gene assays and mRNA- as well as miRNA expression analysis.

The second part of the study intended to deepen the investigation regarding underlying regulatory effects occurring in host cells upon infection. For that purpose, primary human

macrophages were isolated from three different donors and infected with the *A. butzleri* reference strain. Subsequent to infection RNA was isolated and miRNAome was studied by means of RNAseq.

Apart from a recent study presenting a global profile of innate immune response to *C. concisus* infection in differentiated THP-1 macrophages including the identification of 13 significantly regulated miRNAs (Kaakoush et al., 2015), there is no data of the miRNA response of human macrophages towards infection by members of the familiy of *Campylobacteraceae.* Therefore, data generated in this study broadens the knowledge about the role of miRNAs in infectious diseases and provides a basis for the development of alternative therapeutic strategies.

2. Materials & Methods

2.1. Bacterial strains and culture conditions

Employed bacterial strains are listed in table 1. *C. jejuni* was grown at 37°C, all *A. butzleri* strains were grown at 30°C on Mueller–Hinton blood agar for 48 h (MHB; Oxoid) before being transferred in Brucella Broth to grow an overnight liquid culture at 37°C and 30°C, respectively. This liquid culture was then adjusted to an OD_{600} of 0.01 and cultured for another 24 h (*A. butzleri*) and 48 h, respectively (*C. jejuni*). Microaerobic atmosphere (5% O_2, 10% CO_2) was generated using the Anoxomat system (Mart Microbiology). To prepare the bacteria for infection experiments, cultures were centrifuged, washed with PBS (Sigma-Aldrich) and resuspended in cell culture medium (RPMI + 10% FCS superior, both Biochrom). For quantification of bacteria the number of colony forming units was determined by plating serial dilutions on MHB agar plates which were incubated for 48 h at 30°C (*A. butzleri*) and 37°C, respectively (*C. jejuni*).

Table 1: Bacterial strains used in this study

Strain	*Source*	*supplier*
A. butzleri CCUG30485 (type strain)	Human isolate reference strain	Culture Collection University of Göteborg, Sweden
A. butzleri F0 also referred to as FR1, H2	Human isolate	NRZ *Helicobacter*, National Reference Centre for *Helicobacter pylori*; University Medical Center Freiburg, Germany
A. butzleri 88	Chicken meat isolate	Bavarian Health and Food Safety Authority, Oberschleißheim, Germany
A. butzleri 89	Chicken meat isolate	Bavarian Health and Food Safety Authority, Oberschleißheim, Germany
A. butzleri 94	Chicken meat isolate	Bavarian Health and Food Safety Authority, Oberschleißheim, Germany
A. butzleri 102	Pork isolate	Bavarian Health and Food Safety Authority, Oberschleißheim, Germany
C. jejuni 81-176		ATCC, # BAA-2151

2.2. Cell culture

2.2.1. Cell line THP-1

The monocytic cell line THP-1 (DSMZ ACC 16) was cultured in RPMI (Biochrom) supplemented with 10% FCS and Gentamycin (10 µg/ml) (Biochrom) at 37°C in a 5 % CO_2 humidified atmosphere. Cells were kept at a density of 0.1-1 x 10^6 cells /ml in a 75 cm² cell culture flask (Sarstedt) and split every 2 to 3 days 1:2 or 1:3, respectively. THP-1 cells were used up to passage 20.

Cell numbers were determined by mixing 50 µl of trypanblue (Biochrom) and 50 µl of cell suspension. After an incubation time of 1 min 10 µl of the trypanblue-cell-suspension was transferred in a Neubauer-cell-counting-chamber. The mean of 3 to 4 counted squares was calculated by 2 x 10^4 (equals 1 ml of cell suspension).

To perform infection experiments, cells were differentiated into macrophages by stimulation with phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) 48 h prior to infection. As a consequence, cells stopped the cellcycle, became adherent to cell culture plate surfaces and changed their morphology. For that purpose, cells were resolved in a 10 µM PMA-solution in RPMI and 10% FCS without antibiotics and 4 – 6 x 10^5 cells were seeded in a 6-well cell culture plate (TPP). After 6 h the stimulus was removed by washing the adherent cell-layer with PBS. Fresh RPMI + 10% FCS without antibiotics was provided for another 48 h before being used for infection experiments.

2.2.2. HeLa cells

HeLa cells, a human cervix adenocarcinoma cell line (ATCC, CCL-2TM), were cultivated at 37°C and 5 % CO_2 humidified atmosphere in RPMI and 10% FCS in 75 cm² cell culture flasks. Gentamycin was added at a concentration of 10 µg/ml. The media was changed every 2 to 3 days and cells were passaged when reached 90% of confluency using Accutase (PAA) at a concentration of 0.04 ml/cm². Cells were used for transfection experiments (2.8.2.2.) up to passage 20.

2.2.3. Primary cells: Isolation & cultivation of primary human macrophages

To isolate macrophages from human blood, buffy coats of healthy human donors were obtained at the German Red Cross in Berlin Wannsee. Sixty to 75 ml of buffy coat were diluted in 210 ml PBS containing 0.5% BSA (Sigma-Aldrich). Fifteen ml of Ficoll Paque Plus (GE Healthcare) were pipetted in a 50 ml Falcon tube, gently covered with 35 ml of the diluted buffy coat and centrifuged at 750 x *g* for 20 min at 20°C in a swingout-bucket rotor

without the brake. After centrifugation, 3 separate layers appeared with the mononuclear cell layer at the interphase. The upper layer was removed and the interphase layer was transferred to a new 50 ml Falcon tube which was filled up with BSA/PBS, mixed and centrifuged at 350 x *g* for 10 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 25 ml BSA/PBS and centrifuged for another 15 min at 160 x *g* at 4°C. Supernatant was again removed carefully, the cell pellet was resuspended in 25 ml BSA/PBS and centrifuged at 300 x *g* for 10 min at 4°C. Finally, the cell pellet was resolved in 10 ml Gibco macrophage SFM medium (Life Technologies) and combined with all generated cell pellets.

Cells were stained with trypanblue and counted in a Neubauer-counting-chamber. Subsequently, 5 x 10^7 cells were seeded in a 75 cm² cell culture flask in 10 ml Gibco medium for 1-2 h at 37°C and 5% CO_2 humidified atmosphere to enrich monocyte population by plastic adherence. After 1-2 h incubation, the supernatant (containing non-adherent cells) was discarded and adherent monocytes were washed 3 times with 15 ml pre-warmed PBS. Subsequently, 10 ml of Gibco SFM medium including Gentamycin and 50 ng/ml macrophage-colony stimulating factor (M-CSF, a cytokine which influences hematopoetic cells to differentiate into macrophages) (Pan Biotech) was added. Two days after isolation the adherent cell layer was washed 3 times with pre-warmed PBS and fresh Gibco macrophage SFM medium containing Gentamycin without M-CSF was provided for another 3 days.

To determine the percentage of CD14+ cells in the isolated cell population, fluorescence activated cell sorting (FACS) was performed using a FACSCalibur flow cytometer (Becton Dickinson GmbH). In the cytometer, one cell at a time passes a laser light. Detectors capture the fluorescence emitted from the fluorochrome-stained cells and detect the light scattered forward and to the side from the cells. Forward scattered light provides information about cell sizes and light scattered to the side correlates with cell density (e.g. granularity). Differences in size, scattering and respective fluorescence signals allow to distinguish cell populations.

For FACS analysis, the adherent cell layer was washed 3 times with pre-warmed PBS and cells were detached from cell culture flask surface by adding 3 ml Accutase (PAA) for 10 – 20 min. The reaction was stopped by adding another 3 ml RPMI including 10% FCS and cells were subsequently scraped off the flask surface using a sterile cell scraper. Cell-suspension was centrifuged at 200 x *g* for 5 min, resuspended in 200 µl RPMI + 10% FCS and divided in 2 sub-samples (each contained appr. 1 x 10^6 cells) off which one was incubated with a CD14 Antibody (UCH-M-1: sc-1182, Santa Cruz Biotechnology) for 20 min (1:100). The second sample remained untreated and served as a negative control to calculate unspecific binding of the secondary antibody. After incubation, cells were washed

with 2 ml RPMI and centrifuged at 450 x *g* for 5 min. Supernatant was removed and both cell samples were resuspended in 50 µl RPMI medium containing the secondary antibody (IgG2a Goat Anti-Mouse, PE labeled, Southern Biotech) (1:200) and incubated for 20 min at 4°C, protected from light. After incubation, samples were washed with 2 ml RPMI and centrifuged at 450 x *g* for 5 min. The supernatant was discarded and cells were resuspended in 300 µl RPMI and analyzed with the FACSCalibur flow cytometer and integrated software at the Institute of Animal Nutrition, Freie Universität Berlin.

If FACS analysis proved that the cell population contained more than 80% CD14+ cells, macrophages were seeded in 6-well plates and used for infection experiments. For that purpose, medium in the macrophage containing cell culture flasks was removed and the adherent macrophage cell layer was washed 3 times with PBS and treated with 2 – 3 ml Accutase for approximately 20 min at 37°C and 5% CO_2. Reaction was stopped by adding another 3 ml RPMI including 10% FCS, cells were scraped off flask surface using a sterile cell scraper and transferred to a Falcon tube. Cells were counted in a Neubauer-chamber as described above before cell-suspension was centrifuged at 80 x *g* for 10 min. Subsequently, supernatant was discarded and the cell pellet was resolved in a respective amount of fresh Gibco macrophage SFM media to seed 6 x 10^5 cells in 1.5 ml media/ 6 well in a cell culture plate. Cells were incubated for another 24 h at 37°C and 5% CO_2 before further experimental use. Monocytes were isolated from three different human donors and infection experiments were reproduced in 3 independent experimental set ups.

2.3. *In vitro* Infection experiments of primary human macrophages for RNAseq

Approximately 4 - 6 x 10^7 bacterial cells (*A. butzleri* reference strain CCUG30485, prepared as described above) were inoculated on 4 - 6 x 10^5 primary human macrophages (MOI =100) and incubated at 37°C and 5% CO_2. Non-infected (but Gentamycin treated) cells served as a negative control. After 3 h of infection, cells were washed three times with PBS and incubated with fresh media containing 300 µg/ml Gentamycin for another 2 h to remove remaining extracellular bacteria. Samples were taken 1 h, 5 h and 24 h after infection. For the 24 h time point, cells were treated with 20 µg/ml Gentamycin for the remaining incubation time. For RNA extraction, cells were washed 3 times with PBS, lysed with RNA Lysis Buffer (mirVANA, Life Technologies) and total RNA was isolated according to the manufacturer's instruction.

2.3.1. Quality control of isolated RNA

Quantity and quality of RNA was first determined by measuring absorbance at 260 and 280 nm with a Nano Drop 1000 spectrophotometer according to the manufacturer's instructions (Thermo Scientific). The ratio of absorbance at 260/280 nm was used to assess the protein and solvent contamination in RNA samples. Samples with a ratio of 1.8 to 2.0 were further analyzed for their RNA integrity with an Agilent 2100 BioAnalyzer and RNA 6000 Nano Kits (Agilent) according to the manufacturer's protocol. RNA with integrity value (RIN) above 9 was used for further investigation.

2.3.2. Deep sequencing of infected human macrophages and bioinformatic investigation of generated data

Deep sequencing of the collected RNA was performed at the Institute of Clinical Molecular Biology at Christian-Albrechts University Kiel, Germany using a HiSeq2000 device (Illumina). The raw data was processed for bioinformatic investigations at Saarland University, Chair for Clinical Bioinformatics, working group of Prof. Dr. Andreas Keller.

2.4. Infection experiments for mRNA determination (cytokines, caspase 8) by RT-qPCR

To reflect the ability of *A. butzleri* to induce the production of typical inflammatory cytokines, *A. butlzeri* liquid culture of three different strains (prepared as described above) was inoculated to infect 4 – 6 x 10^5 THP-1 derived macrophages at a MOI of approximately 100 for 2 h at 37°C and 5% CO_2. *C. jejuni*, a pathogen of the family *Campylobacteraceae* was used as an established stimulus to infect THP-1 derived macrophages, respectively.

For the investigation of mRNA expression levels of the apoptotic marker caspase 8, THP-1 cells were infected with the *A. butzleri* reference strain for 1 h, 5 h and 24 h as described above (2.3).

After incubation, cells were washed with PBS, lysed with RNA Lysis Buffer (miRVana Isolation Kit, Life Technologies) and total RNA was isolated according to the manufacturer's instruction. Total RNA was eluted in 35 to 45 µl elution buffer and stored at -80°C until further investigation. RNA concentration and quality was checked as described above. Infection experiments were reproduced three times to generate three biological replicates per treatment.

2.4.1. cDNA synthesis

Synthesis of cDNA from collected RNA was accomplished by DNAse digestion and reverse transcription reaction (Fermentas). A master mix was prepared for DNAse treatment of RNA samples (table 2). Same concentration of RNA was used to ensure intra-experimental comparability.

Table 2: Reaction set up for DNAse treatment

Component	*Volume per reaction*
10x Buffer + MgCl2	1 µl
RNAse-free DNAse	1 µl (1U)
H_2O	X
RNA	X (1 µg)
Total volume	10 µl

Samples were incubated in a thermocycler (Verity) for 30 min at 37°C before 1 µl of 25 mM EDTA was added and subsequently incubated for another 10 min at 65°C. The whole reaction volume was used as a template for reverse transcription.

Reverse transcription of DNase treated RNA (Fermentas):

1 µl of random hexamer (0.2 µg) were added to the DNAse treated RNA and incubated at 70°C for 5 min. Subsequently, samples were stored on ice and final compounds were added (table 3).

Table 3: Reaction set up for reverse transcription

Component	*Volume per reaction*	*Final concentration*
5 x RT-buffer	4 µl	1x
10 mM dNTP mix	2 µl	0.5 mM
H_20	1 µl	
MMLV-RT (200 U/µl)	1 µl	200 U

After a short centrifugation step, samples were incubated at 25°C for 10 min, at 42°C for 60 min and at 70°C for 10 min. After reverse transcription 80 µl of nuclease free H_2O was added, mixed, centrifuged and divided into two aliquots of 50 µl. cDNA samples were stored at -20°C or used directly for RT-qPCR.

2.4.2. RT-qPCR Primer establishment

Cytokines involved in *Campylobacteraceae*-infection and following inflammation processes in host cells (primarily macrophages) were selected according to literature. Primers were designed using the open software Primer 3 (Koressaar and Remm, 2007; Untergrasser et al., 2012) as well as the ncbi.com database and ordered lyophilized at metabion or Sigma-Aldrich. Primer sequences are listed in table 4. A 100 pmol/l - stock solution was prepared by diluting the lyophilisate in the respective volume Tris EDTA-buffer.

Table 4: Oligonucleotides used for RT-qPCR (Annealing temperature 60°C):

hsa Primer	*Sequence 5'-3' fw*	*Sequence 5'-3' rev*	*Product size bp*
IL-1α	ATCAGTACCTCACGGCTGCT	TGGGTATCTCAGGCATCTCC	189
IL-1β	TCCAGGGACAGGATATGGAG	TCTTTCAACACGCAGGACAG	133
IL-6	GAAAGCAGCAAAGAGGCACT	TTTTCACCAGGCAAGTCTCC	109
IL-8	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCCTT	196
IL-10	AATAAGGTTTCTCAAGGGGCT	AGAACCAAGACCCAGACATCAA	348
IL-12α	TCAGCAACATGCTCCAGAAG	TACTAAGGCACAGGGCCATC	234
IL-12β	GGACATCATCAAACCTGACC	AGGGAGAAGTAGGAATGTGG	123
TNFα	CCCTGAAAACAACCCTCAGA	AAGAGGCTGAGGAACAAGCA	217
iNOS	ACAAGCCTACCCCTCCAGAT	TCCCGTCAGTTGGTAGGTTC	158
Caspase 8	GACCACGACCTTTGAAGAGC	TCCTGTCCATCAGTGCCATA	180
TBP	CCACAGCTCTTCCACTCACA	GCGGTACAATCCCAGAACTC	136
B2M	GTGCTCGCGCTACTCTCTCT	GGATGGATGAAACCCAGACA	135
GAPDH	CCATCTTCCAGGAGCGAGAT	CTAAGCAGTTGGTGGTGCAG	249
β-ACT	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	234

All primers exhibited an annealing temperature of 60°C. For Primer validation, a cDNA pool was used to produce DNA products of the respective gene of interest by means of RT-qPCR (Step One plus). For that purpose a master mix was prepared (table 5).

Table 5: Reaction set up for RT-qPCR

Component	*Volume per reaction*	*Final concentration*
Forward primer	0.2 µl	1x
Reverse primer	0.2 µl	
Sensimix Hi ROX	5 µl	
H_20	3.6 µl	
Total volume	9 µl	

Per reaction, 9 µl of the mastermix and 1 µl of the cDNA pool template were pipetted and ran in 40 cycles with the initiation step at 95°C for 10 min, cycling at 95°C for 15 sec, 60°C (annealing) for 20 sec and 72°C for 20 sec. A meltcurve was generated at 95°C for 15 sec, starting at 60°C + 0.5°C for 1 min. The fluorescent signal was detected at 72°C.

The generated PCR product was transferred to an agarose gel (1.5%) including 0.01% Ethidium bromide (0.5 µg/ml) (Roth GmbH) and ran for 45 min at 80 V in TAE buffer (Roth GmbH). PCR products were cut out of the gel, purified with QIAquick Gel Extraction Kit (Qiagen) and used for investigation of primer efficiency. For that purpose, serial dilutions (1:10) of the eluted PCR products were prepared und used as a template. The purified PCR products were subsequently sequenced (GATC) to ensure product specificity. If efficiency proved to be around 100 % (+/- 10%), primers were used for RT-qPCR arrays.

2.4.3. RT-qPCR arrays

Stock solution (100 µM) of primers was diluted to obtain a 4 µM working solution and a mix of forward and reverse primer was prepared by combining both dilutions. A mastermix was prepared for each sample and control (table 6).

Table 6: Reaction set up for RT-qPCR arrays (for a full 96 well plate)

Component	*Volume per reaction* *9 µl*
Diluted cDNA	50 µl
Sensimix Hi Rox	260 µl
H_2O	158 µl
Total volume	468 µl

The mastermix (9 µl) was pipetted into the respective wells and 1 µl of forward-reverse-primer-mix was added per reaction. Three technical replicates were measured per sample. Water was used as a negative control.

Fourty cycles were run in the StepOne plus Cycler as described above.

2.5. Survival assays (Gentamycin protection assay)

To investigate the pathogens ability to survive the hostile environment of macrophages, the number of living intracellular bacteria 5 h and 22 h after macrophage infection was determined as described elsewhere with slight modifications (Hickey et al., 2005). THP-1

derived macrophages (4 x 10^5 cells per well) were infected at a MOI of 100 for 3 h at 37°C and 5% CO_2. After incubation, cells were washed three times with PBS and incubated with RPMI + 10% FCS containing 300 µg/ml Gentamycin for another 2 h to remove remaining extracellular bacteria. After this 5 h incubation time, cells were either lysed (by the addition of 1 ml 1% SDS in PBS for 10 min) or incubated for another 17 h with the addition of 20 µg/ml Gentamycin before lysis (22 h time point). Total numbers of living bacteria were determined by plating serial dilutions of respective lysates on MHB agar, which were incubated for 48 h at 30°C. THP-1 cell numbers were determined in parallel at the respective time points to finally calculate numbers of living intracellular bacteria per macrophage. For that purpose, infected THP-1 cells were detached from the cell culture plate with Accutase (PAA) and viable cells were stained with trypan blue (Biochrom) and counted using a c-chip Neubauer cell-counting-chamber (Roth GmbH). Each experiment was performed in triplicates and average bacterial and THP-1 cell numbers were calculated.

2.6. Motility assays

Strain-dependent variations in motility were investigated by using motility assays as described elsewhere with slight modifications (Lavrencic et al., 2012). Briefly, 1 µl of bacterial liquid culture (approximately 1 x 10^6 cells) of different *A. butzleri* isolates was inoculated on semisoft agar plates (Brucella broth with 0.4% agar) parallel to the *A. butzleri* type strain CCUG 30485 and incubated at 37°C under microaerobic conditions for 48 h. Swarming halos were measured and compared to the swarming halo of the parallel inoculated type strain.

2.7. Apoptosis

2.7.1. Caspase activity

To revise *A. butzleris* impact on apoptotic processes triggered via the extrinsic as well as the intrinsic apoptotic pathway, THP-1 derived macrophages were infected in a 96-well plate with five different isolates of *A. butzleri* (MOI=100) as well as heat killed culture of the type strain CCUG 30485 (60°C, 40 min) as described above (3 h of infection, followed by a treatment with 300 µg/ml Gentamycin for another 2 h and for the 24 h time point investigation an additional incubation time of 19 h with the supplementation of 20 µg Gentamycin per ml). Caspase 8 and caspase 3/7 activities were analyzed 5 h and 24 h after infection using the luminescence based Caspase glo 8 assay system and Caspase glo 3/7 assay system (Promega), respectively. Mitomycin C (Roche), a known inducer of apoptosis was used as a positive control at a concentration of 5 µg/ml. Non-infected cells served as a negative control.

For normalization of caspase activity, numbers of viable THP-1 were determined using Calcein AM (Sigma-Aldrich) as described elsewhere (Malhotra et al., 2012). For this purpose, a 4 mM Calcein-solution was prepared in cell culture medium and incubated for 30 min at 37°C and 5% CO_2 after rinsing the cell layer with PBS. Calcein AM is a cell-permeable dye. In live cells the non-fluorescent Calcein AM is converted to fluorescing Calcein after acetoxymethyl ester hydrolysis by eukaryotic intracellular esterases. After acquiring the Calcein fluorescence, the cell layer was washed three times with PBS before Caspase glo reagents were added and luminescence was determined using the FLUOstar OPTIMA (BMG Labtech).

In addition to acivity assays, activation of caspase 3 was demonstrated by staining infected cells with an antibody recognizing cleaved and therefore activated caspase 3 (Cell Signaling Technology, (Asp175) (5A1E) Rabbit mAb, #9664). For that purpose, THP-1 derived macrophages were seeded in 8-well chamber slides (Sarstedt) (2 x 10^5/ well) and infected with viable as well as heat killed *A. butzleri* (reference strain) as described above for 5 h and 24 h. LPS (1 µg/ml) (Sigma, *Salmonella enterica* serotype typhimurium), Mitomycin C (5 µg/ml) (Roche) and non-infected cells served as controls. Subsequent to infection, cells were stained with active caspase 3 antibody. 4′,6-Diamidin-2-phenylindol (DAPI, stains DNA) as well as phalloidin (binds F-actin) staining (Sigma-Aldrich, Phalloidin Atto 488) were used to visualize DNA and the eukaryotic cytoskeleton, respectively. For that purpose, infected cells were washed three times with PBS and fixed by adding 3% Formaldehyde (Roth) to each well and incubated for 15 min. After fixation cells were washed 3 times with PBS and permeabilized with 0.5% Triton X100 in PBS for 10 min. After permeabilization cells were washed with PBS and stained with caspase 3 antibody (1:400) and respective secondary antibody (1:200) (Thermo scientific, Goat-anti-Rabbit, #35561) according to the manufacturer's protocol. Subsequently, 250 µl Phalloidin solution was added per well for 20 min at room temperature. Cells were washed twice with PBS and DAPI was added and incubated for 5 min at room temperature. Wells were washed with PBS for 5 min on a shaker before well separation was removed and slides were mounted with 50% Glycerol in PBS and visualized with the fluorescent inverted microscope DMI6000 B (Leica).

2.7.2. mRNA expression caspase 8

For investigation of mRNA expression levels after *A. butzleri* infection, a timecourse experiment was performed and THP-1 derived macrophages were infected with the *A. butzleri* reference strain CCUG 30485 for 1 h, 5 h and 24 h as described above (2.3.). Total RNA was isolated as described above and cDNA synthesis and RT-qPCR were performed as described above. Caspase 8 primer was synthesized by Sigma-Aldrich.

2.7.3. TUNEL assay

Trevigen TACS 2 TdT-Fluor In Situ Apoptosis Detection Kit (R&D Systems) was used as an end-point apoptosis assay. To visualize DNA strand breaks, THP-1 derived macrophages were seeded in 8-well chamber slides (Sarstedt) (2 x 10^5/ well) and infected with viable and heat killed *A. butzleri* strain CCUG30485 for 24 h as described for the caspase activity assay. Non-infected cells served as a negative control. Mitomycin C treated cells (5 µg/ml) as well as nuclease treated cells (intra assay control, Trevigen) were used as positive controls. DNA strand breaks were labeled with modified nucleotides using an exogenous terminal transferase according to the manufacturer's protocol (Trevigen, R&D Systems) and visualized by means of a green FITC staining signal using the fluorescent fully automated inverted research microscope DMI6000 B (Leica).

2.8. miRNA expression

2.8.1. *In silico* prediction of miRNA candidates interacting with caspase 8 mRNA

A potential miRNA regulation of caspase 8 was investigated by *in silico* prediction of putative miRNA candidates using the mirmap software (http://mirmap.ezlab.org) (Vejnar and Zdobnov, 2012), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (Rehmsmeier et al., 2004) and mirbase software (www.mirbase.org) (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008). These tools calculate miRNA - mRNA binding interactions based on sequence complementarity as described in 1.1. RNAhybrid additionally includes calculation of thermodynamic features of the miRNA-mRNA interaction.

2.8.2. Reporter gene assays

2.8.2.1. Plasmid generation

Generation of reporter plasmids containing the human caspase 8 3' UTR target sites of miR-106a-5p was performed as described earlier (Sharbati et al., 2011) with slight modifications. For that purpose, the 2 identified (RNAhybrid) target sites of miR-106a-5p in the 3' UTR of caspase 8 as well as the mutagenized target sites were amplified with synthetic oligonucleotides and cloned in pTK-Gluc (NEB GmbH). For that purpose, the target sites were extended and both target sites were combined leaving 20 nucleotides as a spacer region in between the two target sites as shown in figure 1 A. Preparation of the reporter plasmid containing the mutagenized target sites is illustrated in figure 1B. The plasmid pTK-Gluc contained the coding sequence for *Gaussia* luciferase and a multiple cloning site. The sequence with the 2 target sites was ordered as synthetic DNA, denaturated at 90° C for 1

min and hybridization via stepwise reduction of temperature to 22°C in 10x NEB ligation buffer and ligated using the restriction enzymes *Not*I and *Xba*I. A total of 200 ng of linearized pTK-Gluc and 150 ng of the hybridized target site were ligated in 20 µl employing T4 DNAse Ligase (NEB GmbH) for 30 min at 22°C, followed by heat inactivation for 10 min at 65°C.

Transformation in *Escherichia (E.) coli* K12 was performed as described by Hanahan et al. (1983). *E. coli* was cultivated in LB liquid medium and positive clones harboring the caspase 8 target sites (or mutagenized target site, respectively) were selected through Ampicillin resistance via cultivation on LB agar plates (Roth) with 100 µg/ml Ampicillin (Sigma-Aldrich). Endotoxin free plasmids were purified with a plasmid preparation kit (Macherey Nagel GmbH).

A

```
extended target site 1:   gatttTCTACTTTATTAATTGTTTTGCACTTTTTtataagagct
extended target site 2:   cacgtatggtGGCTCATGTCTATAATCCCAGCACTTTGggagg
combined target sites, sense and antisense:
5' GGCCGCgatttTCTACTTTATTAATTGTTTTGCACTTTTTtataagagctcacgtatggtGGCTCATGTCTATAATCCCAGCACTTTGggagg '3
      3' ctaaaAGATGAAATAATTAACAAAACGTGAAAAAatattctcgagtgcataccaCCGAGTACAGATATTAGGGTCGTGAAACcctccAGATC '5
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B

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combined mutagenized target sites, sense and antisense:
5' GGCCGCgatttTCTACTTTATTAATTGTTTTcgcaaaaaTtataagagctcacgtatggtGGCTCATGTCTATAATCCCcgcaaaaaGggagg '3
      3' ctaaaAGATGAAATAATTAACAAAAgcgtttttAatattctcgagtgcataccaCCGAGTACAGATATTAGGGgcgtttttCcctccAGATC '5
```

Fig. 1 Reporter plasmids. (A) Preparation of caspase 8 3' UTR target sites for the reporter plasmid. Green: recognizing sequence for the restriction enzyme *Not*I and *Xba*I, red: extension of target sites, blue: target site 1**,** orange: spacer region in between target sites**,** black: target site 2 **(B)** Preparation of mutagenized caspase 8 3' UTR target sites sequence for the reporter plasmid.

2.8.2.2. Co-transfection of HeLa cells

HeLa cells were cultured as described earlier (2.2.1) and subcultured 3 days prior to transfection at a density of 2 - 3 x 10^3 cells/cm^2 in a 175 cm^2 flask to reach the optimal confluency of 70 - 80% for nucleofection. Amaxa Cell Line Nucleofector Kit R (Lonza) and Nucleofector program I-013 were used for transfection and electroporation according to the manufacturer's instruction. Cells were transfected with oligonucleotides as listed in table 7.

Table 7: Oligonucleotides used for transfection

The following combination of oligonucleotides were used to transfect 1x10^6 HeLa cells:

pTKGluc plasmid (1 µg) containing the caspase 8 target sites	• synthetic miR-106a-5p (50 pmol) (Ambion) • pTKCluc plasmid (150 ng) (New England Biolabs GmbH) containing *Cypridina* luciferase serving as a control reporter gene which was used for normalization
	• non-target siRNA (50 pmol) • pTKCluc plasmid
pTKGluc plasmid (1 µg) containing the mutagenized caspase 8 target site	• synthetic miR-106a-5p • pTKCluc plasmid
	• non-target siRNA • pTKCluc plasmid
pmaxGFP vector (2 µg) serving as a control for transfection efficiency	

Transfected cells were incubated in 1.5 ml RPMI+10 % FCS in a 6 well plate at 37°C and 5% CO_2 for 24 h before supernatant was taken to analyze luciferase activity of reporter genes.

2.8.2.3. Luminescence measurement

Luciferase activity of *Gaussia* as well as *Cypridina* luciferase was analyzed with the Biolux assay kit (New England Biolabs GmbH) in white 96-well plates (Greiner GmbH) using the FLUOstar Luminometer (BMG Labtech). To normalize the obtained data, luciferase of reporter plasmid (caspase 8, pTKGluc) was divided by luciferase activity of the control plasmid (pTKCluc).

2.8.3. miR-Q RT-qPCR: miRNA expression analysis

THP-1 cells were infected with *A. butzleri* for 1 h, 5 h and 24 h and total RNA was isolated as described above (2.3). Non-infected cells served as a negative control. MiR-Q assays as established by Sharbati-Tehrani et al. were used with slight modifications to quantify expression levels of miRNAs (Sharbati-Tehrani et al., 2008). MiR-Q PCR is a highly sensitive

reverse transcription PCR to analyze expression levels of small RNAs. Briefly, RNA was converted to cDNA by reverse transcription using a miRNA-specific RT-6 primer which has 6 complementary bases that matches the miRNA of interest at the 3' end and in parallel produces a 5' overhang. To quantify the miRNA by qPCR, a second miRNA-specific oligonucleotide (short-mir-rev-primer) was used, which hybridizes to a specific sequence at the 3' end of the generated cDNA. The use of additional oligonucleotides allowed the provision of another overhang. The final amplification was performed by two universal primers, which bind to the created overhangs.

2.8.3.1. miR-Q reverse transcription

Collected RNA was diluted to 50 ng/µl with nuclease free water and 2 µl (=100 ng) were incubated with 1 µl RT6-miR-106a primer and 3 µl of nuclease free water at 70°C for 5 min.

A Master mix was prepared for the following reactions as shown in table 8. Per sample, 4 µl were added and incubated at 37°C for 5 min, 42 °C for 120 min and 70°C for 10 min.

Table 8: Reaction set up for miR-Q reverse transcription

	Volume per reaction	*Final concentration*
5x RT buffer	2 µl	
dNTP Mix (10mM)	1 µl	1 mM
RT MMuLV (200 U/µl)	0.5 µl	100 U/ reaction
H_2O	0.5 µl	

To normalize the data, same conditions were used to generate SNORD47–specific cDNA.

2.8.3.2. miR-Q qPCR reaction

A Master mix was prepared (table 9) and 8 µl were incubated with 2 µl of the generated cDNA (2.8.3.1.) at hold 95°C for 2 min, cycling at 95°C for 15 sec, 60°C for 20 sec and 72°C for 20 sec, melt at 95°C for 15 sec starting at 60° + 0.5°C for 1 min. The fluorescent signal was detected at 72°C.

Table 9: Reaction set up for miR-Q qPCR

	Volume per reaction
short-miR-106a-rev (100 fmol/µl)	0.4 µl
MP primer fw (10 pmol/µl)	0.1 µl
MP primer rev (10 pmol/µl	0.1 µl
SYBR Green Hi-Rox Fast Enzyme	5 µl
H_2O	2.4 µl

The same assay conditions were used for SNORD47, a housekeeper to normalize the expression data of miR-106a.

Table 10: Oligonucleotides used for miR-Q experiments

miR-106a	
RT6-miR-106a 5' - 3'	TGTCAGGCAACCGTATTCACCGTGAGTGGTGCTACC
short miR-rev oligonucleotide sequence 5'–3'	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGAAAAG TGCTTACAGTGCAGGT
SNORD47	
RT6-SNORD47 5' – 3'	TGTCAGGCAACCGTATTCACCAACCTC
short miR-rev oligonucleotide sequence 5'–3'	CGTCAGATGTCCGAGTAGAGGAACCAATGATGTAATGAT TCTGC
Universal miR-Q oligonucleotides	
MP fw	TGTCAGGCAACCGTATTCACC
MP rev	CGTCAGATGTCCGAGTAGAGG

2.9. Validation of novel miRNAs in primary human macrophages

Three potential novel miRNA candidates were selected from the sequencing results to be analyzed for expression and differential regulation in primary human macrophages during *A. butzleri* infection.

For that purpose, oligonucleotides were designed and synthesized (Sigma-Aldrich) as described earlier (Sharbati-Tehrani et al., 2008) and miR-Q assay conditions were established using synthetic cDNA as a standard template, which was diluted from 2 fmol to 2 zmol. MiR-Q assays were performed as described above (2.8.3) using a temperature gradient from 58°C to 62°C to determine optimized annealing temperature for the mir-Q PCR reaction and to check primer efficiency.

To ensure specificity of amplification a melt curve was generated subsequently to amplification. Obtained miRNA products were transferred to an agarose gel (2 %) as described above.

For analysis of miRNA expression levels in infected macrophages compared to the non-infected controls, miR-Q assays were performed as described earlier using the established assay conditions with an annealing temperature of 62 °C for the miR-Q qPCR reaction. Parallel to amplification of the potential novel miRNAs, housekeeping genes SNORD44 and SNORD47 were amplified and used for normalization.

Table 11: Oligonucleotides used for validation of novel miRNAs

novel-miR-259	annealing temperature: 62°C
RT6-novel-miR-259 5' - 3'	tgtcaggcaaccgtattcaccgtgagtggtGCTAGA
short-novel-miR-259-rev 5' – 3'	cgtcagatgtccgagtagaggggaacggcgCTCTGACCTCTGACCCTCT
novel-miR-55	
RT6-novel-miR-55 5' – 3'	tgtcaggcaaccgtattcaccgtgagtggtTCTGCG
short-novel-miR-55-rev 5' – 3'	cgtcagatgtccgagtagaggggaacggcgAGGGCCTGCTCCCACCCCGC
novel-miR-134	
RT-6-novel-miR-134 5'-3'	tgtcaggcaaccgtattcaccgtgagtggtAACTCT
short-novel-miR-134-rev 5'-3'	cgtcagatgtccgagtagaggggaacggcgAAAAGCTGTCCACTGTAGA
cDNA sequences	
novel-miR-259 5' - 3'	tgtcaggcaaccgtattcaccgtgagtggtGCTAGAGGGTCAGAGGTCAGAG
novel-miR-55 5' – 3'	tgtcaggcaaccgtattcaccgtgagtggtTCTGCGGGGTGGGAGCAGGCCCT

2.10. Analysis of RT-qPCR and miR-Q

The ΔΔCT method (Pfaffl, 2001) was used to calculate the relative fold difference of the mRNA and miRNA expression levels compared to the negative control. To determine CT values thresholds were set at 0.1 or 0.2. To determine specificity of amplified PCR products a melt curve was generated and analyzed subsequent to amplification.

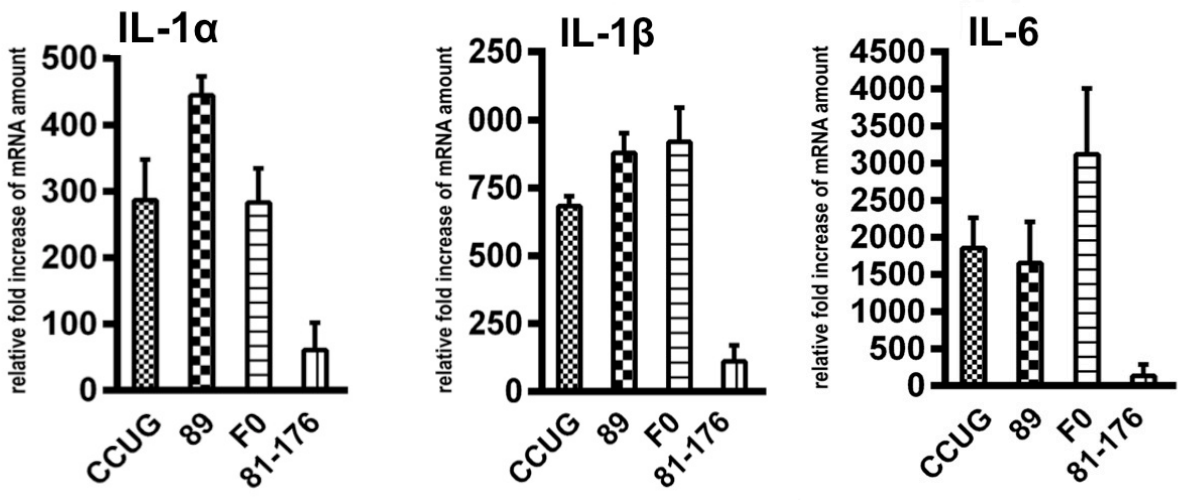
2.11. Statistical Analysis

Unpaired t-tests were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Asterisks in figures summarise p values (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

3. Results

3.1. *A. butzleri* infection induces a pro-inflammatory response in THP-1 derived macrophages

To evaluate if *A. butzleri* infection leads to induction of inflammatory cytokines, an array of 9 different cytokines typically produced during bacterial infections was established and mRNA expression was analyzed by means of RT-qPCR. Relative fold change of mRNA amounts was calculated compared to the negative control using the ΔΔCT method. The pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, IL-12β and TNFα were highly induced by all tested strains while the expression of iNOS and IL-12α remained unaffected (figure 2). The anti-inflammatory cytokine IL-10 was also induced but to a much lesser extent compared to the pro-inflammatory counter- parts (figure 2). Although cytokine expression levels in macrophages varied after infection with different strains – type strain (human isolate), F0 (human isolate), 89 (chicken isolate) – the overall cytokine profiles exhibited by macrophages in response to *A. butzleri* infection did not seem to be strain specific since no specific pattern of induction was observed. *C. jejuni* was used in parallel as a pathogenic control. Except for IL-12β, *C. jejuni* infection revealed induction of investigated cytokines, although to a lesser extent than infection with *A. butzleri* isolates did.



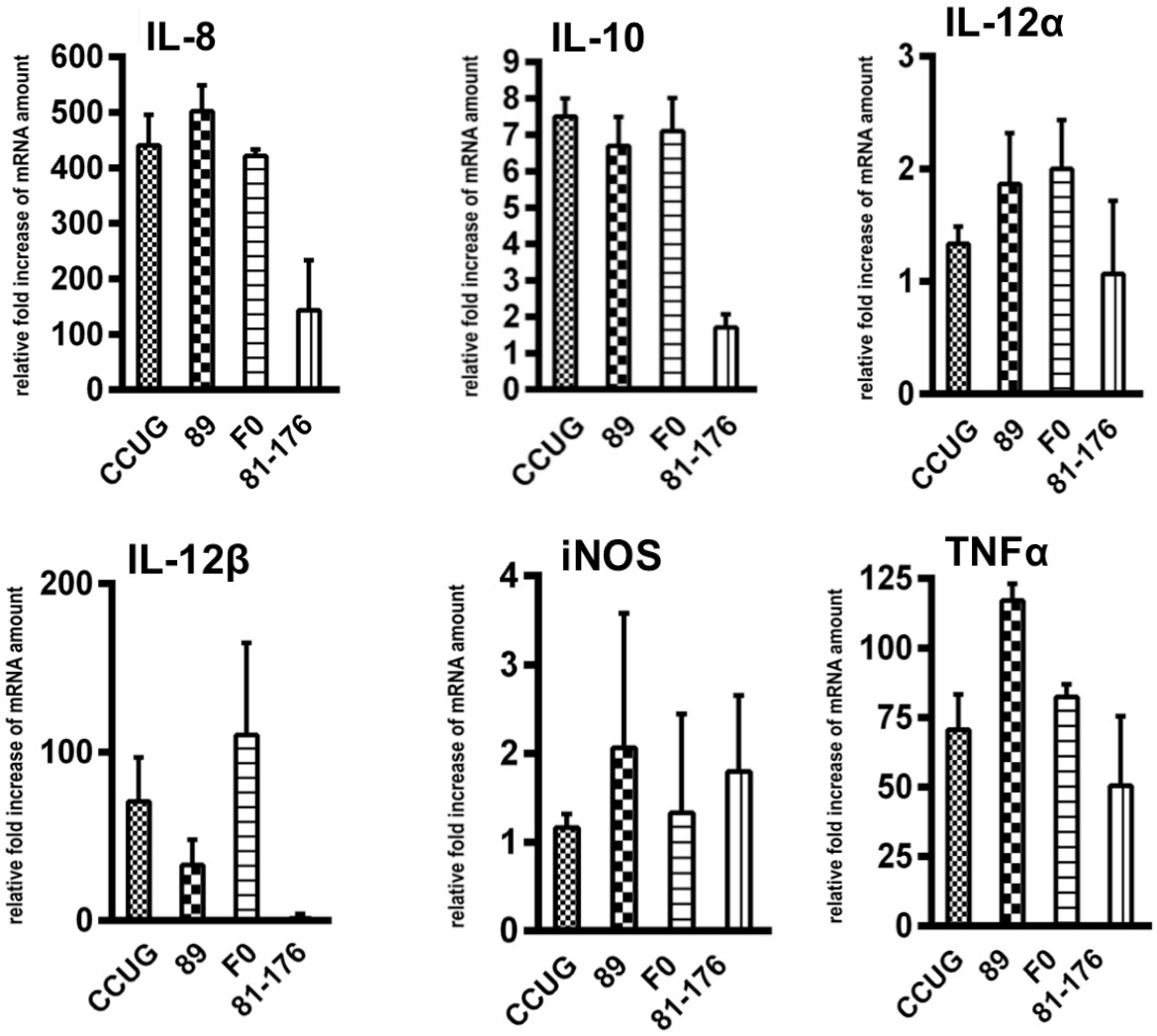
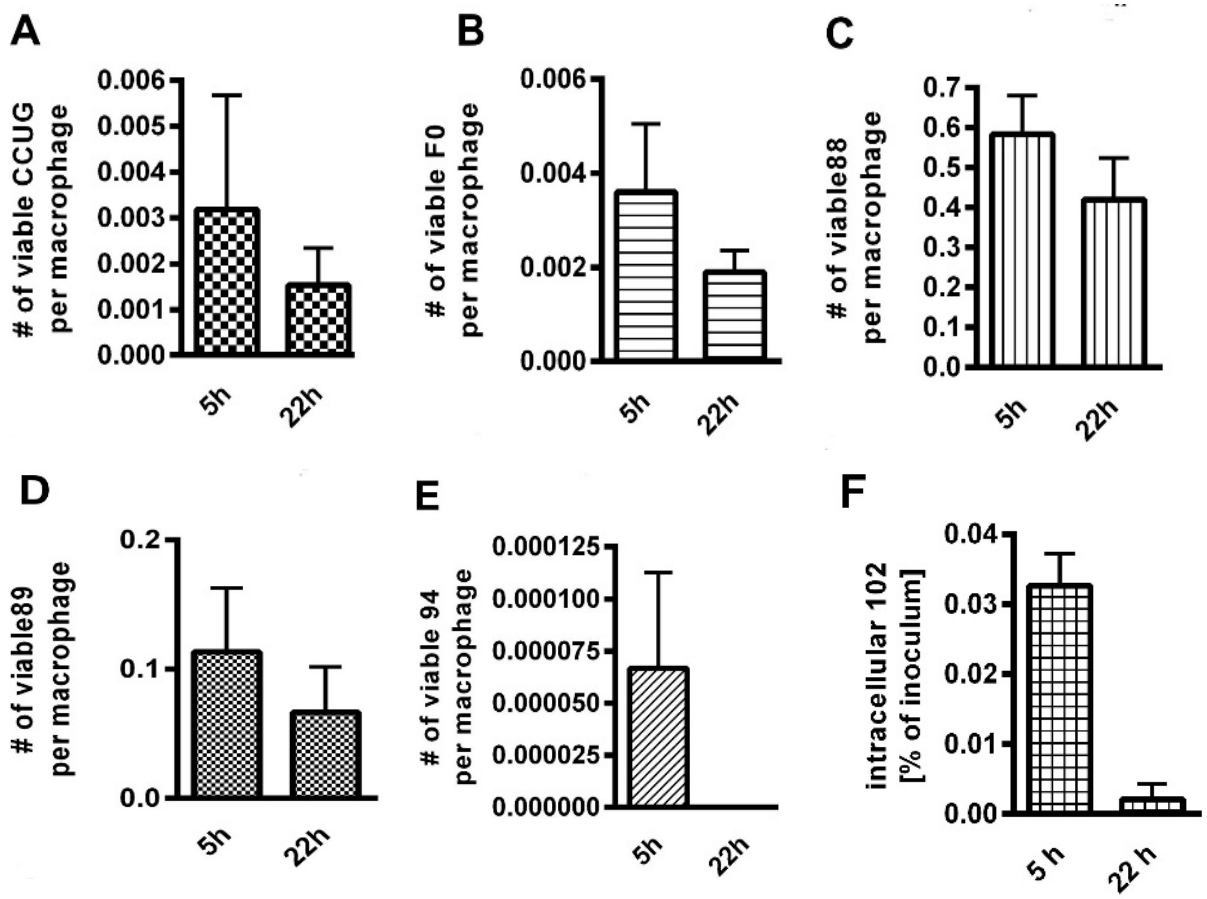


Fig. 2 Relative fold induction of mRNA amounts of cytokines expressed 2 h after infection of THP-1 derived macrophages. Shown figures reflect data from 3 independent infection experiments with *A. butzleri* strains 89 (chicken isolate), F0 (human iso- late) and the human type strain (CCUG) as well as a pathogenic representative of *Campylobacteraceae*, *C. jejuni* strain 81-176. Macrophages responded with high mRNA expression levels of pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, IL- 12β and TNFα but only moderate induction of the anti-inflammatory cytokine IL-10. Except for IL-12β *C. jejuni* infection also induced the expression of the investigated cytokines although to a smaller degree. Columns show the mean of 3 biological replicate measurements while bars indicate the SD. (Published in zur Bruegge et al., 2014)

3.2. *A. butzleri* is able to survive in macrophages for at least 22 h with distinct strain-dependent differences

To revise the ability of *Arcobacter* to escape host immune defense by surviving and replicating inside macrophages as described for *C. jejuni* (Kiehlbauch et al., 1985) and many other pathogens, a Gentamycin protection assay was performed. To deepen the investigations, strains 88 and 94 (isolated from chicken) as well as strain 102 (isolated from

pork) were additionally used. Except for isolate 94 all *A. butzleri* strains were able to survive up to 22 h in THP-1 derived macrophages although surviving intracellular bacteria were depleted to a small fraction of the inoculum (0.0015–0.42 living intracellular bacteria per macrophage of originally inoculated 100 bacterial cells per macrophage) (figure 3 A-F). Bacterial cell numbers of all strains decreased between the 5 h and 22 h time point with the highest survival rate for isolate 88 (figure 3 C). Most interestingly, tremendous differences in intracellular bacterial numbers per macrophage were observed at 5 h post infection among the different *A. butzleri* isolates (figure 3 G). Significant differences were found for isolates 88 (0.583 bacteria per macrophage, $p < 0.001$) and 102 (0.223 bacteria per macrophage, $p < 0.001$) followed by isolate 89 (0.113 bacteria per macrophage, $p < 0.01$) compared to the human type strain CCUG30485 (0.003 bacteria per macrophage).



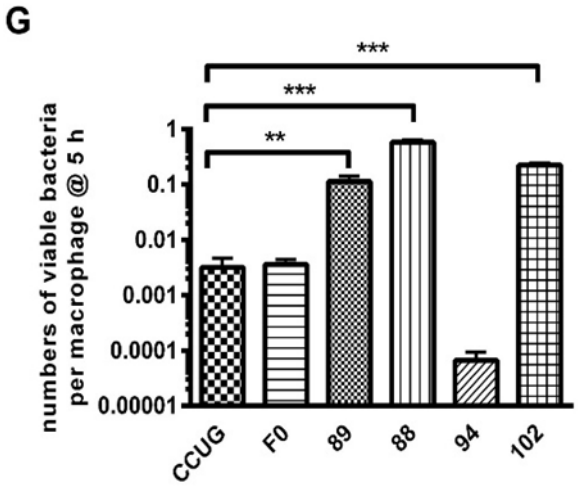


Fig. 3: *A. butzleri* are able to survive in THP-1 derived macrophages. (A-F) A Gentamycin protection assay revealed the ability of 5 out of 6 different *A. butzleri* isolates to survive inside macrophages for up to 22 h although bacteria were depleted to a minimal amount of originally inoculated cell numbers. Macrophages were lysed at 5 h and 22 h after infection and numbers of living bacteria per macrophage were determined. (C,D,F). Highest invasion and survival capabilities were achieved by chicken isolate 88 (0.583 bacteria per macrophage 5 h p.i.; 0.42 bacteria per macrophage 22 h p.i.) followed by pork isolate 102 and chicken isolate 89. (G) Most striking differences were observed among the isolates comparing bacterial cell numbers found intracellular 5 h after infection. Columns show the mean of 3 replicate measurements while bars indicate the SD (**: $p < 0.01$; ***: $p < 0.001$). (Published in zur Bruegge et al., 2014).

3.3. Invasion and survival capabilities might be correlated with strain-dependent differences in motility

Since *A. butzleri* possess two short flagellins (Ho et al., 2008) which could also play a role in colonization and invasion of host cells, *A. butzleri* strain specific differences in the invasion and survival ability might be correlated with variations in motility. Therefore motility assays were performed on semisoft agar plates to explain the vast differences in intracellular bacterial cell-numbers found in THP-1 derived macrophages at 5 h post infection. Swarming halos were measured and compared to swarming halos of in parallel inoculated type strain. After 48 h of cultivation on semi-soft agar plates at 37°C, isolates 102 (2.69 cm diameter swarming halo, $p < 0.001$), 89 (2.2 cm swarming halo, $p < 0.05$) and 88 (2.1 cm swarming halo) showed higher motility compared to the human type strain (1.93 cm swarming halo) (figure 4). The motility, especially of isolates 102 and 89, correlated with their higher capability to infect and invade macrophages 5 h after infection in the survival assays (figure 3 G). Accordingly, strain 94 possessed lowest motility rates (1.83 cm swarming halo) among tested strains being associated with lowest invasiveness and intracellular numbers (figures 3 G and 4).

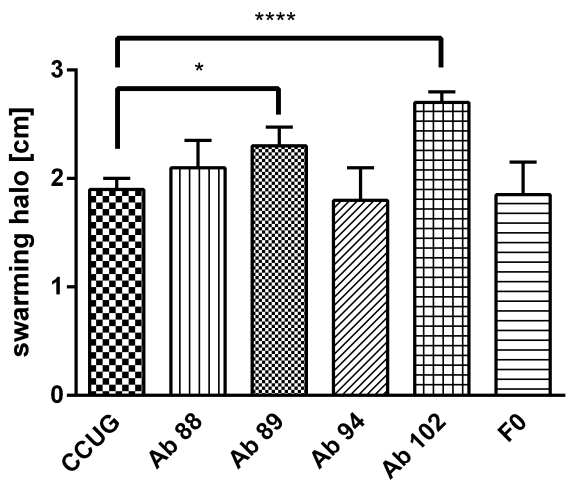
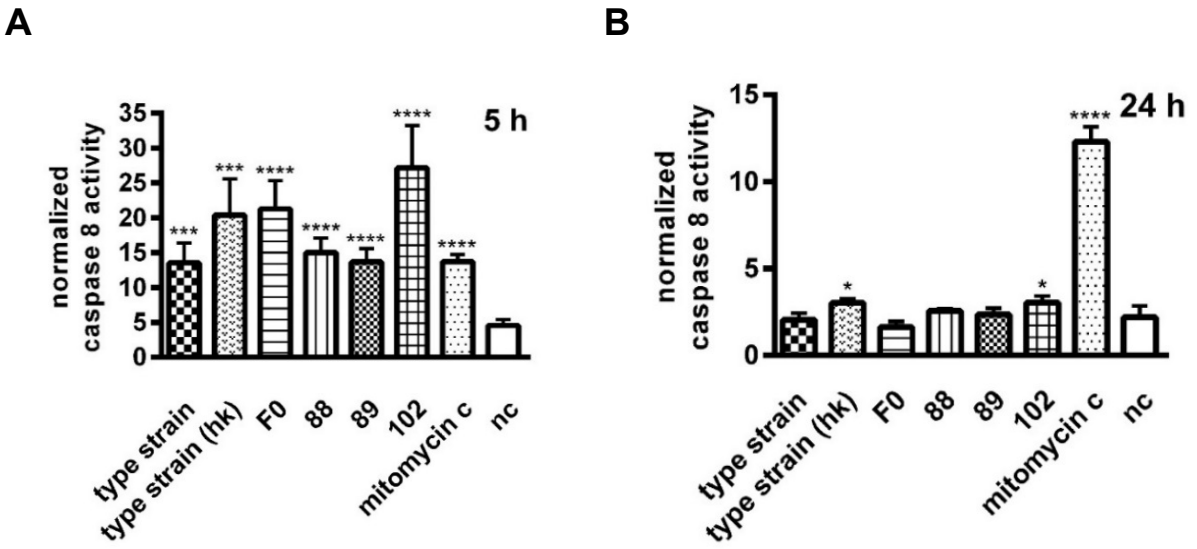


Fig. 4: Strains of *A. butzleri* exhibit differences in motility. Motility assays were performed on semisoft agar plates to correlate invasion and survival capabilities with strain-dependent differences in motility. Swarming halos of each strain were measured and compared with swarming halos of the parallel inoculated reference strain. After 48 h of cultivation on semi-soft agar plates at 37°C isolates 102, 89 and 88 showed higher motility compared with the human type strain whereas isolate 94 exhibited lower motility. Columns show the mean of 8 replicate measurements while bars indicate the SD (*: $p < 0.05$****: $p < 0.0001$). (Published in zur Bruegge et al., 2014)

3.4. *A. butzleri* induces caspase activity in early stages of infection without obvious initiation of apoptosis in THP-1 derived macrophages

3.4.1. Caspase 8 activity is increased 5 h but not 24 h after *A. butzleri* infection

The ability of *A. butzleri* to induce caspase 8 activation was analyzed at 5 h and 24 h after infection. As illustrated in figure 5 A the apoptotic marker caspase 8 showed increased and significant activity in early stages of infection caused by all strains as well as the heat killed type strain ($p < 0.001$). At 24 h after infection activity levels of caspase 8 dropped down to the level of non-infected cells (figure 5 B). Interestingly, and in contrast to increased caspase 8 activity, 5 h after infection the mRNA expression of caspase 8 was downregulated but rose up again to the basal level reaching levels of the negative control at 24 h after infection (figure 5 C) suggesting a counter regulation mechanism driven by macrophages.



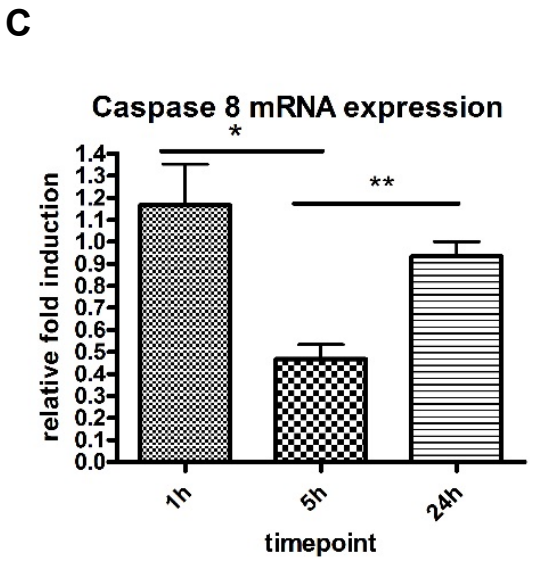


Fig. 5 Caspase 8. (A – B) All strains induced activity of caspase 8 five h after infection. The activity decreased to activity levels of non-infected cells 24 h after infection. Columns show the mean of 5 biological replicate measurements while bars indicate the SD. Significant differences were calculated relative to non-infected cells. (C) Infection of THP-1 derived macrophages with *A. butzleri* type strain and subsequent mRNA analysis of caspase 8 mRNA revealed a significant downregulation in the course of infection. 24h after infection mRNA level increased again and reached the level of non-infected cells (fold induction relative to negative-control). (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$; ****: $p < 0.0001$). (Published in zur Bruegge et al., 2014)

3.4.2. Caspase 3 and 7 activity is induced 5 h but not 24 h after infection

To determine whether *A. butzleri* initially induced caspase 8 activation leads to activation of executioner caspases 3 and 7 and finally macrophage apoptosis, caspase 3 and 7 activity assays were performed. Similar to caspase 8, activities of caspases 3 and 7 were significantly induced ($p < 0.01$) by all strains at 5 h compared to non-infected cells (nc) (figure 6 A) but were significantly ($p < 0.01$) lower than the activity of the negative control at 24 h after infection (figure 6 B).

Staining of cleaved and therefore activated caspase 3 confirmed the observations made in the caspase activity assay since active caspase 3 could be detected in viable and heat killed *A. butzleri* infected cells 5 h but not 24 h after infection whereas stimulation with Mitomycin C and LPS led to continuing cleaved caspase 3 signals (figure 6 C).

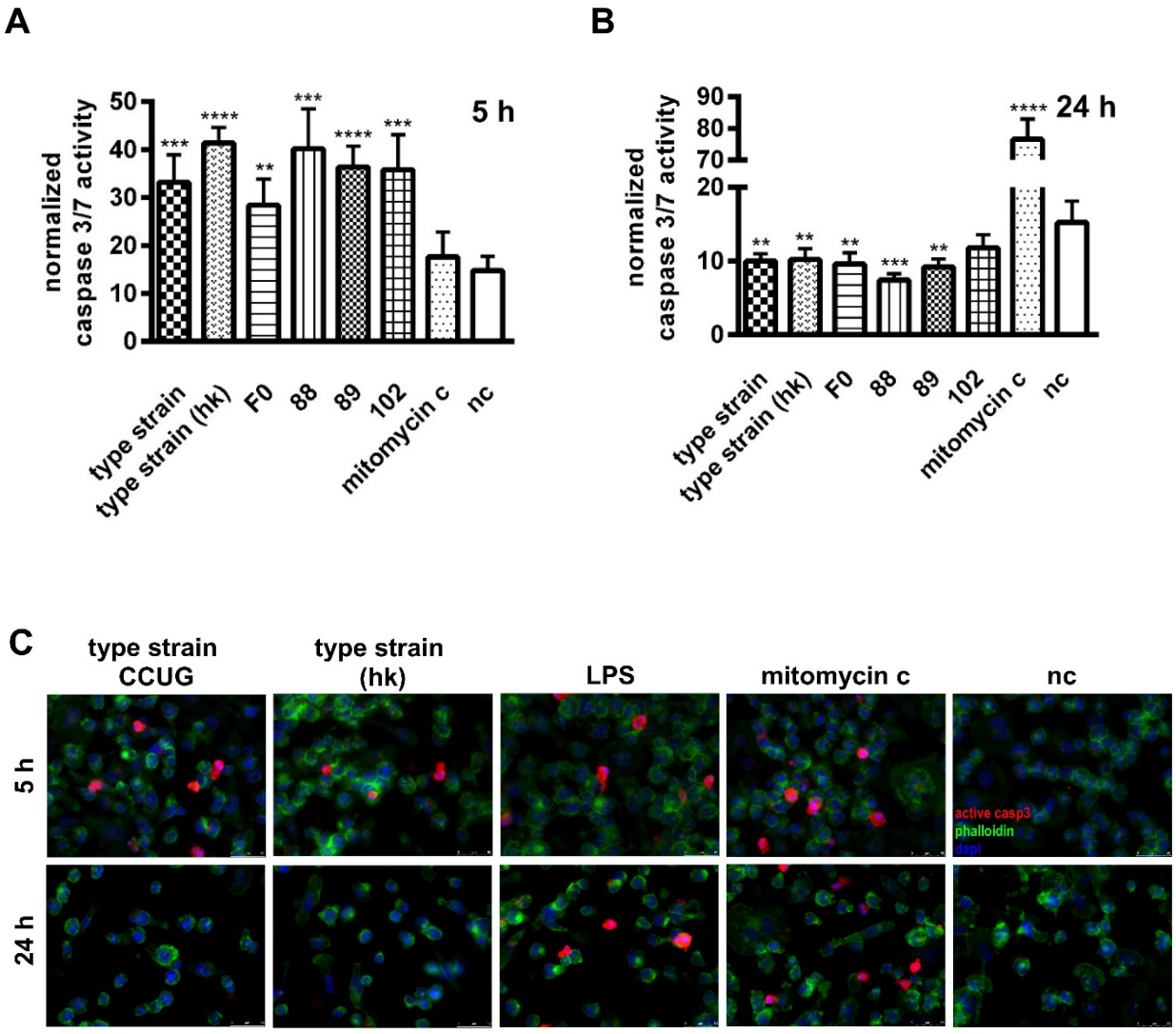


Fig. 6 Caspase 3 & 7. (A – B) All strains induced activity of caspases 3 and 7 at 5 h after infection. The activity declined to activity levels of non-infected cells 24 h after infection. Columns show the mean of 5 biological replicate measurements while bars indicate the SD. Significant differences were calculated relative to non-infected cells (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$; ****: $p < 0.0001$). (C) THP-1 cells were infected with viable and heat killed *A. butzleri* (reference strain) to detect cleaved caspase 3 with fluorescent antibody staining. A distinct signal for cleaved and therefore active caspase 3 (red) could be detected 5 h but not 24 h after infection with viable and heat killed *A. butzleri* whereas Mitomycin C and LPS treatment of cells resulted in continuous caspase 3 signal for up to 24 h. (green= phalloidin staining of eukaryotic cytoskeleton, blue=DAPI staining of DNA). (Published in zur Bruegge et al., 2014)

3.4.3. TUNEL assays do not indicate end-point apoptosis occuring in THP-1 derived macrophages 24 h after infection

Due to caspase activation at 5 h but not 24 h after infection and potential counter regulation mechanisms of host cells pointed out by the mentioned downregulation of caspase 8 mRNA, a TUNEL assay was performed to determine end-point apoptotic effects. For this purpose, viable as well as heat killed *A. butzleri* type strain were used as an example to follow up if

infection does finally lead to DNA damage in macrophages. Images in figure 7 do not reveal obvious apoptotic effects in response to *A. butzleri* 24 h after infection (viable and heat killed) compared to non-infected cells reflecting the previous observations regarding caspase activity in early but not later stages of infection.

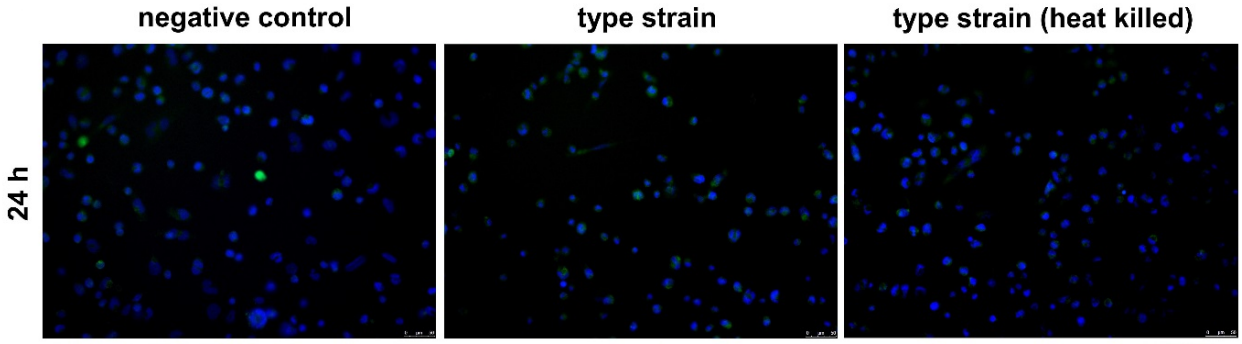


Fig. 7 TUNEL assays do not indicate obvious DNA strand breaks (green FITC staining co-localized with blue DAPI staining of cellular DNA) occurring at 24 h after infection with viable and heat killed *A. butzleri* type strain compared with the negative control. Scale bars indicate 50 µm. (Published in zur Bruegge et al., 2014).

3.5. miR-106a binds caspase 8 target sites in reporter gene assays but is not upregulated in *A. butzleri* infected THP-1 cells

Based on downregulation of caspase 8 mRNA expression observed in *A. butzleri* infected THP-1 derived macrophages (3.4.1.), a potential miRNA regulation was investigated by *in silico* prediction of possible miRNA candidates. Reporter gene assays and miR-Q assays were performed to validate the bioinformatic calculation.

For that purpose, the mirmap software (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008) was employed. Human caspase 8 mRNA was selected as a target and a search was performed. Search filter was set for miRNAs with a mirmap score above 95.

Beneath other miRNAs, mirmap revealed miR-106a-5p to be a potential candidate targeting caspase 8 mRNA and RNAhybrid identified 2 promising target sites within the 3' UTR of caspase 8 mRNA without G:U wobbles in the seed sequence (Helix constraint nucleotides 2-8) (figure 8).

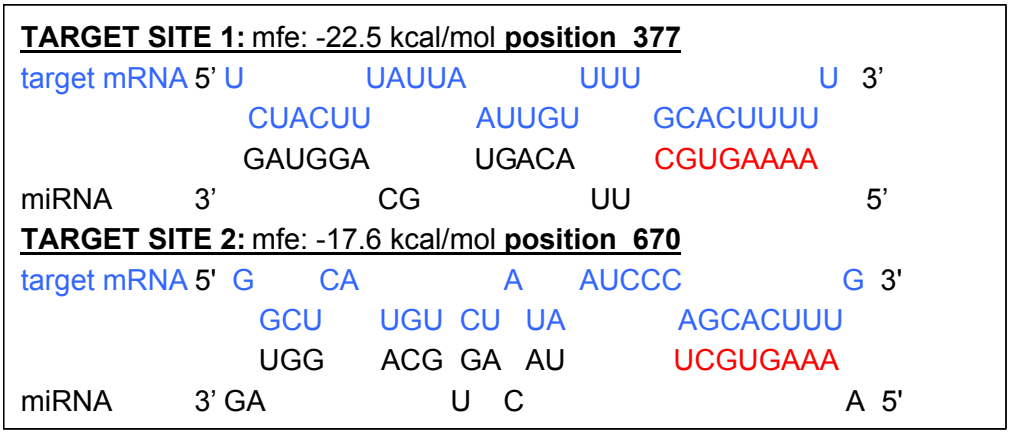


Fig. 8 RNAhybrid-predicted target sites within the 3'UTR of caspase 8 mRNA. (red= miRNA seed sequence, blue= target sites mRNA caspase 8)

In the next step reporter gene assays were performed to validate the predicted miRNA-mRNA interaction. For that purpose HeLa cells were co-transfected with the synthetic miR-106a-5p as well as a reporter plasmid (*Gaussia luciferase*, pTKGLuc) containing two caspase 8 target sites of miR-106a-5p. If miR-106a was binding to the caspase mRNA target sites reporter luciferase activity was downregulated. A plasmid containing the two mutagenized target sites as well as non-target siRNA served as controls. An additional co-transfected reporter plasmid (*Cypridina luciferase*, pTKCLuc) was used for normalization. The experiment was repeated three times and luciferase activity was measured in triplicates. The calculated mean of the luciferase activity of the mutagenized target site (mutCasp8GLuc) co-transfected with non-target siRNA (NT) was set at 100 % for calculation of downregulation of reporter activity in transfected cells. Figure 9 indicates a downregulation of reporter activity of caspase 8 target site (Casp8GLuc) and miR-106a co-transfected cells compared to all controls. Strongest downregulation (38.35%) of miR-106a transfected cells containing the caspase 8 target sites could be observed compared to miR-106a transfected cells containing the mutagenized target site (mutCasp8GLuc) ($p<0.001$) followed by 18.9% of downregulation for caspase 8 and miR-106a transfected cells compared to the mutagenized plasmid and non-target siRNA transfected cells ($p<0.05$).

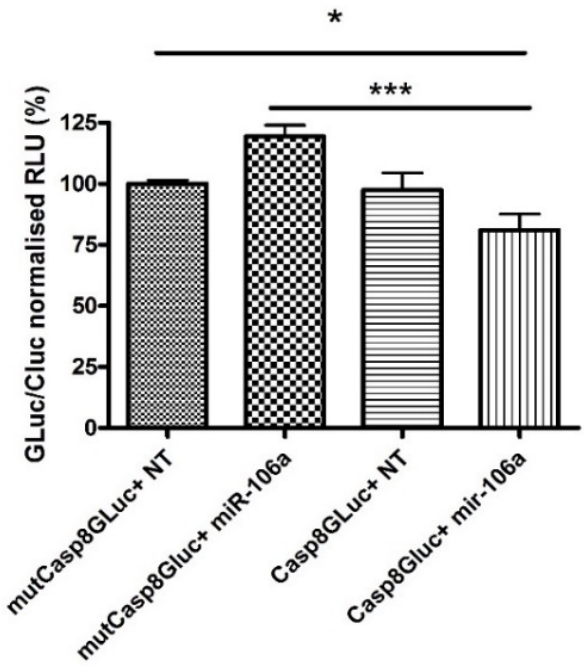


Fig. 9 Reporter gene assays were performed in HeLa cells to investigate the interaction of miR-106a and its two predicted targets sites in the 3' UTR of caspase 8 mRNA. Co-transfection of a Luciferase-plasmid containing these target sites as well as the synthetic miR-106a results in downregulation of luminescence compared to the control (plasmid containing mutagenized target sites of caspase 8 mRNA co-transfected with miR-106a). (*: $p < 0.05$; ***: $p < 0.001$)

Since binding of miR-106a to caspase 8 target sites and subsequent downregulation of luciferase activity could be demonstrated in the previous steps, miR-Q assays were performed to finally prove the expression and upregulation of miR-106a in THP-1 cell-derived macrophages upon *A. butzleri* infection. Therefore, cells were infected with *A. butzleri* type strain CCUG30485 and samples were taken 1 h, 5 h and 24 h after infection. Total RNA was isolated and miR-Q assays were performed as described above (2.8.3). Fold differences were calculated to respective negative controls (non-infected cells). As illustrated in figure 10, miR-106a could be detected in all samples. However, no differences in expression levels of infected cells compared to non-infected controls could be observed.

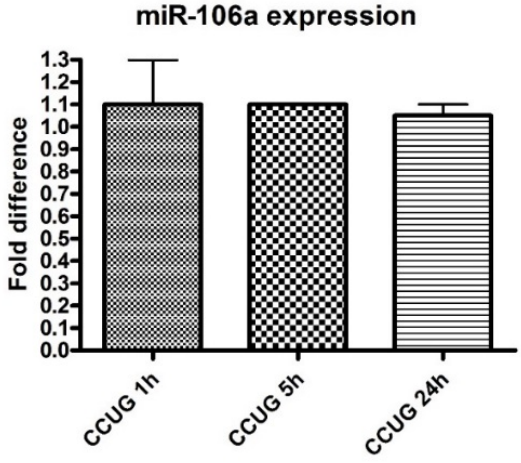


Fig. 10 Analysis of miR-106a by means of RT-qPCR (miR-Q). *A. butzleri* (reference strain) infected THP-1 cells did not display an upregulation in response to infection (fold difference to non-infected cells).

3.6. miRNA sequencing of macrophages

miRNAome of *A. butzleri* infected primary human macrophages was studied by means of RNAseq to identify differentially expressed miRNA candidates and potential mRNA targets. For that purpose primary macrophages were isolated from buffy coats from three different human donors and infected with *A. butzleri.*

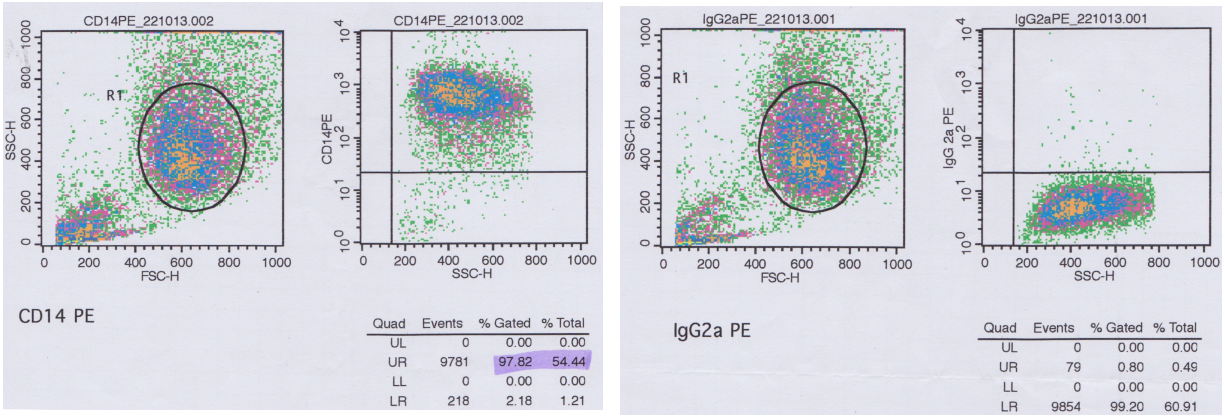
3.6.1. FACS analysis proves high efficiency of macrophage isolation and enrichment from human buffy coats

Since monocytes only represent a small fraction of leukocytes (2-6%), an enrichment step was necessary to obtain a high percentage of monocytes in the leukocyte cell composition.

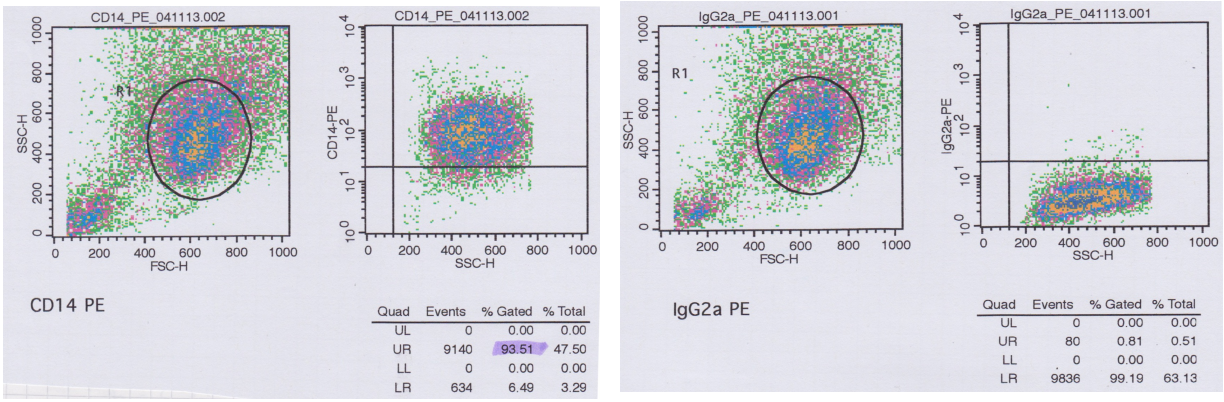
To establish an efficient protocol (low cost, high outcome) for the isolation of primary macrophages from buffy coats, three methods were compared and outcome of CD14+ cells were determined by Immuncytochemistry. Isolation techniques based on either density centrifugation or antibody crosslinking. The protocol for density centrifugation included subsequent adherence to cell culture flasks in a differentiation medium. The two other essayed protocols based on tetrameric antibody complexes that cross-linked non-desired cells to multiple red blood cells (Rosette Sep, StemCell Technologies) or to magnetic nanoparticles (Easy Sep, StemCell Technologies). Subsequent to the establishment of the technique, cells were cultured either in serum supplements (HL-1, Lonza or PAN NTA, Pan Biotech) or in serum free medium (Gibco serum free macrophage medium, Life Technologies) to avoid the use of FCS (undefined composition). To evaluate the best supplement, morphology was compared and cells were stained with life/dead staining. Density centrifugation and subsequent adherence to cell culture flask was the chosen method for further experimental use because of similar outcome of CD14+ cells comparing the three isolation techniques. Best macrophage morphology and cell viability was achieved using Gibco serum free macrophage medium.

Because of these pre-investigations, the final protocol to isolate primary macrophages from buffy coats was used as described in 2.2.3. Subsequent to cultivation, flow cytometry was performed to analyze CD14 expression, a cell surface marker for monocytes and macrophages, to reveal the purity of the isolated monocyte population. For that purpose, cells were stained with a CD14 antibody as well as a secondary fluorochrome labelled antibody and analyzed with a cytometer. The fluorochrome stained cells were gated and analyzed for CD14 antibody staining. Macrophage fraction in cell culture isolated and cultivated from Donor 1 constituted 97.02% (figure 11 A), from Donor 2 = 92.70% (figure 11 B) and Donor 3 = 81.0% (figure 11 C).

A



B



C

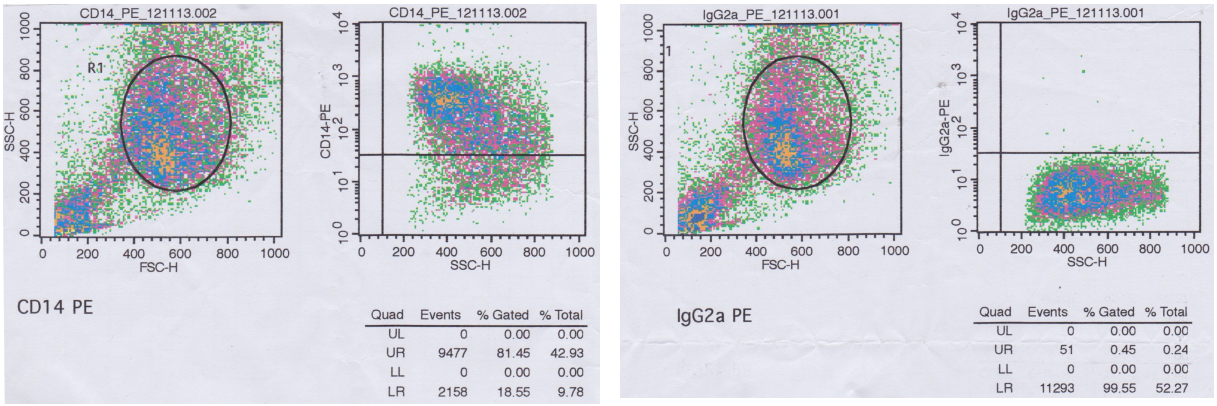
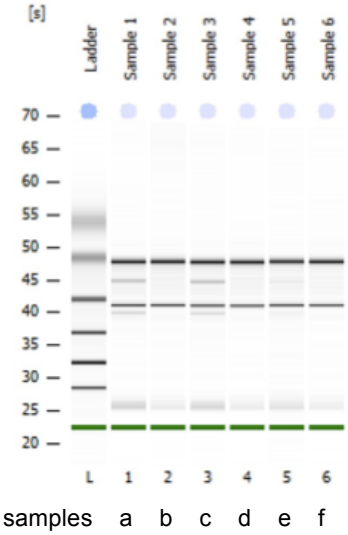


Fig. 11 (A) FACS Plot indicates 97.82% CD14+ of gated cells with 0.8% unspecific binding of the secondary antibody (IgG2a) isolated from Donor 1. **(B)** Isolated primary cells of Donor 2 yield 93.51% CD14+ cells minus 0.81% due to unspecific binding of the secondary antibody. **(C)** 81.45% CD14+ cells isolated from Donor 3 with 0.45% unspecific binding of the secondary antibody.

3.6.2. Isolated RNA exhibits high quality with RNA integrity numbers above 9

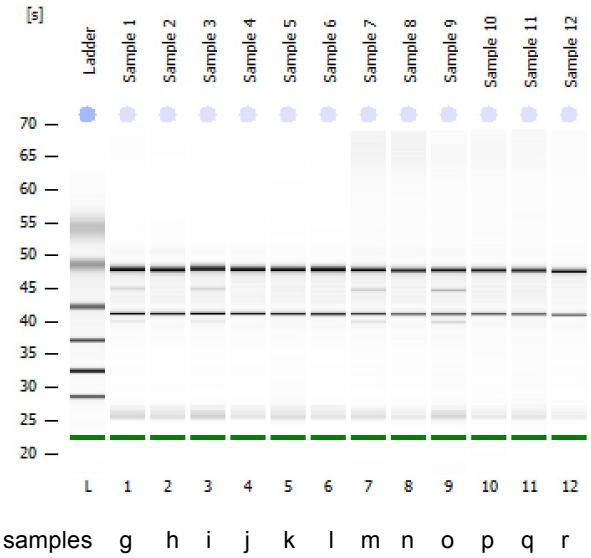
To investigate the miRNAome of infected macrophages, total RNA was collected from infected CD14+ cells and non-infected controls at three different time points (1 h, 5 h, 24 h) and RNA integrity was analyzed with the Agilent 2100 Bioanalyzer.

A



a – f: primary human macrophages donor 1
a: infected 1 h (RIN: 9)
b: non infected control 1 h (RIN 10)
c: infected 5 h (RIN 9.1)
d: non infected control 5 h (RIN 10)
e: infected 24 h (RIN 9.3)
f: non infected control 24 h (RIN 9.9)

B



g – l: primary human macrophages donor 2
g: infected 1 h (RIN 9.3)
h: non infected control 1 h (RIN 9.6)
i: infected 5 h (RIN 9.2)
j: non infected control 5 h (RIN 9.7)
k: infected 24 h (RIN 9.4)
l: non infected control 24 h (RIN 9.6)
m – r: primary human macrophages donor 3
m: infected 1 h (RIN 9.5)
n: non infected control 1 h (RIN 10)
o: infected 5 h (RIN 9.1)
p: non infected control 5 h (RIN 10)
q: infected 24 h (RIN 9.6)
r: non infected control 24 h (RIN 9.8)

Fig. 12 Bioanalyzer revealed all RNA samples to exhibit RIN numbers above 9. (A) Samples of Donor 1. (B) Samples of Donor 2 and 3.

Gel-like images of the electropherograms (figure 12) allow an evaluation of the RNA quality in different samples and furthermore calculates a RNA integrity number, considering the whole electropheric trace, with 1 standing for the most degraded RNA profile and 10 possessing highest integrity. All samples exhibited an RNA integrity number of 9 or above. Furthermore, ribosomal RNA (rRNA) bands (eukaryotic 28s and 18s subunits) can be

visually evaluated and should exhibit a 28s/18s ratio of ~ 2. The additional bands in samples a, c, g, i, m, o arose from prokaryotic rRNA in 1 h and 5 h infected cells (figure 12 A & B).

3.6.3. Sequencing and bioinformatic analysis reveals several miRNAs to be regulated upon *A. butzleri* infection

All RNA samples met the quality needs to be processed for RNA sequencing at the Institute of Clinical Molecular Biology at Christian-Albrechts University Kiel using an Illumina HiSeq2000 device. Analysis of the raw data was performed by Prof. Dr. Andreas Keller, Saarland University. The bioinformatic investigation involved raw data analysis, the investigation of expression levels of known human miRNAs and identification of potential novel (unknown) human miRNAs expressed by human macrophages in response to *A. butzleri* infection.

3.6.4. Annotated human miRNAs

An Anova analysis of the data obtained from the *A. butzleri* infected primary cells reflected several human miRNAs to be significantly regulated. An additional t-test displayed significant differences in expression levels among the different time points of infection and compared to the non-infected control (table 12).

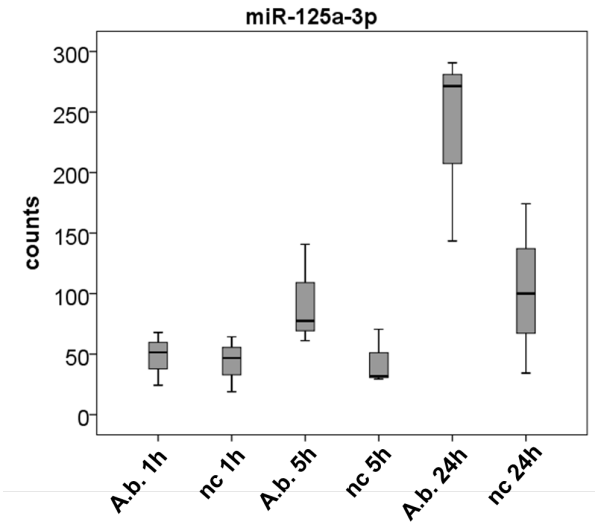
According to previous reports regarding regulatory functions of miRNAs, they could be grouped into miRNAs commonly affected by various bacterial agents and identified as key players in host innate immune response (figure 13) and those which have not yet been associated with bacterial diseases (figure 14). Following, differentially expressed miRNA in these two groups will be presented.

Table 12: miRNAs found to be regulated upon *A. butzleri* infection

miR	*sequence*	*p-values different time points of infection*
125a-3p	acaggugagguucuugggagcc	0.014 (1 h vs. 24 h), 0.035 (5 h vs. 24 h), 0.038 (24 h vs. nc)
146a-5p	ugagaacugaauuccauggguu	0.048 (1 h vs. 24 h)
155-3p	cuccuacauauuagcauuaaca	0.38 (1 h vs. 24 h), 0.038 (5 h vs. 24 h), 0.035 (5 h vs. nc)
155-5p	uuaaugcuaaucgugauaggggu	0.02 (1 h vs. 5 h), 0.041 (1 h vs. 24 h), 0.02 (5 h vs. nc)
212-3p	uaacagucuccagucacggcc	0.041 (1 h vs. 24 h), 0.034 (24 h vs. nc)
181b-5p	aacauucauugcugucgguggguu	0.015 (5 h vs. 24 h), 0.025 (1 h vs. 24 h)
181c-5p	aacauucaaccugucggugagu	0.030 (1 h vs. 5 h), 0.031 (5 h vs. 24 h), 0.034 (24 h vs. nc)
181d-5p	aacauucauuguugucgguggguu	0.003 (5 h vs. 24 h), 0.009 (1 h vs. 24 h)
21-3p	caacaccagucgaugggcugu	0.016 (5 h vs. 24 h), 0.021 (1 h vs 24 h), 0.012 (24 h vs. nc)
99b-3p	caagcucgugucuguggguccg	0.04 (5 h vs. 24 h), 0.037 (1 h vs. 24 h)
27a-5p	agggcuuagcugcuugugagca	0.049 (1 h vs. nc)
let-7a-3p	cuauacaaucuacugucuuuc	0.038 (1 h vs. 24 h), 0.05 (24 h vs. nc)
26b-5p	uucaaguaauucaggauaggu	0.049 (5 h vs. 24 h), 0.0008 (1 h vs. 24 h), 0.044 (1 h vs. nc), 0.048 (5 h vs. nc), 0.004 (24 h vs. nc)
148b-3p	ucagugcacuacagaacuuugu	0.026 (5 h vs. 24 h), 0.024 (1 h vs. 24 h), 0.004 (24 h vs. nc)
3613-5p	uguuguacuuuuuuuuuguuc	0.019 (1 h vs. 5 h), 0.018 (5 h vs. nc), 0.019 (24 h vs. nc)
590-3p	uaauuuuauguauaagcuagu	0.027 (5 h vs. 24 h)
941	cacccggcugugugcacaugugc	0.031 (5 h vs. 24 h), 0.008 (1 h vs. 24 h)
2116-3p	ccucccaugccaagaacuccc	0.025 (5 h vs. nc), 0.032 (24 h vs. nc)
671-3p	uccgguucucagggcuccacc	0.015 (1 h vs. 5 h), 0.017 (1 h vs. 24 h), 0.003 (5 h vs. nc), 0.009 (24 h vs. nc)
30d-3p	cuuucagucagauguuugcugc	0.033 (5 h vs. 24 h), 0.032 (1 h vs. 24 h), 0.038 (5 h vs. nc)
30d-5p	uguaaacaucccgacuggaag	0.025 (1 h vs. 24 h), 0.035 (24 h vs. nc)
339-5p	ucccuguccuccaggagcucacg	0.041 (1 h vs. 24 h)
629-5p	uggguuuacguugggagaacu	0.021 (5 h vs. 24 h), 0.03 (5 h vs. nc)
193a-5p	ugggucuuugcgggcgagauga	0.041 (5 h vs. 24 h)

3.6.4.1. miRNAs associated with bacterial infection

MiR-125a, miR-146a, miR-155, miR-212, miR-181b, miR-181c, miR-181d, miR-21, miR-99b, miR-27a, let-7a, miR-26b and miR-148b were differentially expressed in *A. butzleri* infected human macrophages and have previously been reported to play a role in host immune response towards bacterial infection (figure 13). Expression levels of miR-125a-3p were increased in *A. butzleri* infected cells 5 h and 24 h compared to respective controls. A continous increase could be observed over the time of infection although slightly elevated expression levels were also found in 24 h non-infected cells. Significant differences were calculated for expression levels 1 h versus 5 h of infection ($p < 0.05$) as well as 24 h versus respective negative control ($p < 0.05$). Expression of miR-146a-5p also increased over the time of infection ($p < 0.05$ for 1 h versus 24 h of infected cells), again elevated miRNA expression was also found in non-infected cells at 24 h. MiR-155-3p was significantly upregulated in infected macrophages 5 h post infection ($p < 0.05$ for 1 h vs. 5 h, 5 h vs. 24 h and 5 h vs. non-infected cells). 24 h after infection the expression level was similar to those of non-infected cells, whereas for miR-155-5p expression level at 24 h remained significantly elevated ($p < 0.05$ 1 h vs. 24 h, 1 h vs. 5 h, 5 h vs. non-infected). The expression level of neither miR-155-3p nor 5p was affected 1 h after infection. Overall miR-155-5p was induced to a higher degree compared to miR-155-3p. MiR-212-3p was increased 24 h post infection compared to the non-infected control as well as compared to the other time points of infection ($p < 0.05$ 1 h vs. 24 h, 24 h vs. non-infected control). Similar to miR-212-3p, miR-181b/c/d-5p as well as miR-21-3p and 99b-3p were found to be upregulated 24 h after *A. butzleri* infection compared to other time points of infection. In case of miR-181b and miR-181d, expression in 24 h non-infected cells was also increased. MiR-27a-5p showed an increased expression 1 h after infection compared to the non-infected control ($p < 0.05$). MiRNA let-7a-3p ($p < 0.05$ 1 h vs. 24 h , 24 h vs. nc) and miR-26b-5p decreased over the time of infection ($p < 0.05$ 1 h vs. nc, 5 h vs. nc, $p < 0.01$ 24 h vs. nc, $p < 0.001$ 1 h vs. 24 h) whereas miR-148b-3p exhibited reduced expression levels 24 h after infection ($p < 0.5$ 1 h vs. 24 h, 5 h vs. 24 h, $p < 0.01$ 24 h vs. nc), 1 h and 5 h after infection expression was not affected. Highest miRNA copy numbers were found for miR-146a and miR-155-5p.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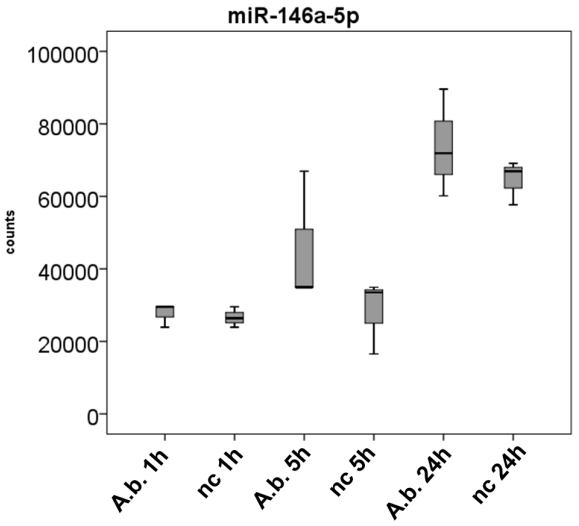
A.b. 24h
nc 24h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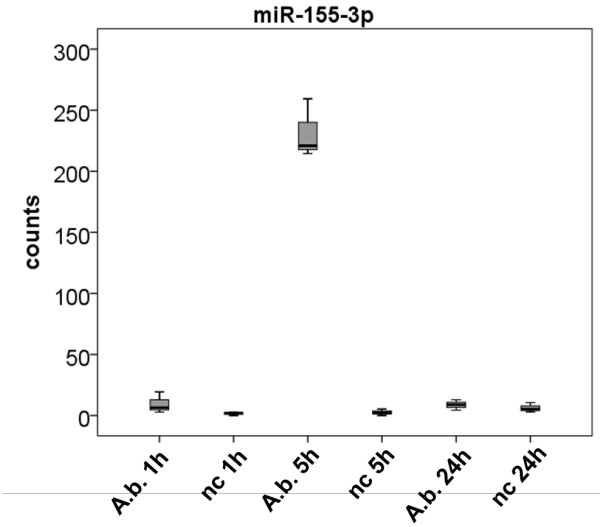
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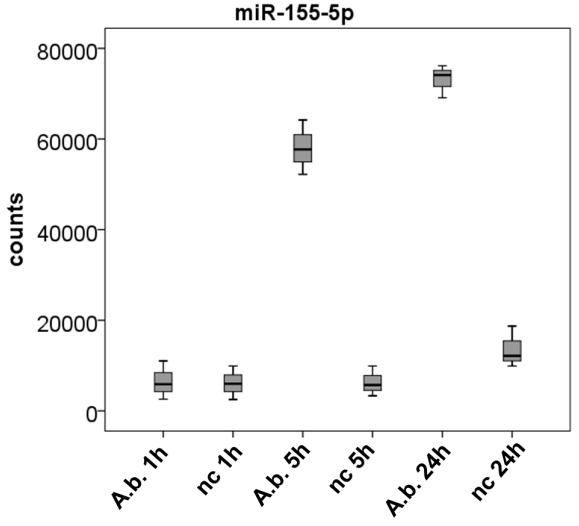
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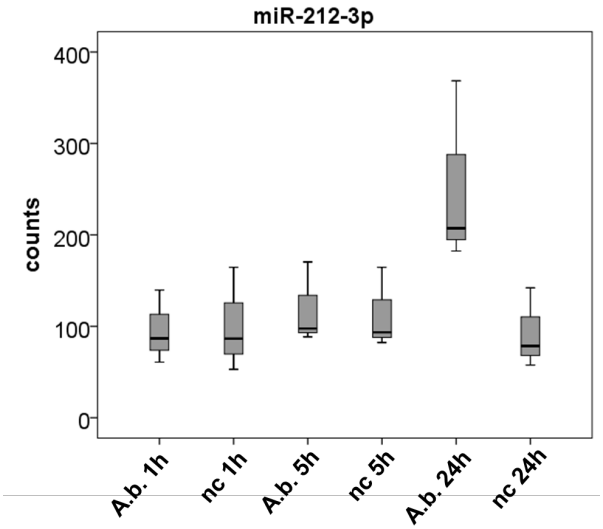
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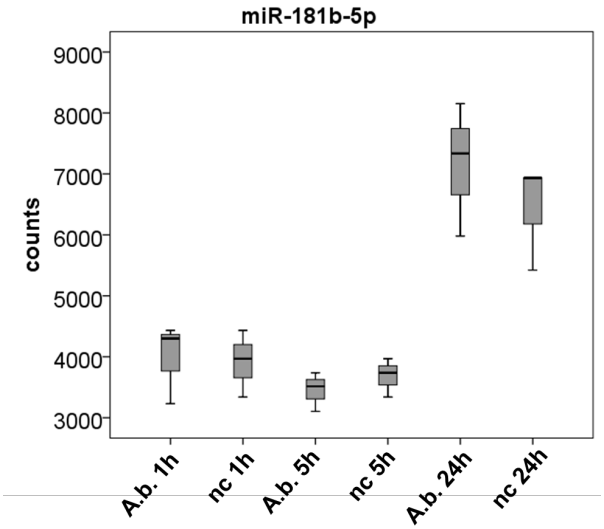
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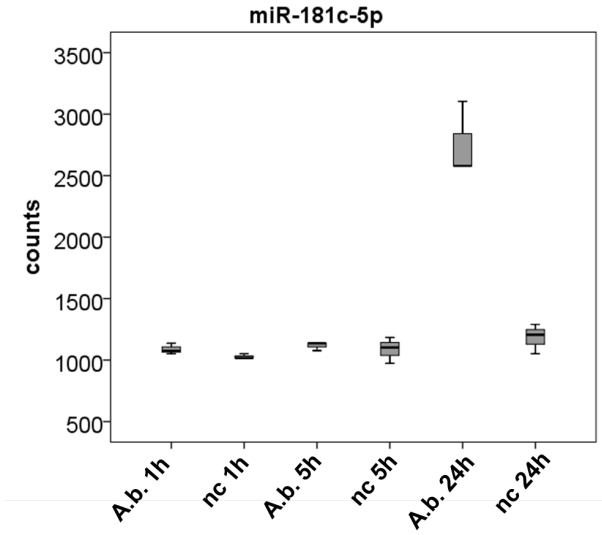
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A.b. 5h
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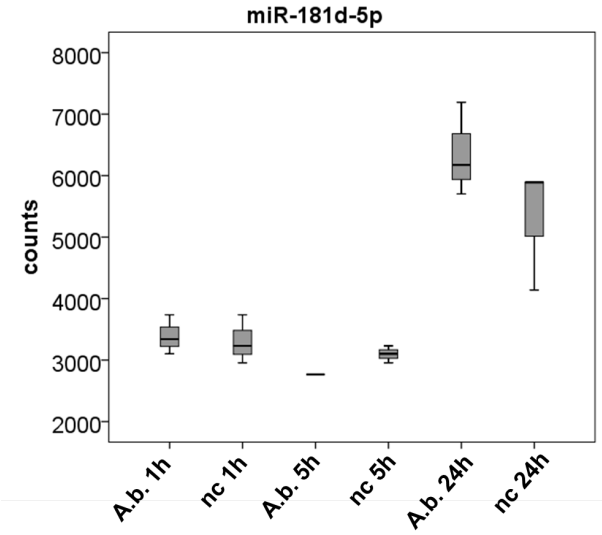
nc 24h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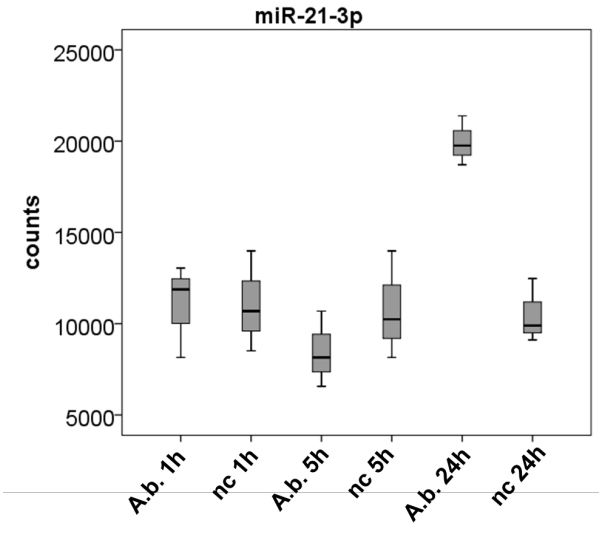
A.b. 24h
nc 24h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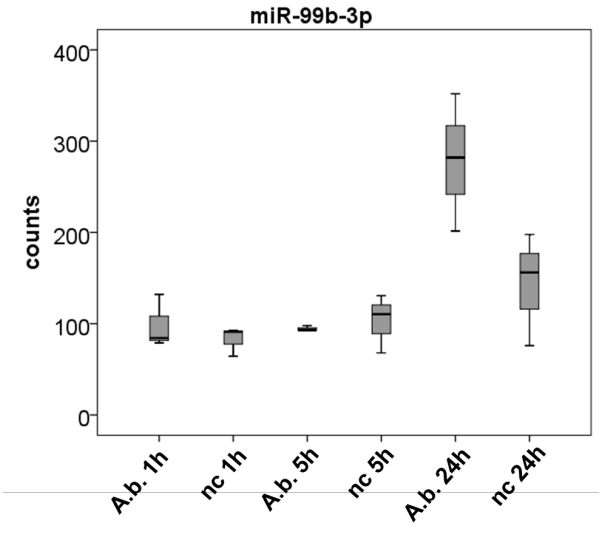
A.b. 5h
nc 5h
A.b. 24h
nc 24h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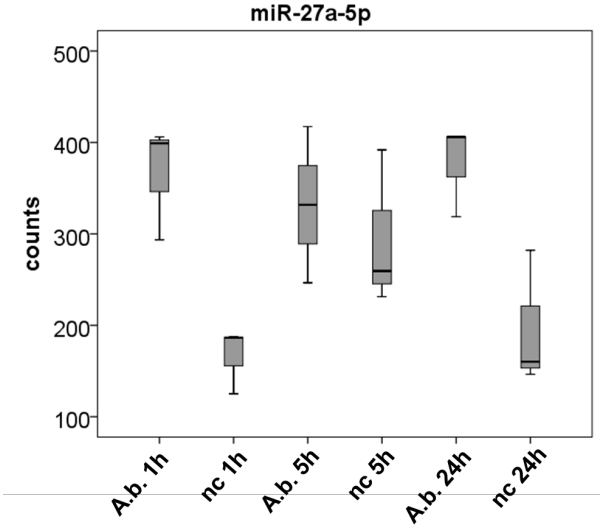
nc 24h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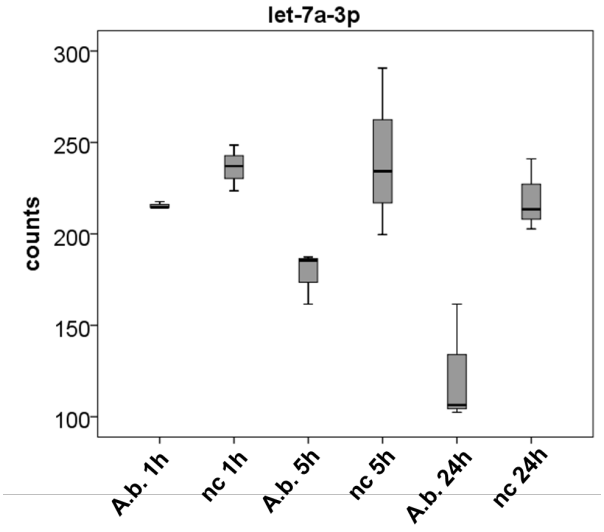
A.b. 5h
nc 5h
A.b. 24h
nc 24h


let-7a-3p
counts
300
250
200
150
100
A.b. 1h
nc 1h
A.b. 5h
nc 5h
A.b. 24h
nc 24h

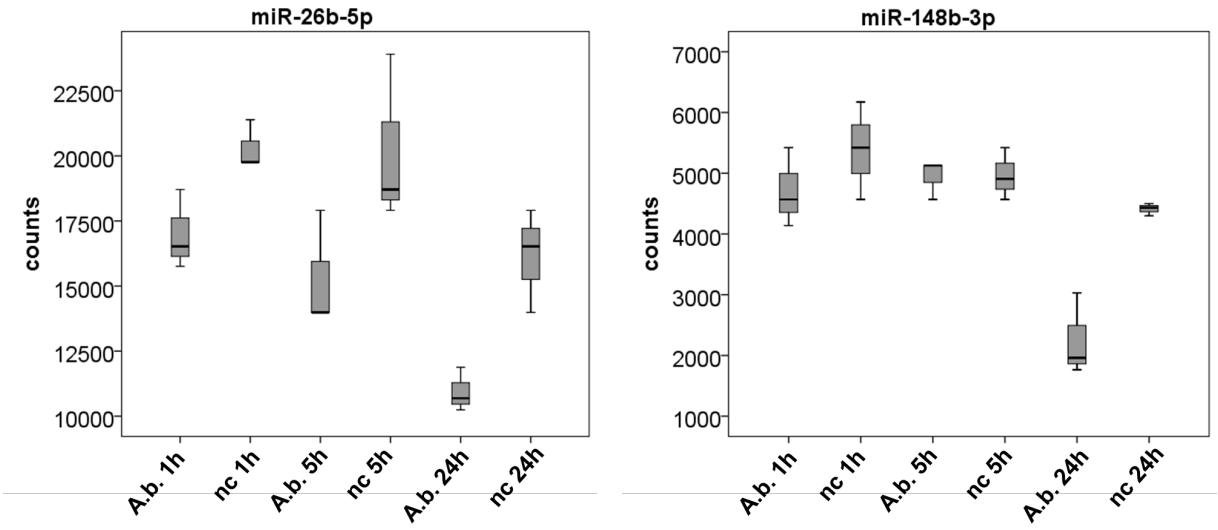


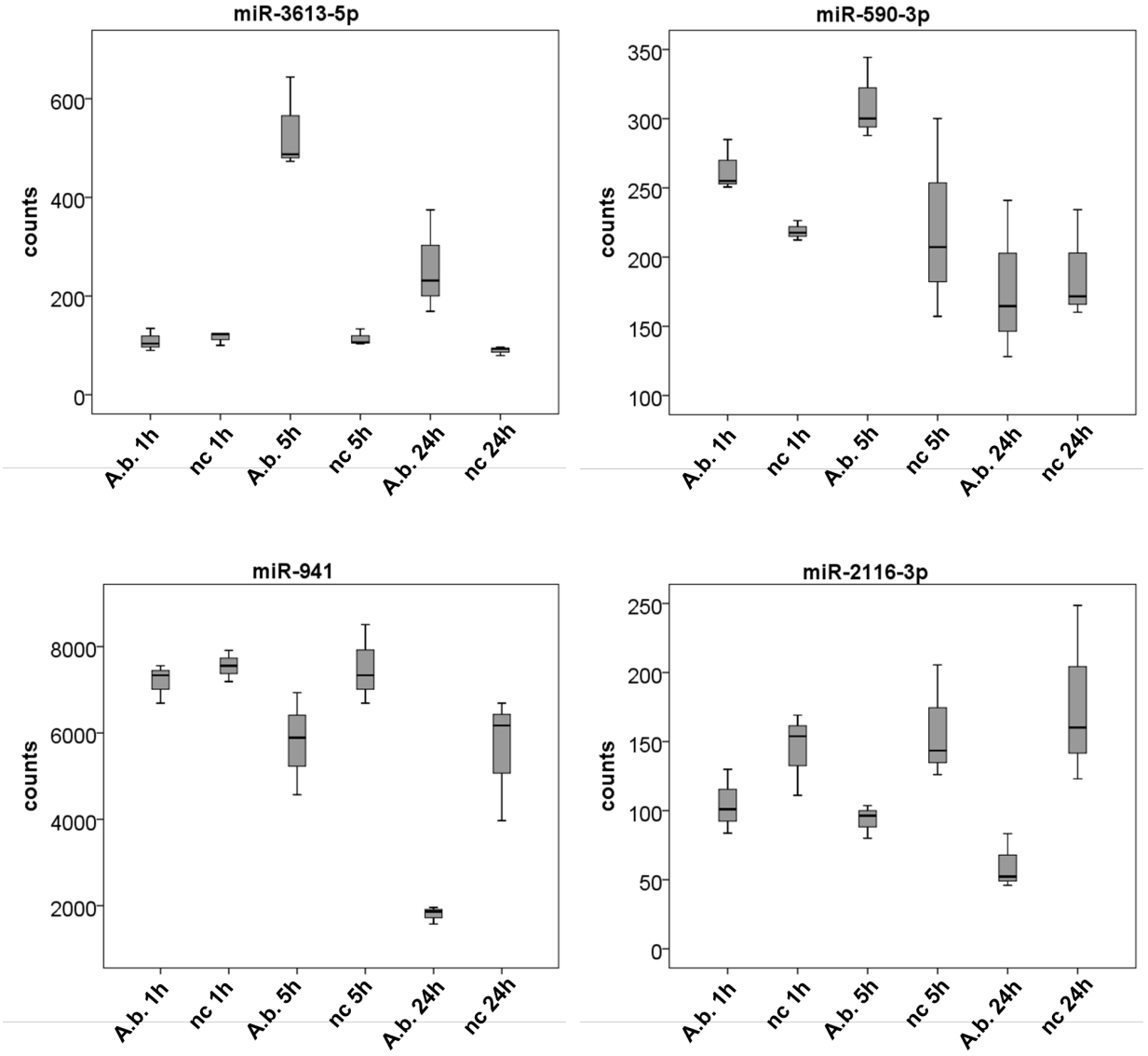
Fig. 13 miRNAs associated with bacterial diseases, expressed in primary human macrophages as a response to *A. butzleri* infection. Cells were infected at a MOI of 100. Samples were taken 1 h, 5 h and 24 h after infection (A.b. = *A. butzleri* reference strain CCUG30485, nc = non infected cells).

3.6.4.2. miRNAs not yet associated with bacterial infection

MiR-3613, miR-590, miR-941, miR-2116, miR-671, miR-30d, miR-339, miR-629 and miR-193a exhibited significant differences in expression levels in infected macrophages. So far, none of these miRNAs have been described being expressed in response to bacterial infection in human host cells.

As indicated in figure 14, miR-3613-5p exhibited elevated expression levels 5 h and 24 h post infection compared to respective controls ($p < 0.05$). Expression remained unaffected 1 h after infection ($p < 0.05$ 1 h vs. 5 h). MiR-590-3p was induced 1 h and 5 h after infection compared to respective controls. Nevertheless, t-tests revealed significant differences only for expression levels 5 h vs. 24 h of infected cells ($p < 0.05$). MiR-941 was downregulated over the time of infection. Significant differences were calculated 1 h and 5 h vs. 24 h of infection as well as 24 h compared to 24 h non-infected cells ($p < 0.05$). Expression of miR-2116-3p and miR-671-3p was downregulated during infection 5 h and 24 h compared to respective controls ($p < 0.05$). Additionaly, in case of miR-671-3p expression decreased significantly 1 h vs. 5 h and 1 h vs. 24 h ($p < 0.05$). Expression of miR-30d-3p decreased over the time of infection ($p < 0.05$ 1 h vs. 24 h, 5 h vs. 24 h). Additionally, 5 h after infection expression was significantly reduced compared to the non-infected control ($p < 0.05$). Expression of miR-30d-5p exhibited a similar pattern as expression of miR-30d-3p did. Nevertheless, miR-30d-5p was induced to a much higher degree compared to miR-30d-3p. MiR-339-5p exhibited reduced expression levels 24 h after infection ($p < 0.05$ 1 h vs. 24 h). Expression of miR-629-5p increased from 5 h to 24 h of infection ($p < 0.05$) and was reduced

5 h after infection compared to non-infected cells ($p < 0.05$). MiR-193a was reduced in 5 h and 24 h infected cells compared to respective controls.



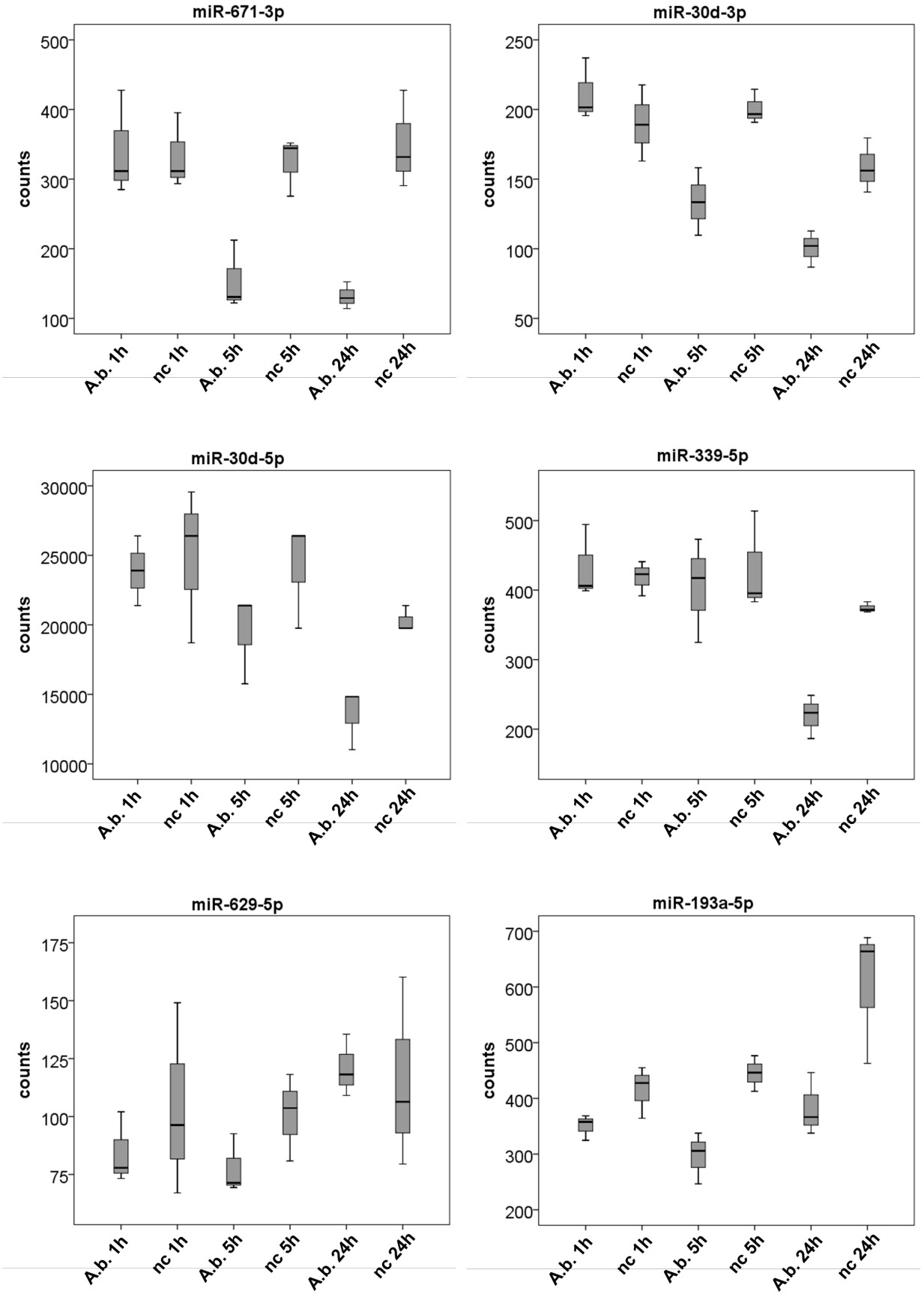


Fig. 14 miRNAs not yet associated with bacterial diseases, expressed in primary human macrophages as a response to *A. butzleri* infection. Cells were infected at a MOI of 100, samples were taken 1 h, 5 h and 24 h after infection.

To unravel the role of differentially expressed miRNAs which have not yet been reported to play a role in bacterial infection, *in silico* prediction was used to identify mRNA targets (miRmap, mirmap score < 80). The list of potential target genes was applied to the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) to identify pathways in which these potential mRNA targets accumulate. DAVID calculates a modified Fisher Exact p-value to measure the gene enrichment in pathways. This will be summarized below and in table 13. Full data of the pathway analysis is attached in the appendix.

miR-3613-5p

Beneath metabolic pathways DAVID analysis revealed a potential role of miR-3613-5p in the DNA damage-induced p53 signaling pathway where miR-3613-5p possibly targets and thereby downregulates genes ATR, CDK4/6, p53R2 and sestrins, which would result in decreased DNA repair and damage prevention.

miR-2116-3p

MiR-2116-3p potentially targets 11 mRNAs influencing apoptotic processes and p53 signaling.

miR-671-3p

Five mRNAs involved in the MAPK-signaling pathways are potential targets of miR-671-3p.

miR-30d-3p and miR-30d-5p

MiR-30d-3p potentially influences endocytosis (19 putative mRNA targets) and regulation of actin cytoskeleton (21 putative mRNA targets). MiR-30d-5p potentially targets endocytosis (8 putative targets) and MAPK signaling (10 putative targets).

miR-339-5p

Targets of miR-339 enrich in endocytosis (19 potential mRNA targets) and apoptosis (12 potential mRNA targets), including caspase 8.

miR-629-5p

Beneath metabolic pathways and signaling involved in cancer development, DAVID revealed 5 mRNAs involved in the formation of the proteasom to be targets of miR-629-5p. This includes also PSMB10 which is involved in the formation of immunoproteasomes.

miR-193a-5p

MiR-193a potentially targets 14 mRNAs which enrich in endocytosic processes. Additionally, caspase 8 is a potential target of miR-193a.

miR-590-3p

DAVID analysis of potential target mRNA suggests miR-590-3p to play a role in endocytosis, regulation of actin cytoskeleton, antigen processing and presenting as well as the p53 signaling pathway. Furthermore, DAVID revealed a potential role in the TLR-signaling pathway. However, the analysis did not reveal a significant enrichement of targets for these pathways.

miR-941

MiR-941 is potentially involved in regulation of apoptosis and endocytosis as well as in TLR-signaling. DAVID did not reveal a significant enrichment for these pathways.

Table 13: Pathways with enriched mRNA targets potentially influenced by *A. butzleri* induced miRNAs

Pathway	*miRNAs*	*Targets*	*P-value*
Endocytosis	miR-30d-3p	SH3GL3, DNM3, FLT1, ERBB4, STAM2, VTA1, PRKCI, ARF6, HLA-B, KIT, ZFYVE20, RAB31, AP2B1, RAB11FIP2, CHMP1B, RAB22A, RAB11B, VPS36, RNF41	0.063
	miR-30d-5p	NEDD4, ACAP2, RAB11A, EEA1, NEDD4L, KIT, ARAP2, CHMP2B	0.085
	miR-193a	FAM125B, ERBB3, VTA1, CXCR2, PSD2, HLA-F, CBLB, ADRB2, RAB11FIP3, CHMP1B, ACAP2, DNAJC6, ITCH, ARAP3	0.023
	miR-339	FAM125B, ERBB3, VTA1, CXCR2, PSD2, HLA-F, CBLB, ADRB2, RAB11, FIP3, CHMP1B, ACAP2, DNAJC6, ITCH, ARAP3	0.043
	miR-590	CBLB, VTA1, WWP1, DNM3, PARD6G	
	miR-941	VPS37B, ZFYVE20, SMURF1, EPN3	
Apoptosis/ p53-signaling pathway	miR-3613	CDK6, RRM2B, ATR, SESN3	0.037
	miR-2116	IRAK2, IRAK1, IRAK3, PRKAR2A, DFFA, PIK3CB, CASP8, CHP2, EXOG, PPP3R2, PIK3R1	0.076
		EI24, CD82, RRM2, SERPINE1, CASP8, RCHY1, RRM2B, MDM4, PERP, CDK2, SESN3	0.017
	miR-339	IRAK2, IRAK1, IRAK3, PRKAR2A, DFFB, CYCS, CASP8, CHP2, PRKACA,	0.021

		APAF1, PIK3R3, ATM	
	miR-590	SESN3	
	miR-941	DFFA, IRAK1, PPP3R1	
Regulation of actin cytoskeleton	miR-590	WASL, CYFIP1, ENAH, FGF23, ITGAV, CRKL	
	miR-30d-3p	GNA13, FGF7, PIK3CB, ROCK2, DIAPH2, SSH2, GNA12, ITGA1, ACTN2, PPP1CB, NCKAP1, DOCK1, CHRM2, TIAM1, SOS1, SOS2, WASL, CRK, FGF2, MYLK, APC	0.079
MAPK-signaling pathway	miR-671	RASGRF1, FGF11, CACNB3, FGF1, CRK	0.063
	miR-30d-5p	MAP3K7, CASP3, MAP3K5, TAOK1, MAP3K2, PLA2G12A, NF1, PLA2G2C, PPP3CA, FGF20	0.099
Antigen presenting and processing	miR-590	KLRD1, NFYB	
TLR receptor signaling	miR-590	STAT1, TMED7	
	miR-941	TLR6, IFNAR1, IRAK1	
Formation of immunoproteasom	miR-629	PSMA2, PSMB10, PSMD13, PSMB2, PSME4	0.023

3.6.4.3. miR-106a

MiR-106a did not exhibit significant differences in expression levels in primary human macrophages during *Arcobacter* infection (figure 15).

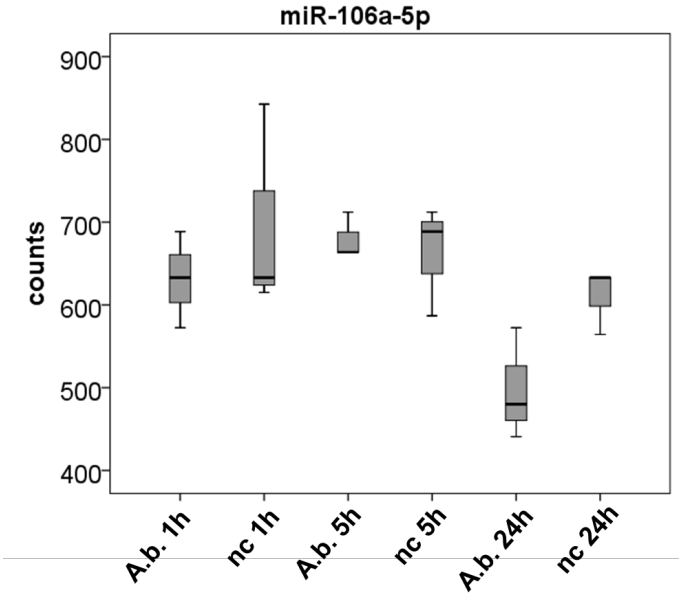


Fig. 15 counts of miR-106a in primary human macrophages after *A. butzleri* infection as well as counts found in non-infected cells at the respective time point.

3.6.5. Potential novel human miRNAs

Raw data analysis indicated potential novel miRNAs, which have not yet been described. To follow up the expression of respective sequences, primers were designed and expression in macrophage samples was investigated by means of miRNA specific RT-qPCR (miR-Q). Decreased numbers of sequence counts were found for the potential novel-miR-55 in infected cells compared to the non-infected control for all time points of infection (figure 16) and a decrease in counts was determined over the time, whereas investigation by RT-qPCR revealed a slight upward trend over time as well as compared to respective negative controls (figure 17). Sequencing data also indicated a slight induction for the potential novel-miR-134 5 h and 24 h after infection (figure 16) whereas a slight induction was only traceable for the 24 h time point in RT-qPCR experiments (figure 17). Expression of potential novel-miR-259 derived from sequencing data indicates a downregulation for all investigated time points compared to the respective controls (figure 16). RT-qPCR data did not reveal differences in expression levels compared to the controls for any investigated time point (figure 17).

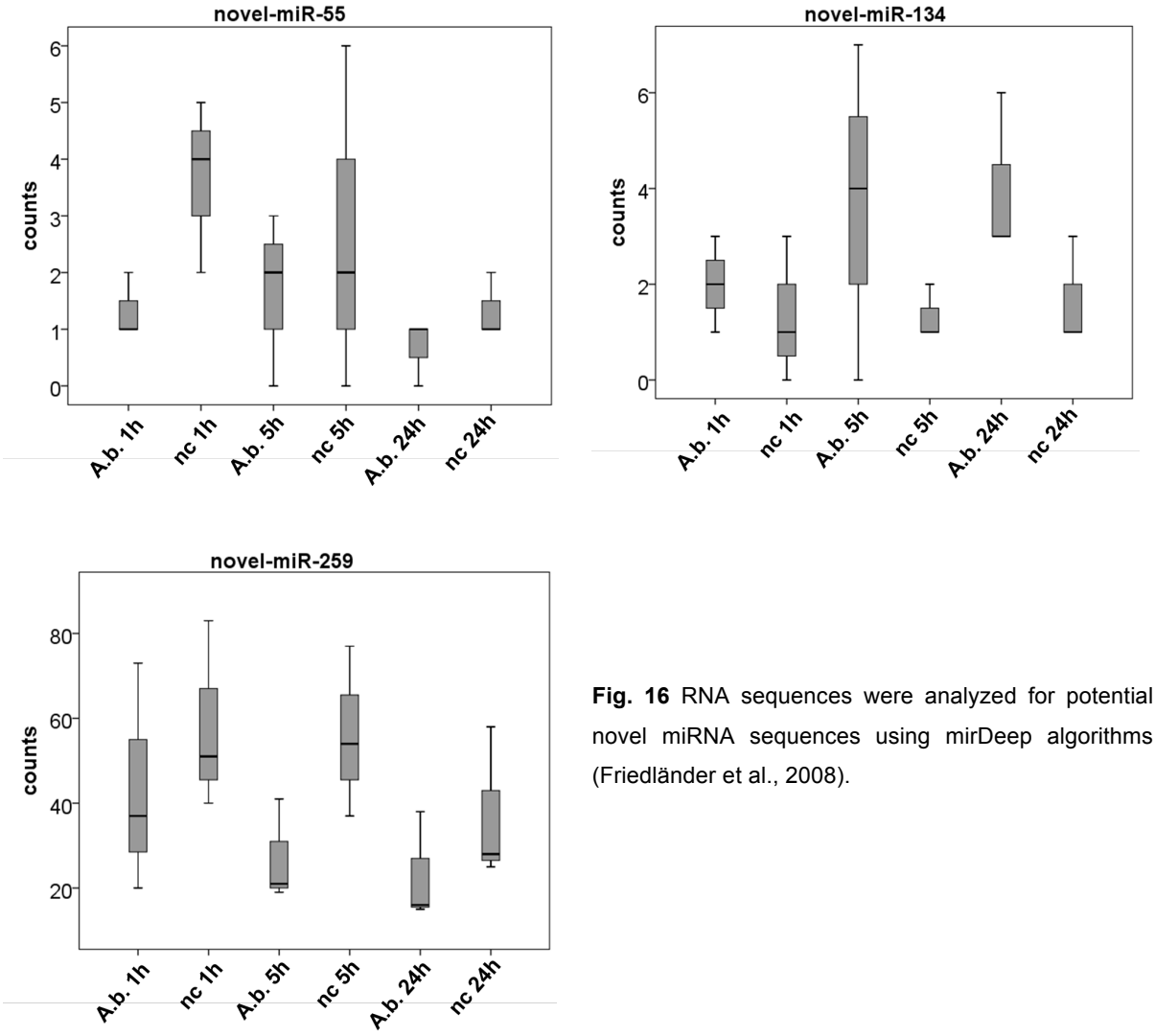


Fig. 16 RNA sequences were analyzed for potential novel miRNA sequences using mirDeep algorithms (Friedländer et al., 2008).

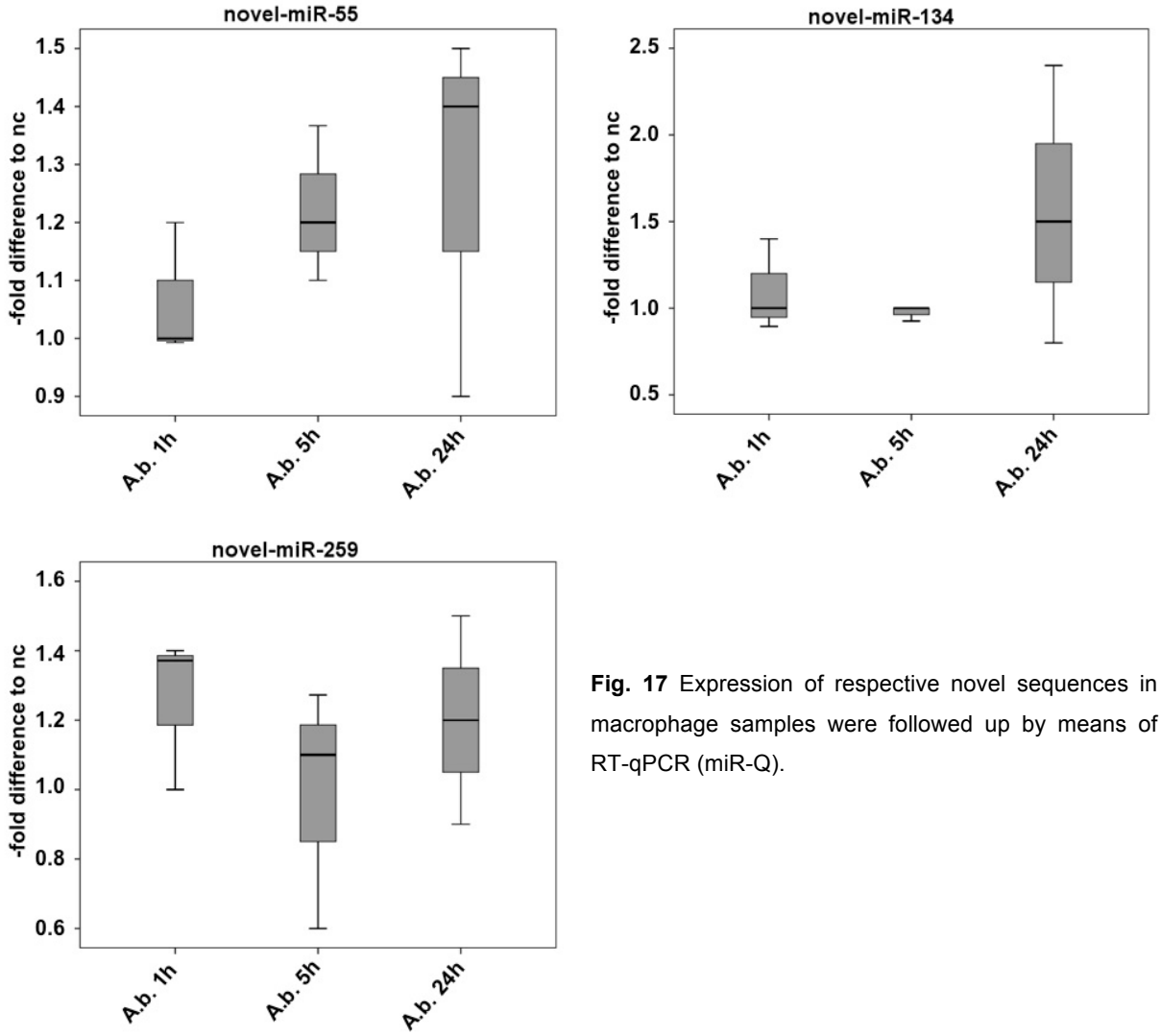


Fig. 17 Expression of respective novel sequences in macrophage samples were followed up by means of RT-qPCR (miR-Q).

4. Discussion

4.1. *A. butzleri* and basic mechanisms in THP-1 derived macrophages upon infection

Since *Arcobacter* was first described as a new zoonotic agent in 1991 (Kiehlbauch et al., 1991) the relevance of *Arcobacter* associated with disease still remains to be evaluated. Case reports identifying *Arcobacter* spp. as the causing agent of severe diseases are sparse (Figueras et al., 2014) which might be due to missing routine diagnostic. Therefore, evaluation of the pathogenic potential is hardly possible. As macrophages have a fundamental role in the first line of host defense during bacterial infection and because there is no data on *A. butzleri*-macrophage interaction the aim of this study was to investigate *A. butzleris* impact on human macrophages to gain further insight into the response of innate immune cells towards *A. butzleri* infection.

The question first addressed was if *A. butzleri* leads to activation of the inflammatory machinery as known for *C. jejuni* (Hu et al., 2006; Jones et al., 2003). Infection of THP-1 derived macrophages resulted in high induction of all kinds of pro-inflammatory cytokines but only moderate induction of the anti-inflammatory cytokine IL-10. This result suggests that cells of the innate immune system recognize *A. butzleri* via TLRs which leads to synthesis and release of cytokines, alert of further immune cells and might result in systemic inflammation. A previous study of the infected human intestinal cell line Caco-2 demonstrated that *A. butzleri* also induces epithelial cell secretion of IL-8 confirming this suggestion (Ho et al., 2007).

As phagocytosis of *C. jejuni* and its intracellular survival and replication in mononuclear phagocytes has been demonstrated in several studies and is considered as a virulence mechanism employed by the pathogen to escape host immune defense (Kiehlbauch et al., 1985; Hickey et al., 2005, Jones et al., 2003), the ability of *A. butzleri* to survive the hostile environment of macrophages was another matter of investigation. The data demonstrate that *A. butzleri* is able to resist macrophage defense for up to 22 h although intracellular bacteria were minimized to a small fraction of originally inoculated cell-numbers. Jones et al. demonstrated intracellular survival of *C. jejuni* in THP-1 cells for up to 24 h after infection. Similar to our observations bacterial cell numbers were diminished to a smaller fraction (Jones et al., 2003). Intracellular survival in THP-1 cells has also been proven for other pathogens such as *M. tuberculosis* or *Salmonella* (Sly et al., 2003; Forest et al., 2010). Survival of these pathogens within macrophages is known to be an essential step in pathogenesis.

Most striking were the vast strain-dependent differences in intracellular bacterial numbers found in macrophages at 5 h after infection with highest invasion and survival capabilities

observed for meat isolates. Isolate dependent differences in gene patterns of *A. butzleri* have been described (besides others) by Merga et al. and explained with an adaptation to different host-niches (Merga et al., 2013). The authors claim that adaptations occur to suit different environments and result in genetic differences between the strains. Therefore, variations in survival and sensing systems might be related to the source of origin. Intracellular invasion and survival of bacterial pathogens are considered virulence mechanisms and in case of *A. butzleri* might be supported by the flagellum which confers its motility. Strain-dependent variations in the ability to enter host cells therefore might be correlated with distinct virulence gene expression. Karadas et al. tested adhesion and invasion capabilities of same isolates that were used in this study in intestinal epithelial cells and were not able to correlate different virulence gene patterns (especially adhesion and invasion genes *cadF, cj1349*, *ciaB*) with variations in adhesive and invasive phenotypes (Karadas et al., 2013). To correlate differences in invasion capabilities with variations in motility in this study, *A. butzleri* liquid culture was inoculated on a semisoft agar and swarming halos were measured. Interestingly, the strains that were most invasive in macrophages possessed also stronger motility. Nevertheless, conclusions from this data have to be drawn very carefully since invasion was determined 5 h after infection whereas agar plates for the motility assay were incubated for 48 h under microaerobic conditions. However, Ho et al. demonstrated that different oxygen levels had no impact on flagellin gene transcription (*flaA* and *flaB)* (Ho et al., 2008). Therefore, more in-depth studies are needed to verify this hypothesis.

Another reported strategy to attenuate host defense is to influence apoptotic processes in cells of the innate immune system. Apoptosis is either mediated through the extrinsic or the intrinsic apoptotic pathway depending on the death-stimuli. The extrinsic pathway is triggered through FAS or the TNF-receptor family and activates initiator caspase 8 which leads to downstream activation of executioner caspase 3, 7 and other proapoptotic molecules and finally the programmed cell death. The intrinsic pathway, however, occurs as a response to different intracellular stimuli and is mediated by cytochrome c release of mitochondria. In both pathways, late apoptotic events occur after activation of the executioner caspases and result in exposure of phosphatidylserine on the extracellular surface of the plasma membrane, cleavage and inactivation of PARP (an enzyme involved in DNA repair) and internucleosomal DNA fragmentation. Bücker et al. demonstrated the induction of apoptosis by *A. butzleri* in intestinal epithelial cells which directly contributed to epithelial barrier dysfunction (Bücker et al., 2009). In this study, infection of THP-1 derived macrophages with different *A. butzleri* strains led to increased activity of the initiator caspase 8 as well as executioner caspases 3 and 7 at 5 h but not 24 h after infection. Interestingly, and in contrast to increased caspase 8 activity, mRNA expression of caspase 8 was downregulated 5 h after infection and rose up again to the basal level of the negative control 24 h after infection. This

suggests an induction of apoptosis in the initial stages of infection followed by a counter regulation driven by the host cell to reconstitute survival and undisturbed function during infection. This mechanism has been described by Perera and Waldmann (1998) who observed a rapid repression of synthesis of caspase 8 in activated monocytes during early stages of infection as a counter regulation to ensure macrophage survival and to execute their function especially in an inflammatory microenvironment rich in cytotoxic cytokines such as TNFα and free radical metabolites. Additionally, the conducted end-point apoptosis assay (TUNEL) did not reveal obvious DNA fragmentation of THP-1 derived macrophages 24 h after inoculating *A. butzleri* and therefore underlines this hypothesis. To decipher the underlying mechanism, the involvement of a potential miRNA regulation in this context was further analyzed. The investigation of a miRNA-mediated downregulation of caspase 8 mRNA based on *in silico* prediction to filter out possible miRNA candidates as well as the observation of an LPS induced expression of miR-106a in HeLa cells (Jiang and Li, 2011). In addition, miR-106a was reported to be upregulated in mouse macrophages after LPS stimulation (Zhu et al., 2013). Another study revealed an interaction of miR-106a with FAS, a membrane receptor that induces the activation of the extrinsic apoptotic pathway (Wang et al., 2013), therefore an interplay between miR-106a and other participants of the apoptotic machinery such as caspase 8 is most likely. To follow up the *in silico* prediction of a possible miR-106a–caspase 8 mRNA interaction, luciferase-reporter assays were used to prove the binding of miR-106a to the predicted caspase 8 target sites followed by subsequent downregulation of luciferase activity. Although, results of the reporter assays underline the hypothesis of a miR-106a mediated downregulation of caspase 8, an upregulation of miR-106a in *A. butzleri* infected THP-1 cells was not detectable by means of RT-qPCR. In addition, RNAseq of infected primary human macrophages did not display significant expression levels of miR-106a compared to the non-infected controls. Therefore, the decrease of caspase activity in macrophages in response to *Arcobacter* infection is presumably not caused by miR-106a.

Taken together, this study was the first to demonstrate that *A. butzleri* has an impact on human innate immune cells. There are indications of pathogenic potential demonstrated by cytokine production and the ability to survive inside macrophages for up to 22 h, although, only a minimal fraction of originally inoculated bacteria invaded and survived. However, similar observations were made for *C. jejuni* in THP-1 cells (Jones et al., 2003). Reports regarding the ability of *C. jejuni* to survive within macrophages are quite contradictory and vary from “ability to survive for up to seven days with an approximate 3-log-level increase in bacterial counts” to “eradication by host phagocytes within 24 hours” (Hickey et al., 2005; Watson and Galán, 2008; Kiehlbauch et al., 1985; Wassenaar et al., 1997).

Opposed to the hypothesis of *A. butzleri* possessing pathogenic potential, it is widely known that stimulation of TLRs leads to increase of pro-inflammatory cytokines and does not necessarily result in systemic disorder but might also lead to a fast eradication of invading microbes without manifestation of disease. Furthermore, macrophages showed a mechanism of counter regulation to the apoptotic induction to re-establish their undisturbed function even though burdened with a high bacterial load.

Most surprising observations made in this study were the strain specific differences in invasion and survival capability as well as variations in motility. Although strains can be grouped by similar virulence gene patterns, they do not exhibit the same virulence phenotype (Karadas et al., 2013). This does not blank out the fact that there are isolate dependent differences in phenotypes possessing divergent virulence potentials. In a prevalence study of *Arcobacter* spp. in Belgian pigs, van Driessche et al. revealed a large heterogeneity among *A. butzleri* isolates in individual animals and also determined a large number of genotypes per species on farm level. Colonization of multiple parent genotypes or genomic rearrangement of parent genotypes during passage through the host might portray possible explanations (van Driessche et al., 2004). These strain specific differences could possibly be responsible for the clinical differences observed in *A. butzleri* infected human patients and animals since some infections lead to severe disease whereas others do not exhibit any clinical symptoms. In addition, whole genome sequencing and MLST of several isolates of different sources indicated that there are strain specific differences in the genomic content of different *A. butzleri* isolates and that these variations are isolate dependent and suggest a level of niche adaptation to the source of origin (Miller et al., 2007; Merga et al., 2013). These processes of adaptation result in genetic differences in between the strains which also might enhance virulence. Based on this knowledge we might have to rethink our understanding of evaluation of pathogenicity of a certain emerging organism but rather have to take into consideration that there might be important intraspecies differences in virulence leading to a formation of different subspecies/serovars as known for other enteric pathogens such as *Salmonella enterica* ssp. To finally evaluate if *A. butzleri* is a severe threat to human health and an opportunistic foodborne pathogen or rather plays a minor role in the development of gastroenteritis being only associated to disease in single cases, optimized *in vivo* models have to be developed. Moreover, further investigations are necessary, possibly including bacterial RNAseq, to evaluate if and to what extent the putative virulence factors are functional and favor disease.

4.2. miRNA response of primary human macrophages towards *A. butzleri* infection

The above discussed potential influence of miR-106a on apoptotic events occurring in macrophages raised the question about the general role of miRNAs upon *Arcobacter* infection.

Recent studies have shown that miRNAs are key regulators of innate immunity (O'Neill et al. 2011; O'Connel et al., 2010; Taganov et al., 2006) and play a prominent role in the specific host response towards bacterial pathogens (Hoeke et al., 2013; Sharbati et al., 2011).

Since nothing is yet known about miRNA patterns expressed in macrophages during *A. butzleri* infection, the second part of this work focused on sequencing of small RNAs in *A. butzleri* infected macrophages. Primary human macrophages derived from buffy coats were used instead of THP-1 cells. As miRNAs were shown to be dysregulated in cancer, immortalized cancer cell lines, such as THP-1 cells (Tsuchiya et al., 1980) may not represent a suitable model to study miRNA expression. Furthermore, THP-1 cells have to be artificially differentiated into macrophages. Phorbol-12-myristate-13-acetate (PMA) is widely used to induce macrophage differentiation but the extent of differentiation in comparison to primary cells is unclear (Daigneault et al., 2010). Due to these reasons, primary cells were used in this study to reflect the in vivo situation more closely.

Since miRNAs were reported to exhibit rapid dynamics in their temporal expression in response to bacterial pathogens (Siddle et al., 2015; Lawless et al., 2013, O'Neill et al., 2011), RNA samples of infected macrophages were investigated at three different time points to assess changes in miRNA patterns during the course of infection.

RNA sequencing of infected macrophages revealed several miRNAs, which were differentially expressed in infected cells compared to the non-infected control. Some of these miRNAs have not been reported to play a role in the host response towards bacterial infection before. However, most of the identified miRNAs belonged to a set of candidates, which were also expressed in immune cells in response to bacterial infection in previous studies, mainly via induction of TLR signaling (Eulalio et al., 2012).

TLR signaling is initiated by pathogen-associated-molecular patterns, which bind to TLRs and induce receptor-dimerization followed by engagement of adapter molecules such as MyD88. MyD88 stimulates Interleukin-1 receptor-associated kinases (IRAKs) to activate TNF receptor associated factor 6 (TRAF6). This leads to activation of mitogen-activated protein kinases (MAPKs) and the IκB kinase (IKK) enzyme complex, respectively. Thereby, the activation of transcription factor NFκB is induced, which results in transcription and release of inflammatory cytokines (O'Neill et al., 2013). *C. jejuni* was reported to activate TLR2, TLR4 and TLR9 in a MyD88 dependent manner (Haag et al., 2012; Friis et al., 2009; Bereswill et

al., 2011). These TLRs may also be induced during *A. butzleri* infection, since *C. jejuni* and *A. butzleri* are members of the same bacterial family.

Multiple miRNAs are induced in innate immune cells in a TLR-dependent manner. Among these, miR-155, miR-146 and miR-21 are particularly ubiquitous (O'Neill et al., 2011).

Concordant to these findings, miR-155-3p and 5-p, miR-146a-5p as well as miR-21 were upregulated in this study in response to *A. butzleri* infection. Furthermore, miR-155 was previously reported to be an early responder, since it was highly induced 2 h after stimulation whereas miR-21 was expressed at later times (O'Neill et al., 2011). This was also in agreement with the observations made in the present study, since miR-155 was highly induced 5 h after infection in contrast to miR-21, which was induced 24 h after infection. MiR-155 and miR-146a/b are not only induced in a TLR-dependent manner, but in turn, target several genes involved in TLR signaling (O'Neill et al., 2011). For example TRAF6 and IRAK1, components downstream in the MyD88 dependent pathway regulating NFκB activity, are validated targets of miR-146. Therefore, upregulation of miR-155 and miR-146 results in a negative regulation of immune reactions and maintains a balanced response to bacterial infections (Staedel and Darfeuille, 2013). Additionally, miR-155 is crucial for the development of a normal adaptive immune response that requires B and T cell activation after infection (Lind et al., 2013). For example, miR-155 knockout mice were not able to exhibit vaccine-induced protective immunity against *Salmonella typhimurium* (Rodriguez et al., 2007). MiR-21 was described to be induced in macrophages and other immune cells upon stimulation with purified TLR ligands as well as with *H. pylori* or *Salmonella* (Eulalio et al., 2012). For *H. pylori* infection it was discussed that its influence on miR-21 expression might initiate tumorigenesis in gastric tissue since this miRNA targets RECK, a known tumor suppressor in gastric cancer (Zhang et al., 2008). However, miR-21 was also shown to be induced in human monocytes by *Mycobacterium leprae* (Liu et al., 2012) where it directly downregulated IL-1β expression as well as indirectly upregulated IL-10. In addition, Sheedy et al. (2010) demonstrated that miR-21 negatively regulated TLR4 signaling in LPS treated human peripheral blood mononuclear cells. Treatment with LPS resulted in lower PDCD4 expression, a pro-inflammatory protein that promotes activation of the transcription factor NFκB and suppresses IL-10. Thereby miR-21 enhances the production of the anti-inflammatory cytokine IL-10 and reduces the pro-inflammatory activity of NFκB resulting in a decreased inflammatory response and therefore protection against overwhelmed inflammation (Sheedy et al., 2010).

Involvement in regulation of TLR signaling leading to dampened NFκB activity has also been shown for miR-125 and miR-212 which were upregulated in this study 24 h after infection.

MiR-125a was shown to target TRAF6 (Guo et al., 2014) and has been reported to be involved in the early immune response of macrophages to *Listeria* infection and potentially targets the IL-1 receptor 1 as well as the IL-6 receptor (Schnitger et al., 2011; Staedel and Darfeuille, 2013). MiR-212 was demonstrated to be induced together with miR-132 by the TLR2 ligand peptidoglycan in THP-1 cells, PBMCs and primary macrophages. TLR2 was shown to be induced by *C. jejuni* (Haag et al., 2012) and might possibly also be activated by *A. butzleri*. An early component of the MyD88 dependent pathway, IRAK4, was identified as a direct target of miR-212 (Nahid et al., 2012). Furthermore, the authors observed cross-tolerance by the TLR5 ligand flagellin. However, although *A. butzleri* possesses two short flagellins, a TLR5 mediated induction of miR-212 is questionable since *Epsilonbacteria* fail to activate TLR5 (de Zoete et al, 2010).

MiR-181b/c/d showed elevated expression levels 24 h after infection. Sun et al. (2014) reviewed the function of the miR-181-familiy which is known to play a role in vascular inflammation. They additionally found out that miR-181b targets Importin a-3, a protein that is needed for nuclear translocation of NFκB (Sun et al., 2012). Therefore miR-181b might additionally contribute to attenuated NFκB signaling. In addition, miR-181c was shown to act as a suppressor of CD4+ T-cell activation by targeting IL-2 (Xue et al., 2011).

Another miRNA exhibiting increased expression levels in response to *A. butzleri* 24h after infection was miR-99b. This miRNA was shown to be enhanced by *M. tuberculosis* in macrophages and dendritic cells (Singh et al., 2013). Antagonizing miR-99b expression resulted in significantly reduced bacterial growth in dendritic cells. It was shown that miR-99b downregulates proinflammatory cyotkines as well as TNFα which allows *M. tuberculosis* to evade immune response and survive inside the host cell.

Let-7a was downregulated in the course of *A. butzleri* infection. This goes along with previous reports regarding the expression of let-7a in response to bacterial infections. For example, Matsushima et al. (2011) reported let-7a and other members of the let-7-family to be reduced upon *H. pylori* infection in human gastric mucosa and AZ-521 cells, a human gastric epithelial cell line. Schulte et al. found the let-7-family to be downregulated in HeLa cells as well as in a mouse leukaemic monocyte/macrophage cell line when challenged with *Salmonella enterica*. Their study suggests that repression of let-7 relieves cytokine IL-6 and IL-10 mRNAs from negative post-transcriptional control (Schulte et al., 2011). Another study reported that Caco-2 cells (human colon epithelial cells) responded with a reduced let-7 expression to *Listeria monocytogenes* infection (Izar et al., 2012). Furthermore, How et al. demonstrated that LPS stimulation downregulated expression of let-7a in THP-1 cells and overexpression revealed that let-7a significantly decreased TNFα and IL-1β production in response to LPS (How et al., 2015). Therefore, downregulation of let-7 and concomitant decline of inflammatory reactions appears to be a consistent response of several cell types

upon different bacterial stimuli including LPS as well as gram-positive and gram-negative pathogens.

Another miRNA exhibiting reduced expression in response to *A. butzleri* infection was miR-26b. The function of this miRNA during bacterial infection is yet barely described. However, it has been previously reported as a host response element during viral infection (Zhao et al., 2014). In addition, increased expression of miR-26b was found in exosomes isolated from mice with experimentally induced sepsis (Wu et al., 2013). The role of miR-26b in *A. butzleri* infected macrophages therefore needs further investigation to assign a specific function.

MiR-148a was another candidate with decreased expression levels 24 h after *A. butzleri* infection. It was identified as a negative regulator of innate immune response as well as antigen presenting capacity of TLR-triggered dendritic cells by downregulating CaMKII (Calcium/calmodulin-dependent protein kinase II) and inhibition of cytokines including IL-12, IL-6, TNFα and INF-β (Liu et al., 2010). The authors concluded that miR-148, together with miR-152, acts as a finetuner in regulating the innate response and antigen presenting capacity of dendritic cells, which may contribute to the immune homeostasis and immune regulation. In the current study, *A. butzleri* infected macrophages exhibited reduced expression 24 h after infection. According to the above mentioned hypothesis (Liu et al., 2010), this would lead to increased cytokine expression. On the other hand, miR-148 might target a different set of mRNAs in macrophages compared to dendritic cells.

MiR-27a was the only miRNA showing significant upregulation already 1 h after the infection compared to the negative control. Host miR-27a was demonstrated to be degraded by Cytomegalovirus to ensure its survival and replication (Marcinowski et al., 2012). In bacterial infection, miR-27a was shown to be induced in differentiated THP-1 cells during *Salmonella* infection (Sharbati et al., 2012). However, its function in infected host cells still remains to be elucidated.

Taken together, the majority of miRNAs induced by *A. butzleri*, which have been previously associated with bacterial infection, play a role in preventing an overwhelmed reaction of the immune system towards pathogen invasion. Pro-inflammatory cytokine expression of THP-1 derived macrophages in response to *A. butzleri* was demonstrated in the first part of this study. Thus, the above discussed miRNAs may contribute to prevent pathologic conditions deriving from uncontrolled cytokine productions and ensure a balanced immune homeostasis.

Interestingly, some differentially expressed miRNAs identified in macrophages in response to *A. butzleri* have not yet been reported to be expressed in host cells in response to bacterial pathogens.

In silico analysis employing mirmap software and DAVID functional annotation tool revealed that potential targets of these miRNAs (besides putative targets in metabolism and cancer) were enriched in pathways such as apoptosis/DNA damage, endocytosis, regulation of actin cytoskeleton, antigen processing and presentation as well as TLR signaling pathways and formation of the immunoproteasome. Whereas miR-941, miR-2116-3p, miR-671, miR-30d, miR-193a and miR-339 were decreased in expression after infection, miR-590, miR-3613 and miR-629 exhibited an increased response. Since potential targets of both groups were enriched in similar pathways it will require further studies to elucidate potential interactions.

Highest target accumulation was found for the process of endocytosis and apoptosis. Interestingly, miR-193a was downregulated 5 h after infection and slightly rose in 24 h infected samples. DAVID revealed caspase 8 to be a potential target of miR-193a. Therefore, decline in caspase activity from 5 h to 24 h as observed in THP-1 cells (3.4.1.) might be due to inhibition by miR-193a. Nevertheless, miR-339 was also identified to potentially regulate caspase 8 mRNA but its expression was downregulated 24 h after infection. Therefore, further studies are needed to verify a potential interaction. In addition, although some insight can be gained from the study of computationally predicted miRNA targets, *in silico* target identification still remains difficult since bioinformatic prediction is restricted to the current knowledge of the rules of miRNA interaction and does not consider cell-specific interactions (Helwak, Kudler et al., 2013; Siddle et al., 2015).

A further possibility of a miRNA mediated mode of action is the secretion of miRNAs from a donor cell to a recipient cells where they function as endogenous miRNAs, simultaneously regulating multiple target genes or signaling events (Chen et al., 2012). Recent publications demonstrated that miRNAs circulate in microvesicles or bound to a RNA binding protein in various body fluids including serum and plasma (Chen et al., 2008; Gilad et al., 2008; Mitchell et al., 2008). Several studies identified secretion of miRNAs by immune cells (Hunter et al., 2008; Zhang et al., 2010) and Zhang et al. proved that THP-1 derived microvesicles can deliver miR-150 into human microvesicular endothelial cells resulting in suppression of c-myb a known target gene (Zhang et al., 2010; Xiao et al., 2007). Therefore, miRNAs expressed by primary human macrophages might not only alter the expression of endogenous mRNAs but also of genes in neighboring cells such as intestinal epithelial cells. Adhesion to and in certain cases invasion into intestinal epithelial cells constitute important capabilities of intestinal pathogens for a successful establishment of infection (Pizarro-Cerda and Cossart, 2006). *A. butzleri* has been shown to possess the capability both to adhere to and to invade into epithelial cells (Ho et al., 2007; Karadas et al., 2013). In case of non-phagocytic cells such as epithelial cells this endocytotic internalization process is initiated by the bacteria (Cossart and Helenius, 2014). Thus, a potential mechanism of the host organism to counteract bacterial invasion might therefore be an interplay between macrophages

secreting miRNAs that inhibit endocytotic processes in epithelial cells. In addition it might counteract the induction of apoptosis in intestinal epithelial cells by *A. butzleri* (Bücker et al. 2009) to diminish epithelial barrier dysfunction.

Overall, the miRNA response of macrophages towards *A. butzleri* infection differed compared to recent findings regarding infection of THP-1 cells with *C. concisus*, another member of the family of *Campylobacteraceae* (Kaakoush et al., 2015). MiR-146a was the only miRNA to be upregulated in both studies underlining the observation that differences in expression levels and profiles depend on the stimulus and celltype (O'Neill et al., 2011). Nevertheless, most of the miRNAs expressed in human macrophages in response to *A. butzleri* infection have previously been described to be induced in immune cells by various bacterial pathogens. These miRNAs were shown to be crucial regulators of e.g. TLR-signaling and essential for establishing and maintaining an effective immune response towards infection. In addition, miRNAs not yet associated with bacterial infection were determined in the present study. A possible explanation was given by a recent study. The authors detected the presence of a core miRNA response of dendritic cells to infection with different bacteria. Despite such broadly shared expression, they identified pathogen-specific miRNA responses that reflect mechanisms by which certain pathogens interfere with the host response to infection (Siddle et al., 2015). Therefore, identified miRNAs that were not reported to be involved in bacterial diseases might represent an *A. butzleri*-specific response**.** However, follow-up studies are needed to confirm this hypothesis.

In addition, 3 potential novel miRNAs (novel-miR-55, novel-miR-134, novel-miR-259) were identified in *A. butzleri* infected macrophages and expression was followed up by RT-qPCR. Nevertheless, further experiments are necessary to validate their expression and functional role.

The previous remarks and description of differentially expressed miRNAs in the presented study as well as the subsequent accord to literature illustrate the complex network of miRNA regulation in human gene expression. Although, the use of miRNAs as potential therapeutics constitute a promising strategy to face the rising resistance against conventional antibiotics, miRNA studies have to be extended to generate a complex understanding of the diverse miRNA regulatory functions. Off-target effects constitute a great pitfall of miRNA therapy and can only be eliminated by a widespread and broad knowledge of miRNA functions under different cellular conditions. Therefore, the generated data is important to broaden the existing knowledge about miRNA response of immune cells towards bacterial infection but also provides further insight into the bacterial pathogenesis and regulatory interaction occurring upon *A. butzleri* infection.

5. Conclusion

The first part of the study concentrated on basal mechanisms occuring in human macrophages upon *A. butzleri* infection. In response to infection macrophages behaved in a manner similar compared to macrophages infected with established intestinal pathogens such as *C. jejuni,* especially regarding the expression of inflammatory cytokines and the ability to survive within the host cell. End point apoptosis was not induced by *A. butzleri* although reported for *C. jejuni.* Isolate dependent differences were observed especially for the ability to survive in the host cell. Therefore, isolate-specific variations in virulence might be possible. To answer the question if *A. butzleri* represents a hazardous risk to human health or rather possess inferior relevance in the pathogenesis of severe gastroenteritis, more in depth studies are needed preferably using a suitable *in vivo* model.

The second part investigated miRNA expression in macrophages in response to *A. butzleri* infection. Although a common set of miRNAs that have previously been reported to play a key role in the immune response during bacterial infection was induced, the overall expressed miRNA profile seemed to be Arcobacter-specific. The generated data provides the basis to study underlying mechanisms of host-pathogen interaction occuring upon infection and furthermore smoothes the way for a broader knowledge of miRNA interaction, which is needed to develop miRNA based therapeutics.

In total this work contributes to a better understanding of *A. butzleri* infection and deepens the knowledge about miRNAs and infectious diseases.

6. Summary

6.1. Summary

Cases of severe gastroenteritis caused by the new emerging pathogen *A. butzleri* have been recently reported but data to evaluate the pathogenic potential is sparse. Expanding the knowledge of the interplay between *A. butzleri* and its host is indispensable to evaluate the pathogenicity and to develop strategies to combat *A. butzleri* induced diseases. Macrophages have a fundamental role in the first line of host defense and were therefore matter of investigation.

The first part of this work focused on the inflammatory response of macrophages as well as intracellular survival and modulation of host cell apoptosis as potential virulence mechanisms employed by *A. butzleri*. THP-1 cell derived macrophages were used as an *in vitro* infection model. Induction of IL-1α, IL-1β, IL-6, IL-8, IL-12β and TNFα demonstrated a pro-inflammatory response of infected macrophages towards *A. butzleri*. Gentamycin protection assays revealed the ability of the pathogen to survive and resist the hostile environment of phagocytic immune cells for up to 22 h and apoptosis assays proved the initial induction of adapter- as well as effector caspases which was not followed by DNA damage, suggesting a possible counter regulation. A potential role of miR-106a in this context could not be demonstrated. The considered investigations demonstrate that *A. butzleri* employs virulence mechanisms to attenuate host defense similar to those used by other severe intestinal pathogens such as *Campylobacter jejuni*. Additionally, distinct isolate-dependent differences were observed among the strains suggesting the existence of strain-specific phenotype variations possibly exhibiting different virulent potential.

The second part of this work concentrated on the role of miRNAs upon *Arcobacter* infection. The infection of primary human macrophages of three different donors with *A. butzleri* and subsequent miRNAseq contributed new data to understand the regulatory network of miRNAs expressed during bacterial infection. Analysis of the data revealed the expression of miR-125a, miR-146a, miR-155, miR-212, miR-181b/c/d, miR-21, miR-99b, miR-27a, let-7a, miR-26b and miR-148a. These miRNAs have been reported to be mainly involved in the autoregulative control of a balanced immune response during infectious diseases. Additionally, miRNAs which have not yet been reported to be involved in infectious diseases were expressed in *A. butzleri* infected macrophages (miR-3613, miR-590, miR-941, miR-2116, miR-671, miR-30d, miR-339, miR-629 and miR-193a). Taken together, the data generated in this study contributes new findings to the existing knowledge about the interplay of the new emerging pathogen *A. butzleri* with human host cells which is necessary to evaluate the severeness of the disease and develop appropriate therapeutics.

6.2. Zusammenfassung

Einfluss des zoonotischen Erregers *Arcobacter butzleri* auf humane Makrophagen

Der bakterielle Zoonose-Erreger *A. butzleri*, Mitglied der Familie *Campylobacteraceae*, wurde in jüngeren Veröffentlichungen mit schweren Durchfallerkrankungen in Zusammenhang gebracht. Doch ob und inwiefern *A. butzleri* tatsächlich krankmachendes Potential besitzt ist unklar.

Die vorliegende Studie hatte zum Ziel das bestehende Wissen über den Einfluss von *A. butzleri* auf Wirtszellen zu erweitern um somit das pathogene Potenzial von *A. butzleri* besser einschätzen zu können und die Grundlage für die Entwicklung alternativer Therapiemöglichkeiten zu schaffen. Da phagozytierende Zellen des angeborenen Immunsystems eine elementare Rolle in der ersten Immunantwort des Wirtes auf bakterielle Pathogene inne haben, lag der Fokus der Untersuchung auf der Interaktion von *A. butzleri* und humanen Makrophagen.

Der erste Teil der Arbeit untersuchte die Entzündungsantwort der Makrophagen sowie intrazelluläres Überleben und Beeinflussung des programmierten Zelltodes der Wirtszellen (Apoptose) als potentielle Virulenzmechanismen von *A. butzleri*. Die monozytische Zelllinie THP-1 diente hierfür als Infektionsmodell. Die Induktion der Interleukine IL-1α, IL-1β, IL-6, IL-8, IL-12β und TNFα demonstrierten eine pro-inflammatorische Wirtsantwort der Makrophagen auf die Infektion. Gentamycin protection assays zeigten, dass *A. butzleri* die Fähigkeit besitzt für mindestens 22 h in Makrophagen zu überleben. Des Weiteren ergaben Untersuchungen der Caspaseaktivitäten (Caspase 8, 3 und 7), dass eine Infektion mit *A. butzleri* eine erhöhte Aktivität von sowohl Adapter- als auch Effektorcaspasen initial induziert, diese im Verlauf der Infektion aber wieder abnimmt und letztendlich nicht zum programmierten Zelltod führt. Eine vermutete Rolle von miR-106a in diesem Zusammenhang konnte in dieser Studie nicht bestätigt werden. Die Untersuchungen zeigten, dass die von *A. butzleri* genutzten Virulenzmechanismen denen von etablierten Darmpathogenen wie z.B. *Campylobacter jejuni* ähnlich sind. Zusätzlich zu den Einflüssen von *A. butzleri* auf Makrophagen konnten teils starke isolatabhängige Unterschiede in den Untersuchungen festgestellt werden, so dass eine stammspezifische Variation des Virulenzpotentials naheliegt.

Der zweite Teil der Arbeit untersuchte die miRNA Antwort primärer humaner Makrophagen auf die Infektion mit *A. butzleri*. Dafür wurden Makrophagen von 3 verschiedenen Donoren isoliert und infiziert. Die anschließende Sequenzierung der exprimierten miRNAs diente der Erweiterung derzeit bestehender Kenntnisse über die Expression von miRNAs in Wirtszellen während bakterieller Infektionen. Eine Analyse der Daten ergab, dass eine erhöhte

Expression besonders diejenigen miRNAs betraf, die für autoregulative Feedbackmechanismen verantwortlich sind, um überschießende Immunreaktionen zu vermeiden. Eine veränderte Expression im Vergleich zur Negativkontrolle zeigten in diesem Zusammenhang miR-125a, miR-146a, miR-155, miR-212, miR-181b/c/d, miR-21, miR-99b, miR-27a, let-7a, miR-26b und miR-148a. Zusätzlich reagierten die Makrophagen mit erhöhter Expression einiger miRNAs, die bis jetzt noch nicht mit bakteriellen Infektionen in Zusammenhang gebracht wurden (miR-3613, miR- 590, miR-941, miR-2116, miR-671, miR-30d, miR-339, miR-629 und miR-193a).

Zusammengefasst, tragen die in dieser Studie generierten Daten zum besseren Verständnis der molekularen Interaktion von *A. butzleri* und humanen Makrophagen bei, einerseits unerlässliche Informationen zur Evaluierung der Pathogenität, andererseits die Basis für die Entwicklung angemessener Therapiemöglichkeiten.

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D. Appendix

Table 14: DAVID functional annotation list of *A. butzleri* induced miRNAs and potentially influenced pathways

miRNA	pathway	count	p value	genes	list total
3613-5p	Glycerolipid metabolism	4	0.012	ALDH7A1, AKR1B1, GPAM, ALDH9A1	56
	Ascorbate and aldarate metabolism	3	0.014	ALDH7A1, UGT2B15, ALDH9A1	56
	p53 signaling pathway	4	0.037	CDK6, RRM2B, ATR, SESN3	56
	Tryptophan metabolism	3	0.069	ALDH7A1, CYP1B1, ALDH9A1	56
	Pyruvate metabolism	3	0.069	ALDH7A1, AKR1B1, ALDH9A1	56
	Steroid hormone biosynthesis	3	0.088	CYP1B1, CYP7A1, UGT2B15	56
2116-3p	Wnt signaling pathway	21	0.003	FZD8, DVL3, WNT10B, ROCK1, ROCK2, NLK, CAMK2G, CSNK1A1L, CHP2, PPP3R2, FZD7, PRKCB, WNT2, MAP3K7, SENP2, EP300, PRICKLE2, NFAT5, FRAT2, WNT7A, NFATC3	352
	B cell receptor signaling pathway	12	0.013	MAPK1, CR2, PIK3CB, SOS2, NFAT5, CHP2, PPP3R2, VAV2, NFATC3, PIK3R1, PRKCB, BTK	352
	p53 signaling pathway	11	0.017	EI24, CD82, RRM2, SERPINE1, CASP8, RCHY1, RRM2B, MDM4, PERP, CDK2, SESN3	352
	ErbB signaling pathway	12	0.036	MAPK1, CDKN1B, EREG, PIK3CB, CAMK2G, BTC, CBL, GAB1, SOS2, ELK1, PIK3R1, PRKCB	352
	Melanogenesis	13	0.039	DVL3, FZD8, ADCY1, WNT10B, ADCY2, CAMK2G, CREB1, FZD7, PRKCB, WNT2, MAPK1, EP300, WNT7A	352
	VEGF signaling pathway	10	0.072	MAPK1, PIK3CB, NFAT5, CHP2, MAPKAPK3, PPP3R2, PLA2G2D, NFATC3, PIK3R1, PRKCB	352
	Apoptosis	11	0.076	IRAK2, IRAK1, IRAK3, PRKAR2A, DFFA, PIK3CB, CASP8, CHP2, EXOG, PPP3R2, PIK3R1	352
	Neurotrophin signaling pathway	14	0.084	IRAK2, IRAK1, PIK3CB, CAMK2G, IRAK3, MAPK1, YWHAG, PRDM4, NTRK2, SOS2, GAB1, SH2B3, MAPK7, PIK3R1	352
	Prostate cancer	11	0.085	FGFR1, MAPK1, CDKN1B, EP300, PIK3CB, CREB1, SOS2, NKX3-1, CREB5, CDK2, PIK3R1	352
	Fc epsilon RI signaling pathway	10	0.087	MAPK1, GAB2, PIK3CB, SOS2, VAV2, PRKCE, PLA2G2D, PIK3R1, PRKCB, BTK	352
	Long-term potentiation	9	0.094	MAPK1, ADCY1, EP300, GRIN2B, CAMK2G, PPP1R1A, CHP2, PPP3R2, PRKCB	352
	Pathways in cancer	30	0.097	FGFR1, FGF16, EGLN1, WNT2, PAX8, CASP8, SOS2, NKX3-1, RALA, PIK3R1, FZD8, DVL3, WNT10B, EPAS1, PIK3CB, CBL, SKP2, FGF23, MECOM, DAPK2, CDK2, FZD7, CTNNA2, PRKCB, MAPK1, CDKN1B, EP300, ITGA6, PIAS2, WNT7A	352
671-3p	MAPK signaling pathway	5	0.063	RASGRF1, FGF11, CACNB3, FGF1, CRK	30
30d-3p	Wnt signaling pathway	19	0.011	FZD8, TBL1XR1, DVL3, VANGL1, ROCK2, CAMK2G, SMAD2, FZD4, FZD6, MAP3K7, CSNK2A1, CSNK1E, CACYBP, FRAT1, MAPK8, SIAH1, PRKACB, PLCB1, APC	340
	Prion diseases	7	0.026	C8A, EGR1, NCAM2, IL1B, HSPA5, PRKACB, PRNP	340
	Insulin signaling pathway	16	0.034	IRS2, PIK3CB, PHKB, PRKAB2,	340

	Renal cell carcinoma	10	0.041	PRKCI, MKNK1, PPP1CB, SORBS1, SOS1, PRKAR1A, SOS2, MAPK8, PRKAA2, PRKACB, CRK, AKT3 EPAS1, PIK3CB, SOS1, GAB1, SOS2, TGFA, EGLN1, TCEB1, CRK, AKT3	340
	Colorectal cancer	11	0.051	FZD8, DVL3, PIK3CB, SOS1, SOS2, MAPK8, SMAD2, FZD4, AKT3, FZD6, APC	340
	Alanine, aspartate and glutamate metabolism	6	0.052	ADSS, GOT1, GFPT1, GLS, GAD1, PPAT	340
	ErbB signaling pathway	11	0.062	CDKN1B, ERBB4, PIK3CB, CAMK2G, SOS1, GAB1, SOS2, TGFA, MAPK8, CRK, AKT3	340
	Endocytosis	19	0.063	SH3GL3, DNM3, FLT1, ERBB4, STAM2, VTA1, PRKCI, ARF6, HLA-B, KIT, ZFYVE20, RAB31, AP2B1, RAB11FIP2, CHMP1B, RAB22A, RAB11B, VPS36, RNF41	340
	Melanogenesis	12	0.064	FZD8, DVL3, GNAI3, GNAQ, CAMK2G, CREB1, CREB3L3, KIT, PRKACB, PLCB1, FZD4, FZD6	340
	Regulation of actin cytoskeleton	21	0.079	GNA13, FGF7, PIK3CB, ROCK2, DIAPH2, SSH2, GNA12, ITGA1, ACTN2, PPP1CB, NCKAP1, DOCK1, CHRM2, TIAM1, SOS1, SOS2, WASL, CRK, FGF2, MYLK, APC	340
	Neuroactive ligand-receptor interaction	24	0.085	F2RL2, GABRG1, PTGER2, TACR3, RXFP1, GABRB3, CCKBR, OPRK1, GLRA3, LEPR, NPY2R, GRIN2A, P2RY13, PRLR, CHRM2, NMUR1, CNR1, HTR7, NPFFR2, ADRA2C, GLP2R, HTR2C, GABRQ, GABRP	340
	One carbon pool by folate	4	0.086	MTHFD2, MTHFR, DHFR, MTHFD1L	340
30d-5p	Limonene and pinene degradation	3	0.033	ALDH2, LCLAT1, YOD1	106
	Ether lipid metabolism	4	0.035	PLA2G12A, LCLAT1, PLA2G2C, PAFAH1B2	106
	Natural killer cell mediated cytotoxicity	7	0.056	CASP3, TNFRSF10B, NFAT5, PPP3CA, SH2D1B, SH3BP2, LCP2	106
	ABC transporters	4	0.062	ABCC9, ABCG5, ABCD2, ABCC4	106
	Endocytosis	8	0.085	NEDD4, ACAP2, RAB11A, EEA1, NEDD4L, KIT, ARAP2, CHMP2B	106
	Amyotrophic lateral sclerosis (ALS)	4	0.096	CASP3, MAP3K5, DERL1, PPP3CA	106
	MAPK signaling pathway	10	0.099	MAP3K7, CASP3, MAP3K5, TAOK1, MAP3K2, PLA2G12A, NF1, PLA2G2C, PPP3CA, FGF20	106
339-5p	Lysosome	15	0,016	HGSNAT, PLA2G15, AP4E1, CTSS, M6PR, GNS, SLC11A1, LAPTM5, NAGA, SORT1, NEU1, SCARB2, CTSB, GGA2, ATP6V0D2	324
	Apoptosis	12	0,021	IRAK2, IRAK1, IRAK3, PRKAR2A, DFFB, CYCS, CASP8, CHP2, PRKACA, APAF1, PIK3R3, ATM	324
	Small cell lung cancer	11	0,039	COL4A4, MAX, CDKN2B, CYCS, ITGA2, ITGA3, APAF1, LAMC1, PIK3R3, CDK2, TRAF4	324
	Endocytosis	19	0,043	PARD6B, EPN3, USP8, ERBB4, TGFBR1, CBL, CXCR1, HLA-E, CHMP2B, DAB2, SMAP1, CHMP1B, RABEP1, TFRC, RAB22A, MDM2, PARD6G, ITCH, EHD4	324
	Biosynthesis of unsaturated fatty acids	5	0,047	ACOX1, ELOVL2, ELOVL6, SCD5, YOD1	324
	Heparan sulfate biosynthesis	5	0,079	B3GAT2, NDST3, HS3ST1, EXT1, HS3ST3B1	324

629-5p	Wnt signaling pathway	11	0,003	PLCB3, TCF7, SFRP2, VANGL2, NFAT5, LRP6, FZD3, SMAD2, MAPK10, NFATC2, MYC	120
	ErbB signaling pathway	8	0,004	NRG4, GRB2, GAB1, MAPK10, MAP2K7, ABL2, MYC, AKT3	120
	Colorectal cancer	7	0,013	TCF7, GRB2, FZD3, SMAD2, MAPK10, MYC, AKT3	120
	Proteasome	5	0,023	PSMA2, PSMB10, PSMD13, PSMB2, PSME4	120
	Neurotrophin signaling pathway	8	0,025	YWHAZ, GRB2, GAB1, MAPK10, FOXO3, MAP2K7, AKT3, CALM1	120
	Endometrial cancer	5	0,033	TCF7, GRB2, FOXO3, MYC, AKT3	120
	Insulin signaling pathway	8	0,038	PRKAR2A, PTPRF, TSC1, GRB2, MAPK10, INSR, AKT3, CALM1	120
	Acute myeloid leukemia	5	0,046	TCF7, GRB2, MYC, STAT3, AKT3	120
	Sphingolipid metabolism	4	0,062	SGMS2, KDSR, CERK, GAL3ST1	120
	Adipocytokine signaling pathway	5	0,071	MAPK10, ADIPOQ, STAT3, AKT3, ACSL6	120
	Tight junction	7	0,093	CLDN8, RAB3B, MAGI2, MYH11, CLDN2, TJP2, AKT3	120
193-5p	Calcium signaling pathway	15	0.007	ADCY1, ADCY2, TACR3, ERBB3, ERBB2, CAMK2G, OXTR, NTSR1, PRKX, ITPR2, ADRB2, GNAQ, PLCG2, PRKACB, PLCB1	195
	Melanogenesis	10	0.013	TYRP1, ADCY1, ADCY2, GNAQ, CAMK2G, CREB3L2, CREB3L3, PRKACB, PLCB1, PRKX	195
	Endocytosis	14	0.023	FAM125B, ERBB3, VTA1, CXCR2, PSD2, HLA-F, CBLB, ADRB2, RAB11FIP3, CHMP1B, ACAP2, DNAJC6, ITCH, ARAP3	195
	Vascular smooth muscle contraction	10	0.026	ADCY1, ADCY2, GNAQ, PPP1R12B, CALCRL, PRKACB, PLCB1, PRKX, ARHGEF11, ITPR2	195
	Long-term potentiation	7	0.044	ADCY1, GNAQ, CAMK2G, PRKACB, PLCB1, PRKX, ITPR2	195
	Viral myocarditis	7	0.053	CASP8, HLA-DMB, HLA-DOA, ABL2, MYH10, SGCB, HLA-F	195
	Dilated cardiomyopathy	8	0.061	ADCY1, ADCY2, CACNG8, CACNG4, PRKACB, PRKX, SGCB, TGFB2	195
	GnRH signaling pathway	8	0.079	ADCY1, ADCY2, GNAQ, CAMK2G, PRKACB, PLCB1, PRKX, ITPR2	195
	RNA polymerase	4	0.089	POLR3H, POLR2L, POLR2J, ZNRD1	195
	N-Glycan biosynthesis	5	0.097	MGAT4A, MGAT3, GANAB, ALG10B, DOLPP1	195

D.1. List of publications

D.1.1. Publications

Part of this work was published in:

zur Bruegge J, Hanisch C, Einspanier R, Alter T, Gölz G, Sharbati S (2014): *Arcobacter butzleri* induces a pro-inflammatory response in THP-1 derived macrophages and has limited ability for intracellular survival. Int J Med Microbiol. 304 (8): 1209-1, DOI 10.1016/j.ijmm.2014.08.017

Bohmer M, Sharbati J, **zur Bruegge J**, Einspanier R, Sharbati S (2013): Structural Analysis of microRNA-Target Interaction by Sequential Seed Mutagenesis and Stem Loop 3' RACE. PLoS one; 8 (11): S. e81427, DOI 10.1371/journal.pone.0081427

D.1.2. Oral presentations

zur Bruegge J, Sharbati J, Hoeke L, Einspanier R, Sharbati S: miRNA response of immune cells during bacterial infection. 9th Workshop Molecular Interactions 2013, Berlin

zur Bruegge J, Einspanier R, Alter T, Gölz G, Sharbati S: Evaluation of Quorum Sensing of *Campylobacteraceae* as a mediator for host-pathogen-interaction. Mini-Symposium Biology of Nutrition (SFB 852) 2013, Berlin

D.1.3. Poster presentation

zur Bruegge J, Gölz G, Einspanier R, Alter T, Sharbati S: Cytokine induction and virulence mechanisms of *Arcobacter butzleri* in human macrophages. 17th International Workshop on Campylobacter, Helicobacter & Related Organisms 2013, Aberdeen, Schottland

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D.2. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den Unterschrift