THE EFFECTS OF SOLAR IRRADIATED *SALMONELLA* TYPHIMURIUM AND *CAMPYLOBACTER JEJUNI* ON THE PROLIFERATION AND ACTIVATION OF MACROPHAGES *IN VITRO*

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Date: December 2019

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Finally, I thank God for giving me a passion for helping people. I hope this work will be helpful in providing healthier, happier lives through the development of solar directed efforts of improving the health and well-being of individuals.

DEDICATION

Firstly, I would like to dedicate this thesis to God; without whom…, all this would not have been possible. Secondly, I dedicate this thesis to my loving parents Simon and Jane, my siblings Memory, Meroline, Tendai and Sandra Chiomvu. Finally, to my one and only begotten son Ethan Leareng.

ABSTRACT

Salmonella enterica serovar Typhimurium and *Campylobacter jejuni* are the leading causes of Salmonellosis and Campylobacteriosis that is characterised by gastroenteritis. These waterborne diseases can be easily prevented by home water treatment methods such as solar disinfection (SODIS). The SODIS process involves placing microbiologically unsafe water in clear plastic or glass bottles and exposing them to direct sunlight for approximately six to eight hours. SODIS kills microbes through a combination of DNA-damaging effects of ultraviolet (UV) radiation and thermal inactivation from solar heating. The result is microbiologically safe water. Continuous drinking of SODIS treated water may confer some immunological effects on the consumer. These immunological effects have not been thoroughly explored. Therefore, the objectives of this study were to firstly, characterise the effects of solar irradiation on the viability of *S.* Typhimurium and *C. jejuni*; secondly, to determine the cytotoxicity and modulation of cell death of solar irradiated *S.* Typhimurium and *C. jejuni* on macrophages. Thirdly, to analyse the chemokine and cytokine profiles of macrophages infected with solar irradiated *S.* Typhimurium and *C. jejuni*. Lastly, to analyse the host-cell interactions of macrophages infected with solar-irradiated and non-solar irradiated *S.* Typhimurium *and C. jejuni* using a proteomic approach.

In all the experiments, *S.* Typhimurium and *C. jejuni* were (i) heat/chemically treated, (ii) solar and non-solar irradiated for 4 and 8 hours. A murine macrophage cell line RAW264.7 was co-cultured with the differentially treated bacteria species for 3 and 24 hours. Appropriate controls were included.

The impact of solar irradiated *S.* Typhimurium and *C. jejuni* on intracellular growth, proliferation, cytotoxicity, and apoptosis on macrophages was assessed. Intracellular growth of the both bacterial species was assessed with the gentamicin protection assay, and cytotoxicity was determined by Lactate Dehydrogenase Assay (LDH). The macrophages treated with solar irradiated *S.* Typhimurium and *C. jejuni* showed no intracellular growth after 48 hours post-infection. However, the non-irradiated *S.* Typhimurium survived within the macrophages and were highly toxic to the macrophages (average cytotoxicity of 91%±32). The non-solar irradiated *C. jejuni* were metabolically active but non-culturable, whereas the solar-irradiated *C. jejuni* was metabolically inactive. Thus, solar irradiated *C. jejuni* showed a lower percentage cytotoxicity (2.57% ± 0.32%) in comparison to non-solar irradiated *C. jejuni* at 24 hours post-infection (p.i.) (30.28% ± 0.05%). Flow cytometric analysis showed that the non-irradiated *S.* Typhimurium brought about a statistically significant increase in the percentage of necrotic cells (48% ± 2.99%), whereas bacteria irradiated for 8 hours produced a lower percentage of necrotic cells (25% ± 5.87%). The heat/chemical attenuated samples

had the lowest percentage of necrotic cells (21.15% ± 5.36%) at 24 h p.i. Macrophages treated with solar irradiated and non-solar irradiated *C. jejuni* did not induce necrosis, but apoptotic cell death. At 24 h p.i., the highest proportion of apoptotic cell death was observed in macrophages treated with non-solar irradiated *C. jejuni* whereas the solar irradiated *C. jejuni* showed a lower percentage of apoptotic cell death. Therefore, there is great possibility that *S.* Typhimurium and *C. jejuni* could become avirulent after SODIS treatment and this could prevent gastroenteritis in consumers of SODIS-treated water.

The activation of macrophages infected with solar irradiated *S*. Typhimurium and *C. jejuni* was also assessed in this study. The production of nitric oxide (NO) was determined using the Greiss Reagent Assay, whereas the production of chemokines, cytokines, and growth stimulating factors by the RAW264.7 cells *in vitro* was measured using the Luminex 200. The results showed that both solar and non-solar irradiated *S.* Typhimurium inhibited the production of nitric oxide in the RAW264.7 cells. The heat/chemically attenuated *S.* Typhimurium induced a significant increase ($p<0.0.5$) in the production of NO_2- in the macrophages when compared to the unstimulated RAW264.7. The chemokine and cytokine levels produced by the macrophages were similar in the solar inactivated *S.* Typhimurium and the live untreated *S.* Typhimurium. However, macrophages treated with heat/chemically attenuated *S.* Typhimurium showed an anti-inflammatory response by inhibiting the production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-6, and IL-17 in macrophages. The macrophages treated with solar and non-solar irradiated *C. jejuni* possibly produced an anti-inflammatory effect since the amount of pro-inflammatory cytokines in the samples was significantly reduced during the late infection period (24 h p.i.).

This study also analysed the proteomic profiles of macrophages treated with LPS, non-solar irradiated, solar irradiated, heat/ chemical inactivated *S*. Typhimurium, and *C. jejuni.* This was carried out using SWATH-mass spectrophotometry-based proteomics. Proteins were extracted from infected macrophages after 24 hours p.i. HILIC-based sample clean-up and digestion, DDA LCMS-MS (spectral library), SWATH LCMS-MS, and data processing were carried out. A total of 15,077 peptides matching to 2,778 proteins were identified at 1% FDR with numerous differentially expressed proteins (DEPs) detected in macrophages treated with lipopolysaccharide (LPS), non-solar irradiated *C. jejuni* (NS), heat-attenuated *C. jejuni* (HA) and 4h-solar irradiated (SI4) and 8h-solar irradiated (SI8) *C. jejuni,* respectively. Pathway analysis revealed that most of the upregulated proteins in macrophages treated with solar irradiated *C. jejuni* were involved in oxidation-reduction processes, endoplasmic reticulum stress, transport, antigen processing and presentation of exogenous peptide antigens via MHC class I (TAP-dependant) and ATP-biosynthetic processes. The KEGG-pathways also revealed the roles of some upregulated proteins in lysosomal and phagosome pathways. In

conclusion, our results revealed that there is coordinated up-regulation of MHC-I processing pathways occurred at 24 h p.i. It is likely that proteins from solar irradiated *C. jejuni* may undergo proteasomal degradation, and the peptides are transported to the endoplasmic reticulum (ER) and loaded onto MHC-I molecules. Peptide loading results in class I complexes consolidation and transit to the cell surface where antigens can be presented to circulating CD8 + T cells. Additionally, solar irradiated *C. jejuni* also undergoes degradation in the phagosome. The phagosome has the potential to create antigens that can be expressed on the cell surface of macrophages to stimulate different lymphocytes and induce appropriate immune responses, thus, connecting the innate to adaptive immunity, and this could also have health benefits via the consumption of SODIS treated water.

However, proteomic analysis of *S.* Typhimurium showed no significant differentially expressed proteins in macrophages treated with LPS, non-solar irradiated, and solar irradiated *S.* Typhimurium. This may be due to an overestimation of the extracted protein. However, DEPs in macrophages treated with heat-attenuated *S.* Typhimurium showed that macrophages may have adapted an anti-inflammatory M2 phenotype because the IFN-γ signalling pathway was downregulated. This may have contributed to non-expression of the chemokine IFN-γ in RAW264.7 cells. Moreover, proteins such as Hmox1 and Sqstm1 were upregulated, and this is also characteristic of M2 macrophages.

This study provided new insights on the effect of solar irradiated *Salmonella* Typhimurium and *Campylobacter jejuni* on the proliferation and activation of macrophages *in vitro*.

Keywords:
Campylobacter jejuni, chemokines, cytokines, proteomics, apoptosis, pyroptosis, *Sallmonella* Typhimurium, solar disinfection

ABBREVIATIONS AND ACCRONYMS

7-AAD	7- amino actinomycin D
Ab	Antibody
ABNC	Active-but-non-culturable
AMP	Anti-microbial Peptides
APC	Antigen Presenting Cells
ARS	Arylsufatase
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
C	Complement
CALR	Calreticulin
CANX	Calnexin
CARD	Caspase Activation and Recruitment Domain
CCL	CC-chemokine Ligand
CCR	CC-chemokine Receptor
CD	Cluster of Differentiation
CDT	Cytolethal Distending Protein
CFU	Colony Forming Units
CPD	Cyclobutane Pyrimidine Dimer
CSF	Colony Stimulating Factor
CSIR	Centre for Scientific and Industrial Research
CTL	Cytotoxic T lymphocytes
CXCL	CXC-chemokine Ligand
CXCR	CXC-chemokine Receptor
d	day (s)
Da	Dalton
DAMPs	Damage associated molecular proteins
DAVID	Database for annotation, visualisation and integrated discovery
DC	Dendritic cells
DDA	Data dependent acquisition
DEP	Differentially expressed protein
DIA	Data independent acquisition
DMEM	Dulbecco’s Modified Eagle Medium

DNA	Deoxyribonucleic Acid
DOM	Dissolved Organic Matter
DPBS	Dulbecco's phosphate buffer solution
DTT	Dithiothreitol
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FACS	Flourescent Activated Cell Sorter
FBS	Foetal Bovine Serum
FC	Fold Change
Fcc-R	Fcc Receptor
FDR	False discovery rate
FITC	Flourescein isothiocyanite
GBS	Guillain–barré syndrome
G-CSF	Granulocyte-colony stimulating factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GNS	Acetylglusamine-6-sufatase
GO	Gene ontology
GSDM D	Gasdermine D
h	hour(s)
HILIC-SPE	Hydrophilic interaction chromatography magnetic micro-particles for solid phase
HIV	Human Immunodeficiency Virus
HMGB	High Mobility Group Box
HSP	Heat Shock Proteins
HT	High throughput
IFN	Interferon
IL	Interleukin
iNOS	Nitric oxide synthase
IP-10	Gamma-induced protein 10kDa
IRF	Interferon Regulatory Factor
JAK	Janus kinase
KC	Keratinocyte chemoattractant
kDa	KiloDaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Bertani
LC	Liquid chromatography

LDH	Lactate Dehydrogenase
LDL	Lower Detectable Limit
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAF	Macrophage Activating Factor
MALDI	Matrix Assisted Laser Desorption Ionization
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
MBL	Mannose Recognition Receptors
MCP	Methyl-accepting chemotaxis protein
MCP	Monocyte Chemoattractant Protein
MDG	Millenium Development Goals
MDR	Multi drug resistant
M-H	Mueller Hinton
MHC	Major Histocompatibility Complex
Min	Minute(s)
MIP	Macrophage Inflammatory Protein
MIP	Macrophage inflammatory protein
ml	Millilitres
MPIF	Myeloid Progenitor Inhibitory Factor
MR	Mannose Receptor
mRNA	messenger Ribonucleic Acid
MS	Mass spectroscopy
MSU	Monosodium urate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NALP3	NACHT, LPR, and PYD domains containing protein 3
NER	Nucleotide Excision Repair
NF-kB	Nuclear Factor-kB
NIH	National Institutes for Health
NK	Natural Killer Cell
NO	Nitric Oxide
NOD	Nonobese diabetic
NOD	Nucleotide-binding Oligomerization Domain
NS	Not significant
NTS	Non Typhoidal Salmonellae
NTU	Nephelometric Turbidity Units
OD	Optical Density
p.i.	post-infection

PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PER	Photoenzymatic Repair
PerCP	Peridinin Chlorophyll Protein
PET	Polyethylene Terephthalate
Pg	Picogram
PMN	Polymorphnuclear (cell, leukocyte)
PPP	Pentose phosphate pathway
PQP	Peptide query parameters
PRR	Pattern Recognition Receptor
PSM	Peptide spectrum matches
PTM	Post-translational modification
PTP	Protein tyrosine phosphatase
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RELM	Resistin-like molecule
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCV	Salmonella Containing Vacuole
SEM	Standard error of the mean
SOCS	Suppressors of cytokine signaling
SODIS	Solar disinfection
SP	Surfactant Proteins
SPI	Salmonella pathogenicity island
SR	Sulforhodamine
STAT	Signal Transducter and Activation of Transcription
STRING	Search Tool for Retrieval of Interacting Genes/ Proteins
SUVR	Solar Ultraviolet Radiation
SWATH-MS	Sequential window acquisition of all theoretical mass spectra
T3SS	Type III Secretion System
T4SS	Type IV Secretion System
TAP	Transporter associated with antigen processing
TCA	Tricarboxylic acid cycle
TGF	Transforming Growth Factor
TGM	Transglutaminase
Th	T helper
THBC	Total Heterotrophic Bacterial Count

TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TPP	Tripeptidyl peptidase
UDL	Upper Detectable Limit
ug	Microgram
ul	Microlitre
UNICEF	United Nations Children's Fund
US	United States
UV	Ultraviolet
UV-A	Ultraviolet-A
UV-B	Ultraviolet-B
UV-C	Ultraviolet-C
UVR	Ultraviolet Radiation
v/v	Volume to volume ratio (%)
v/w	Volume to weight ratio (%)
VAD-FMK	Valylalanyl aspartic acid flouromethyl ketone
VBNC	Viable But Not Culturable
W	Watts
WHO	World Health Organisation

PUBLICATIONS

MANUSCRIPTS UNDER REVIEW

1. **Patience Chihomvu**, Cornelius Cano Ssemakalu, Eunice Ubomba-Jaswa & Michael Pillay.

   Solar irradiated *C. jejuni* induces nitric oxide production, chemokine and cytokine release in RAW264.7 murine macrophages.

   Submitted in *Microbial Pathogenesis*
2. **Patience Chihomvu**, Cornelius Cano Ssemakalu, Eunice Ubomba-Jaswa & Michael Pillay. Assessing the cytotoxicicity and apoptosis-inducing ability of solar irradiated *Salmonella* Typhimurium in RAW264.7 cell line

   Submitted in *Journal of Microbiology, Biotechnology and Food Sciences*

CONFERENCE ATTENDANCE

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South African Immunology Society

Chihomvu, P., Ssemakalu, C.C., Ubomba-Jaswa, E., Pillay, M. (2017). Investigating the morphological features and *in vitro* cytopathic effects of solar irradiated and non-solar irradiated *Campylobacter jejuni* on RAW 264.7 cells.

Keystone Symposia. Cells vs. Pathogens: Intrinsic defences and counterdefenses (C4), Hyatt Regency Monterey, Monterey, California, USA (2018)

Chihomvu P., Ssemakalu, C.C., Ubomba-Jaswa, E., Pillay, M. (2018). Investigating the cytopathic, cytotoxic and apoptotic effects of solar-irradiated pathogens on murine macrophages (RAW264.7).

South African Society of Microbiology (SASM), Misty Hills Hotel and Conference Centre, Muldersdrift, South Africa (2018).

South African Society of Microbiology (SASM)

Chihomvu P, Ssemakalu C. C., Ubomba-Jaswa E., Pillay M. (2018). Assessing the effect of solar irradiated *Campylobacter jejuni* on the production of iNOS, pro-inflammatory cytokines and chemokines by RAW 264.7 macrophage cells *in vitro*.

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Chapter 1 Introduction

1.1 Background

Solar disinfection (SODIS) is the use of natural sunlight to treat microbiologically contaminated water and make it available for drinking. Solar disinfection has reduced the spread and contraction of water-related infections, most of which are zoonotic in nature. Zoonosis is commonly defined as a disease that is transmissible from animals to humans. Zoonotic diseases account for 70% of all emerging infectious diseases (Woolhouse & Gowtage-Sequeria, 2005; Kahn, 2010). The spread of waterborne zoonotic diseases is made worse by an unprecedented increase in global livestock population and unethical animal husbandry practices. As a result, there is an increase in the prevalence and environmental load of waterborne zoonotic pathogens (Dufour, 2012). The reduction of zoonotic pathogen shedding in a farm or production facilities for domesticated animals may be accomplished by preventing illnesses in livestock. This can be achieved through minimizing exposure to pathogens, immunization of the livestock, manipulation of the animal gastrointestinal tract microbial ecology and managing animal waste to reduce the release of zoonotic pathogens into the environment (Dufour, 2012).

Although several zoonotic pathogens affect humans, five are known to cause illnesses with high frequency around the world. They include *Cryptosporidium, Giardia, Campylobacter*, *Salmonella* and *E. coli* O157. *Cryptosporidium* and *Giardia* are protozoan species that cause cryptosporidiosis and giardiasis, respectively. These parasitic organisms infect the mammalian intestinal tract (Dufour, 2012). However, this study focuses on *Salmonella* and *Campylobacter.*

Diseases due to *Salmonella*, whether caused by typhoidal or non-typhoidal serovars, have become a significant burden worldwide. The lack of robust diagnostic methods, to date, suggests that this burden is relatively neglected. Improvements in hygiene, sanitation and the availability of clean water are known to help in the prevention and control of enteric diseases. Typhoid (enteric) fever caused by *Salmonella enterica* serovar Typhi remains a serious health problem in many parts of Africa and Asia. Currently over a 65% of isolates in many endemic areas are multi drug resistant (MDR), and exhibited an increased resistance to fluoroquinolones. The situation is aggravated in resource-limited settings where the few remaining active antimicrobials are either unavailable or too costly to be afforded by either the general public or by public health services (Kariuki *et al.*, 2015).

Another water-borne pathogen, *Campylobacter jejuni,* is one of the most common causes of gastroenteritis and poses a significant threat to the health of individuals who consume contaminated water. Infections resulting from *C. jejuni* are more frequently reported as opposed to those caused by either *S.* Typhimurium or *E. coli* O157: H7 (Mead *et al.*, 1999). In contrast to other enteric infections, *C. jejuni* mediated enteritis is often a self-limiting disease, with symptoms ranging from fever, abdominal cramping and acute intestinal inflammation leading to bloody diarrhoea (Siegesmund *et al.*, 2004).

The burden that water-related diseases impose on resource-poor communities worldwide requires multifaceted solutions that encourage participation from all tiers of society. According to WHO (2014a), the use of chlorine tablets as a water treatment option could cost an additional US$2 billion, globally. Improving access to improved water and sanitation, by building pit latrines, introducing piped water into dwellings, installing public taps, boreholes, and digging wells would cost approximately US$22.6 billion (World Health Organization, 2014). Despite the investment towards the provision of water and sanitation at the global level, a significant proportion of the world's population remains without access to adequate water sources. Therefore, low-cost water treatment methods such as solar disinfection (SODIS) have been recommended as a short-term intervention. Moreover, field trials have shown significant health benefits of consuming SODIS treated water (Conroy *et al.*, 1996; Conroy *et al.*, 1999). The effectiveness of SODIS against cholera was assessed in a Kenyan health impact assessment and found that there was an 86% reduction in cholera cases in households regularly using SODIS (Conroy *et al.*, 2001).

However, the epidemiological benefits of drinking SODIS water goes beyond the biology and technique of microbiological inactivation (Ssemakalu *et al.*, 2014). Therefore, the immunological effects of consuming SODIS water have to be considered.

1.2 Rationale and Motivation

The unavailability of safe drinking water in developing nations remains an urgent and unsolved problem. There are approximately 844 million people who are consuming water from unprotected water sources (UNICEF & World Health Organization, 2017). The consumption of untreated water often results in the contraction and spread of water-related diseases such as typhoid, cholera, dysentery, respiratory diseases and in some cases death, especially among infants.

As a consequence, low-cost technologies such as SODIS, have been recommended as interventions in communities where potable water is not available. Although SODIS treatment of water improves its microbiological quality and health of the consumers, there are several

unanswered questions related to its use. For instance, there is very little information regarding the effect that SODIS-treated microorganisms may have on the immunity of the consumers. There is no published data on the influence that SODIS-treated pathogens such as *S.* Typhimurium and *C. jejuni* could have on the activation of cells of the immune system such as the macrophages. Activation of the macrophages enhances both phagocytic and endocytic capabilities which are key towards fighting infectious agents. In addition, once activated, macrophages secrete pro-inflammatory cytokines such as the Type I interferons IFN-γ and TNF-α.

Furthermore, the expression of co-stimulatory molecules for antigen presentation also increases, following macrophage activation (Gordon, 2003). Therefore, solar disinfected water may play a crucial role in the activation of macrophages since it contains a variety of antigenic material. There is no clear evidence of how macrophages respond to SODIS-treated pathogens.

This study will (i) provide new insights on the cellular damage caused by solar irradiation on the pathogens such as *S.* Typhimurium and *C. jejuni;* (ii) determine the quality of antigens produced; (iii) obtain new information on the possible reactivation of solar irradiated pathogens within macrophages, and (iv) determine the ability of solar irradiated *S.* Typhimurium and *C. jejuni to* moderate the pro-inflammatory response of macrophages.

1.3 Problem statement

The water shortage currently being faced by a vast portion of the world's population adversely affects the health of individuals especially those consuming water from microbiologically contaminated sources. This situation has led to the spread of water-borne diseases such as typhoid and gastroenteritis. In order to curb the spread of water-borne diseases, household water treatment methods such as SODIS have been implemented. Therefore, this raises the question of whether the consumption of SODIS-treated water improves the immune response to pathogens, and if it does how do the antigens present in SODIS-water modulate the pro- and anti-inflammatory response of macrophages?

1.4 Aim

To assess the effect of solar irradiated *S.* Typhimurium and *C. jejuni* on the proliferation and activation of macrophages *in vitro*.

1.5 Objectives

1.5.1 To characterise the effects of solar irradiated *S.* Typhimurium and *C. jejuni* on intracellular growth, cytotoxicity and modulation of cell death on macrophages.

1.5.2 To investigate the chemokine and cytokine profiles of macrophages infected with solar irradiated *S.* Typhimurium and *C. jejuni*.

1.5.3 To analyse the host-cell interactions of macrophages infected with solar-irradiated and non-solar irradiated *S.* Typhimurium and *C. jejuni* using a proteomic approach.

1.6 The scope of the research

The study will focus on assessing the activation and proliferation of macrophages infected with solar irradiated *S.* Typhimurium and *C. jejuni*. The study will focus on results obtained from microbiological, analytical biochemistry, molecular tests and immunological tests carried out *in vitro.*

1.7 Limitations

The limitation of this study is that still bottled water will be used in the experiments because it offers a constant environment. However, communities using SODIS treatment hardly use still bottled water. Another limitation is the use of pure cultures in the experimental studies, environmental samples contain mixed cultures, and therefore the behaviour of the pure cultures may not mimic the behaviour of pathogens present in the environment.

Chapter 2 Literature Review

2.1 Solar disinfection (SODIS)

Solar disinfection (SODIS) treatment is a simple process of inactivating microorganisms in contaminated water by exposure to natural sunlight. The process involves filling 1–2-liter clear polyethylene terephthalate (PET) bottles up to two-thirds of its capacity with untreated water preferably with a turbidity below 30 nephelometric turbidity units (NTU). The containers are vigorously shaken to increase the amount of dissolved oxygen and then exposed to the sun for 6-8 hours on a sunny day, or between 2 and 3 days if cloudy (Solar Water Disinfection, 2002). After solar disinfection, the microbiological quality of water significantly improves and it is deemed safe to drink (Solar Water Disinfection, 2002). Solar disinfection is one of cheapest and easiest home water treatment methods in areas where access to electricity and chemical supplies is either limited or unavailable (McGuigan *et al.*, 2012). It has been implemented for over 30 years in over 50 countries in Asia, Latin America and Africa (Fig.1) (McGuigan et al., 2012).

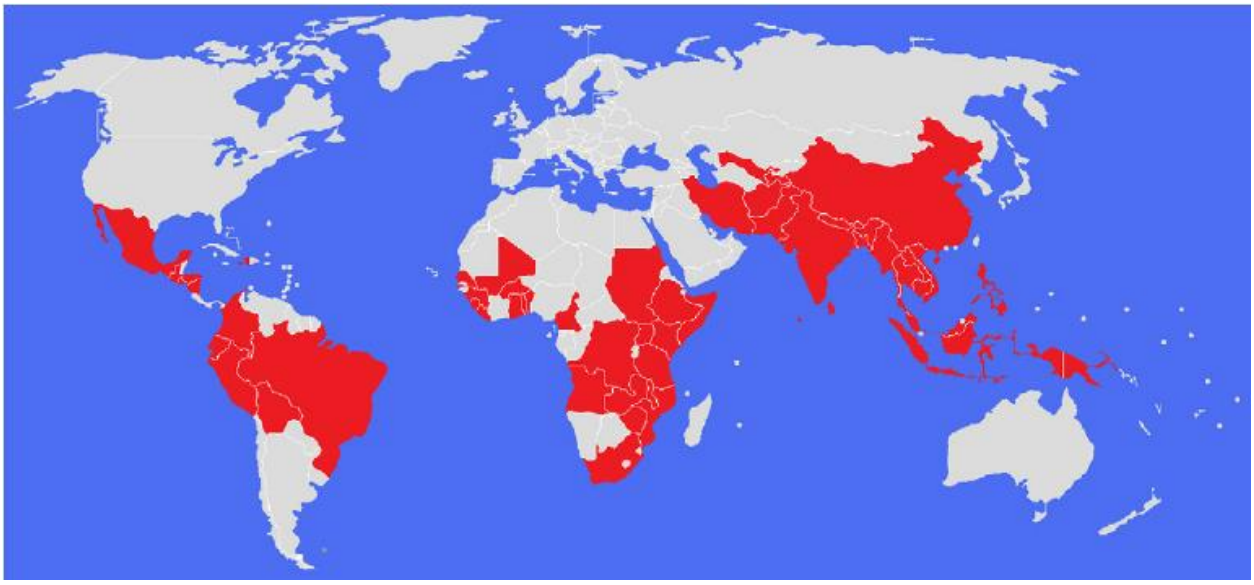

Figure 1 Map of the world indicating the countries where SODIS is used to disinfect water (McGuigan *et al.*, 2012)

SODIS treatment of water is affected by many conditions including UV radiation dosage, water temperature, the type of container (optical transmittance and the geometry, water turbidity, nature of microorganisms and water composition/nutrients) (Acra, 1984; Vivar *et al.*, 2015; Gart *et al.*, 2016).

In 2017, it was reported that approximately 844 million people had no access to safe drinking water (UNICEF & World Health Organization, 2017). Moreover, 263 million people spent over

30 minutes per round trip to collect drinking water from an improved source and 159 million people still used water directly from surface water sources (UNICEF & World Health Organization, 2017). The lack of potable water results in waterborne diseases that are some of the most common causes of illness and death among the poor in developing countries (Malik *et al.*, 2012). Therefore, simple home-water-treatment methods such as chemical disinfection with chlorine and iodine treatments are being implemented to make water potable. However, chemicals can be expensive, may not be readily available and have a short shelf life. Physical water treatment methods such as boiling, UV treatment, and filtration may require materials that are not easily accessible or expensive (Ellis, 1991).

In resource poor communities, solar disinfection can also be used to improve the quality of roof harvested rainwater for drinking purposes. A study in South Eastern Uganda showed that water collected directly from the roofs of buildings was microbiologically unsafe due to the presence of faecal coliforms (Nalwanga *et al.*, 2018). When the same water was solar disinfected it significantly improved the water quality (Nalwanga *et al.*, 2018).

SODIS is more effective in countries with a high annual global solar irradiance as shown in Fig. 2. Therefore, the low irradiance in some regions prevent effective SODIS of water. Climate change has also been reported to contribute to the ineffectiveness of solar disinfection in some regions. For instance, a study carried out by Williamson et al. (2007) at Lake Giles, north-eastern Pennsylvania, USA illustrated how heavy precipitation increased the dissolved organic matter (DOM) in water. The high levels of DOM and consequent browning of surface waters reduced the potential for solar UV inactivation of pathogens, and increased the exposure to infectious diseases in humans and wildlife (Williamson *et al.*, 2017).

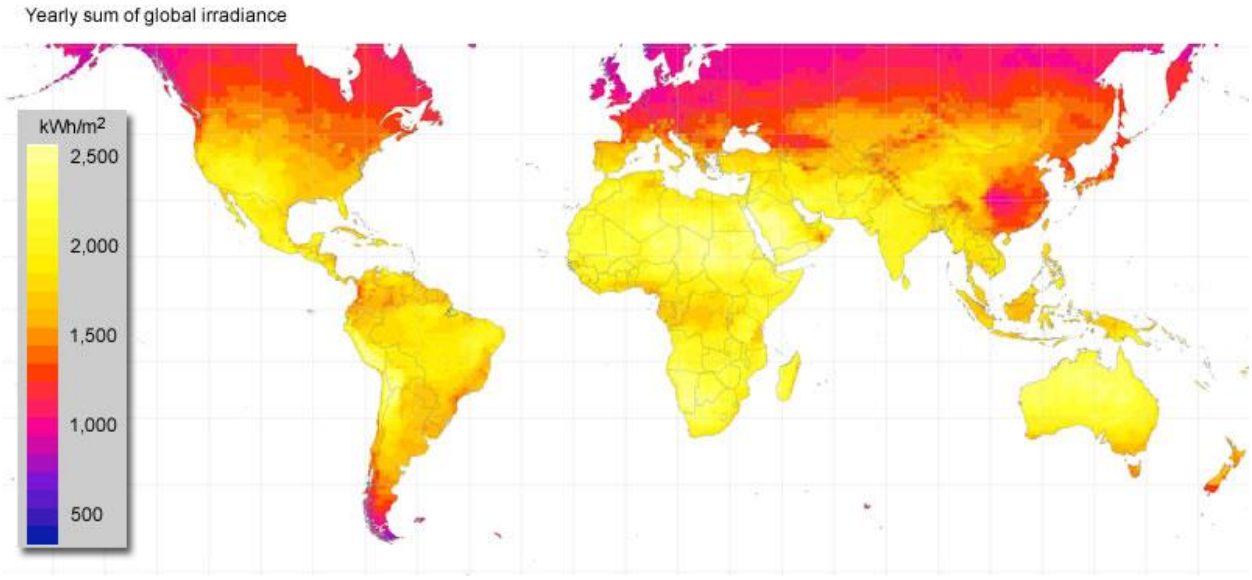

Figure 2 Annual average global solar irradiance in kWh/m^2 (Green Rhino Enenrgy Ltd., 2016)

2.2 Strategies used to enhance SODIS

Solar disinfection has been implemented in places with high UV irradiance (Fig. 2) and found to be a low cost and efficient method to improve the microbiological quality of water (McGuigan *et al.*, 1998). Therefore, several ways to enhance SODIS have been developed. For instance, adding a reflective or absorptive surface such as aluminium foil or placing the SODIS bottles on top of a reflective roof (Kehoe *et al.*, 2001) accelerates SODIS by increasing the UV radiation received by the bottles and by raising the water temperature (Encinas, 2005). It was also postulated that enhanced SODIS efficiency is due to the return of UVA and short-wavelength visible radiation through the transparent SODIS bottles, leading to enhanced cellular damage and the consequent inactivation of the bacteria (Mani *et al.*, 2006). However, thermal inactivation *of E. coli* was only enhanced when water reached temperatures of over 45°C (Mani *et al.*, 2006).

In another study, the effect of temperature on the inactivation efficiency of synthetic secondary wastewater (containing peptone, meat extract, urea, dipotassium phosphate, sodium chloride, calcium chloride and magnesium sulphate) spiked with *E. coli* during solar disinfection showed that total disinfection was attained within one hour at 60°C (Giannakis *et al.*, 2014a). This study recommended that SODIS reactors should be exposed to very high temperatures and prolonged solar irradiation because longer exposure to sunlight favours bacterial inactivation. The synergistic effects of solar irradiation and temperature was also assessed on typical roofing materials from rural households in Alcalá de Henares, Spain by Vivar *et al.* (2015). The study reported that zinc metal sheets were better for SODIS of water than vegetation covers such as bamboo. The high reflectivity and excellent heat transfer properties of zinc accelerated SODIS (Vivar *et al.*, 2015). The high UV radiance and high atmospheric temperatures in summer can raise temperatures to above 40°C resulting in complete inactivation of bacteria without bacterial regrowth (Vivar *et al.*, 2015). It was also postulated that if SODIS starts at midday rather than early in the morning, it can benefit from faster inactivation rates because of higher UV doses and higher water temperatures. They also reasoned that if the metallic support material has been under the sun since the morning, the zinc metal roofing becomes hotter and the water temperature in the bottles can rise more rapidly due to heat transfer (Vivar *et al.*, 2015).

Mirrors have also been shown to enhance the SODIS process of water. One particular study carried out in northern Jordan showed that using mirrors and aluminium foil considerably enhanced solar disinfection of water collected from different wells throughout the region and also shortened the time needed for the process (Hindiyeh & Ali, 2010). It was shown that approximately 90% of the total heterotrophic bacterial count (THBC) were inactivated within the first three hours of SODIS (between 10.00 am and 13.00 pm) due to the high solar intensity at midday (Hindiyeh & Ali, 2010).

The addition of photocatalysts such as titanium oxide and hydrogen peroxide enhance SODIS efficiency. Photocatalysis is an advanced oxidation process involving the production of hydroxyl radicals (OH-) that attack microbial contaminants in water (Malato *et al.*, 2009). The photocatalysts prevent bacterial regrowth in water after SODIS (Fiorentino *et al.*, 2015). A study conducted by Sichel *et al.* (2007) showed that increasing the photocatalytic reactor surface does not enhance the SODIS performance. It was assumed that, once a solar photocatalytic system has received UV dosage disinfection is reached irrespective of the irradiated surface area. **Photocatalytic solar** reactors require lower minimum solar UV irradiance for complete inactivation of microorganisms whereas **solar-only disinfection** requires a higher minimum UV dose for complete inactivation of microorganisms in water (Sichel *et al.*, 2007).

SODIS efficiency may be enhanced by reducing the turbidity of water. However, certain regions in developing nations may be limited by a lack of available technology to first treat turbid water (Dawney *et al.*, 2014). Therefore, inexpensive coagulants are being investigated to address the issue. Reducing turbidity through addition of natural coagulants such as *Moringa* seed powder can significantly improve the SODIS process. Pre-treatment of turbid water with *Moringa* seed powder for 24 hours allowed efficient settling of particles and is followed by a decanting step prior to SODIS (Keogh *et al.*, 2017). The pre-treatment makes the water clearer and removes the risk of regrowth of bacteria (Keogh *et al.*, 2017) because SODIS treatment of water is ineffective in highly turbid water (>30 NTU) (Solar Water Disinfection, 2002). Another study carried out in Sudan showed that sodium chloride (salt) is also an effective coagulant before SODIS treatment (Dawney *et al.*, 2014). Coagulation by addition of salt is effective in areas with specific soil types characterized by the dominant presence of smectite clays, which have a strong shrinking and swelling capacity under repeated dry–wet seasonal cycles (Dawney *et al.*, 2014).

This section showed that to ensure maximum bacterial inactivation during solar disinfection the following recommendations should be made: (1) filtering the water to reduce the turbidity and enhance optical penetration, (2) placing the water in a container with a highly reflective rear surface, (3) not filling the bottle to capacity to allow for agitation of the water for at least one minute to increase the amount of dissolved oxygen in the water and, (4) exposing the bottle to intense natural sunlight (500W/m^2) for as long as possible, ideally at least 8 hours, Finally, it is recommended to consume solar disinfected water as soon as possible (within 48 hours) since some recovery or regrowth of inactivated bacteria may occur mainly in turbid water (Kehoe *et al.*, 2001). This study considered the strategies that are being implemented for SODIS of water and are explained further in the methods and materials section of this thesis.

2.3 Bacterial regrowth after short-term storage

Despite the significant role played by SODIS of water, bacterial regrowth after short-term storage of SODIS treated water is a primary concern (Giannakis *et al.*, 2014b). Two main factors are responsible for bacterial regrowth, namely, (i) the growth of undamaged microorganisms and, (ii) the reactivation and revival of microorganisms (Guo *et al.*, 2011). Regrowth is possible due to several repair pathways that bacteria have developed in response to UV radiation. The pathways include photoenzymatic repair (PER), nucleotide excision repair (NER) (also called dark repair), and recombinational repair (post-replication repair). Photoenzymatic repair involves direct monomerization of cyclobutane pyrimidine dimers (CPDs) by a single enzyme (photolyase) with near-UV or visible light as a source of energy (Sancar, 1994). Giannakis *et al*. (2014b) showed that samples exposed to high temperatures (50-60°C) and high irradiation (1200 W/m^2) experienced no bacterial regrowth. However, bacterial regrowth only occurred in incompletely disinfected samples which were linked to lower radiation (800 W/m^2) and lower temperature (20-40°C) (Giannakis *et al.*, 2014b).

Post-treatment conditions of solar treated samples also have an impact on bacterial regrowth. It is beneficial to keep irradiated samples at 4°C because low temperatures inhibit bacterial regrowth. Re-growth of micro-organisms occurs if storage temperature is around 20°C and if the samples are exposed to only 30-90 minutes of solar irradiation. A further increase in storage temperature leads to excessive growth of bacteria in SODIS-treated water. However, water that is solar irradiated for 210-240 minutes usually showed no signs of bacterial regrowth (Giannakis *et al.*, 2014c).

2.4 Damaging effects of Solar irradiation on bacterial cells

Ultraviolet (UV) radiation is the main region of the electromagnetic spectrum that is most effective in SODIS of water. WHO (1997) has identified three principal components of UV radiation. UV-A (315-400 nm) and UV-B (280-315 nm) reaches the earth's surface and is capable of damaging living organisms including microorganisms (Ocean Health Index, 2015). UV-C (200-280 nm), which is the most energetic and lethal, does not reach the earth’s surface since they are mainly absorbed by the ozone layer and oxygen. Solar radiation has been able to disinfect water that carries a broad range of organisms including bacteria, fungal spores, cysts and viruses (Lonnen *et al.*, 2005; Berney *et al.*, 2006; McGuigan *et al.*, 2006; Boyle *et al.*, 2008; Bosshard *et al.*, 2009; Carratala *et al.*, 2016; Jumat & Hong, 2019).

Several studies have been carried out to assess the effects of solar radiation on bacteria. These date back to the nineteenth century when it was shown that exposure of different media to sunlight prevented the growth of bacteria. It was postulated that this bactericidal action was dependent on a number of factors including intensity, duration, and wavelength of the solar

ultraviolet rays (SUVR) (Downes & Blunt, 1877). It was also shown that ultraviolet radiation synergistically combined with heat also plays a role in the inactivation of pathogenic microorganisms in water (Downes & Blunt, 1877). Several studies have reported on the harmful effects of natural sunlight on bacteria (Ward, 1894; Deller *et al.*, 2006; Ruiz-González *et al.*, 2013). Ultraviolet light effectively kills micro-organisms because bacterial DNA absorbs UV photons between 200 and 300 nm. UV-A causes indirect damage to bacterial DNA, proteins, and lipids through reactive oxygen intermediates (ROS) (Gelover *et al.*, 2006) as shown in Fig. 4. The accumulation of intracellular stable ROS in solar irradiated bacteria is the main cause of **indirect** bacterial inactivation by UV-A (Castro-Alférez *et al.*, 2016). On the other hand, UV-B rays causes **direct** damage to the DNA by inducing the formation of DNA photoproducts including cyclobutane pyrimidine dimer (CPD), the pyrimidine (6–4), and pyrimidinone (6–4PP) as shown in Fig. 3 (Douki, 2013). The accumulation of DNA photoproducts in the cells hinders DNA replication and RNA transcription leading to the destruction of the cells (Britt, 1996; Rincón & Pulgarin, 2004) (Fig. 3).

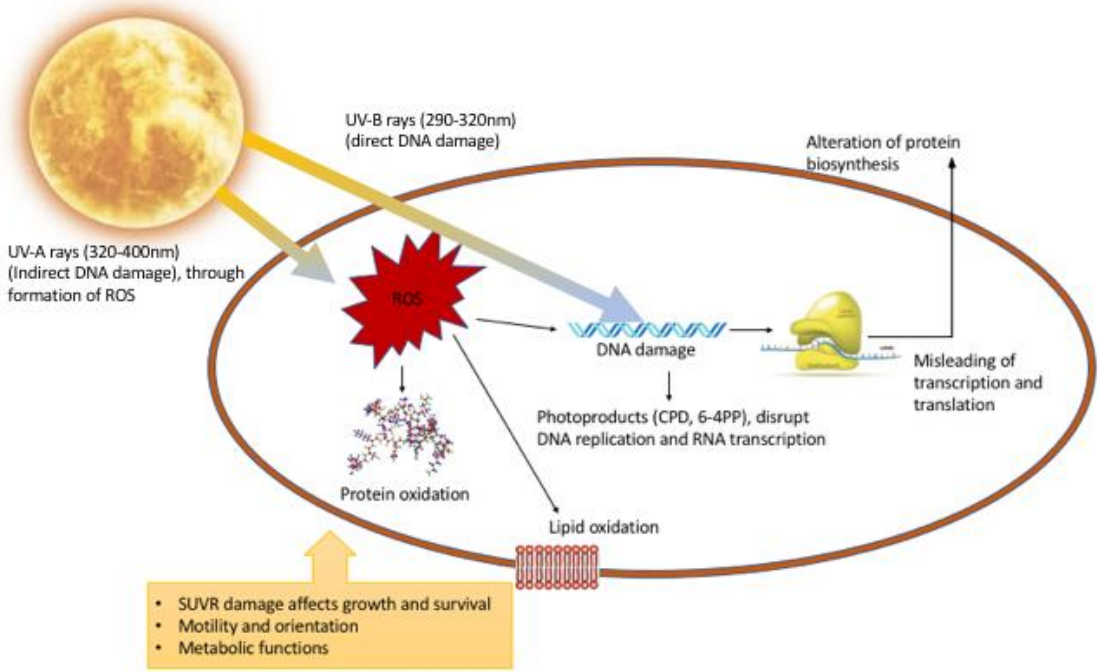

Figure 3 Damaging effects of SUVR on bacterial cells source (Britt, 1996; Rincón & Pulgarin, 2004; Gelover *et al*., 2006; Douki, 2013)

Finally, solar irradiation also affects protein stability and turnover. Radiation resistant bacteria can protect proteins against protein oxidation arising from radiation, whereas the sensitive ones do not. Solar radiation can also induce extensive protein damage due to oxidative stress, such as amino acid modifications, carbonyl group formation, fragmentation, the formation of protein-protein cross-links, and formation of S–S bridges (Chatgilialoglu *et al.*, 2011).

The adverse effects SUVR on bacteria have made SODISof water a success in reducing the spread and contraction of water-related infections, most of which are zoonotic. Zoonosis is a disease that is transmissible from animals to humans. Zoonotic diseases account for 70% of all emerging infectious diseases (Woolhouse & Gowtage-Sequeria, 2005; Kahn, 2010).

2.5 Waterborne zoonoses

Zoonotic pathogens are disease-causing agents (bacterium, a virus, a fungus or other communicable disease agent) that can be transferred between animals and humans. Three criteria are used to classify zoonotic pathogens as *priority one* waterborne pathogens. Firstly, the pathogens must be able to induce clinical illness in susceptible humans. Secondly, they must to be transmitted to humans through water, and lastly, exhibit prevalence in a natural host including one or more livestock species (Dufour, 2012).

Currently there are five priority-one waterborne zoonotic pathogens including *Cryptosporidium, Giardia, Escherichia coli* O157:H7, *Salmonella*, and *Campylobacter.* A large number of zoonotic pathogens must be released in the faeces of livestock and transmitted by water to be effective as disease-causing agents. Moreover, they must survive several physical processes such as filtration, desiccation, dilution, UV inactivation and senescence that occur on land or aquatic environments and they must be consumed in infectious doses (Dufour, 2012). The infectious dose for *S.* Typhimurium is quite large (>10^5 CFU/ml), whereas *C. jejuni* has a very low infectious dose (>500 CFU/ml).

Zoonotic diseases pose significant risks to human health and must be controlled. Maintaining a clean water supply for both humans and animals can control the spread of zoonotic pathogens. Waterborne zoonotic diseases are more rampant during large increases in global livestock population and when unethical animal husbandry is practiced (Dufour, 2012). This study focused on effects of SODIS on two of the priority one waterborne zoonotic pathogens: *Salmonella* and *Campylobacter* sp. that are responsible for salmonellosis and campylobacteriosis, respectively (Kaakoush *et al.*, 2015).

2.6 Salmonella Typhimurium

Salmonellosis is a zoonotic disease that affects both animals and humans. The disease is caused by *S.* Typhimurium with symptoms of fever, acute intestinal inflammation, and diarrhoea within 24 h after infection of the pathogen (Fabrega & Vila, 2013). The clinical feature of an invasive non-typhoidal *Salmonella* (NTS) disease in Africa is typically febrile systemic illness (Graham *et al.*, 2000; Gordon *et al.*, 2002; Peters *et al.*, 2004) resembling enteric fever; however, diarrhoea is usually absent and other clinical features are non-specific as shown in Fig. 4 (Gordon *et al.*, 2002). It is difficult to identify or treat invasive non-typhoidal

Salmonella diseases especially in children because a clinical overlap exists between the symptoms of pneumonia and malaria (Graham *et al.*, 2000; Graham & English, 2009). In low-income settings, empirical diagnosis and treatment remains a challenge to successfully identify and treat invasive NTS disease (Wamola & Mirza, 1981; Lepage *et al.*, 1987; Green & Cheesbrough, 1993).

Invasive non-typhoidal *Salmonella* can also cause bacterial meningitis especially in children (Molyneux *et al.*, 2009). A study in Malawi found that fatalities resulting from salmonella meningitis was about 52% in children and 80% in adults (Molyneux *et al.*, 2009). Moreover, invasive non-typhoidal *Salmonella* infection in African children is also associated with schistosomiasis (Rocha *et al.*, 1971) because the bacteria can adhere to the adult helminth's tegument where they can escape antimicrobial treatment (LoVerde *et al.*, 1980; Melhem & LoVerde, 1984).

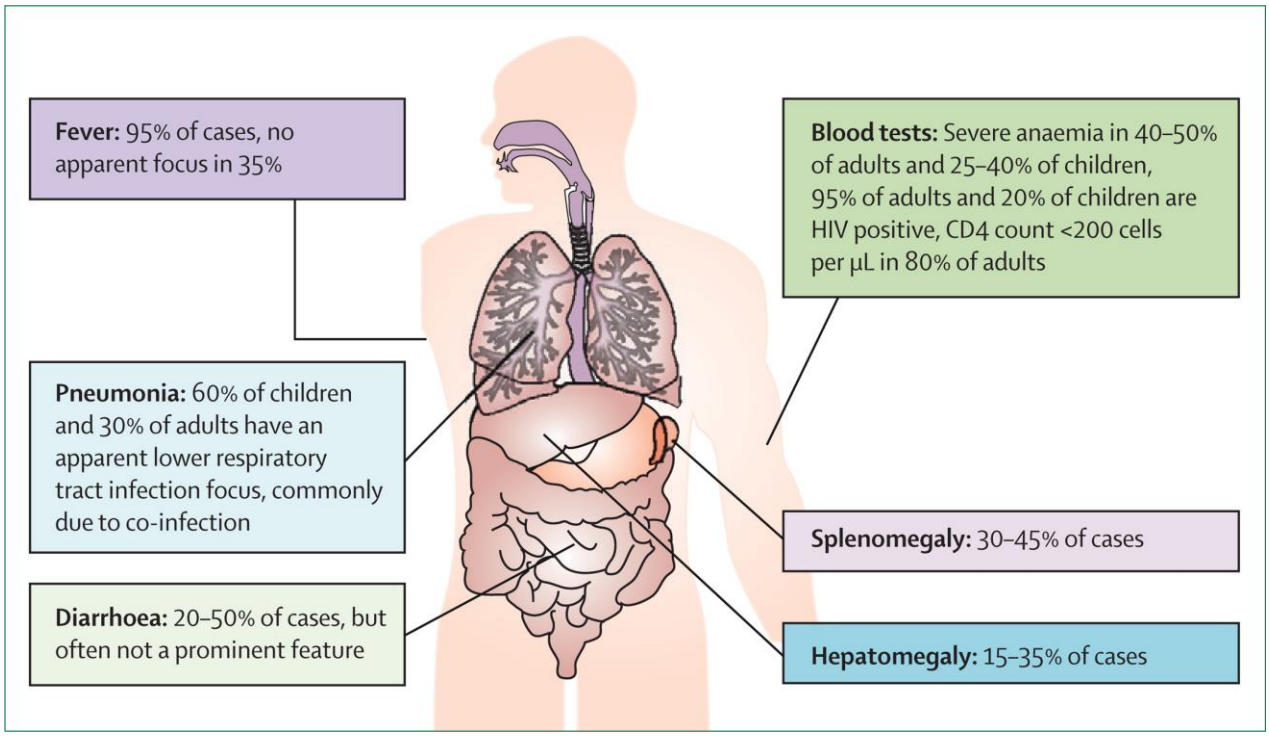

Figure 4 Clinical features of invasive non-typhoidal *Salmonella* (iNTS) disease in adults and children in Africa (Reddy *et al.*, 2010)

2.6.1 Epidemiology of *S.* Typhimurium in Africa

Bacteraemia studies have shown that invasive NTS are among the most common isolates from febrile presentations in adults and children across sub-Saharan Africa, especially in areas where there is a high HIV prevalence (Fig. 5) (Reddy *et al.*, 2010). However, the unavailability of high-quality or affordable diagnostic microbiology facilities in most African countries makes it difficult to document the incidence of iNTS (Clemens, 2009). Therefore,

the total burden of invasive disease caused by invasive non-typhoidal *Salmonella* in Africa has not been accurately measured but is probably substantial, with an estimated annual incidence of 175–388 cases per 100 000 children aged 3–5 years (Berkley *et al.*, 2005; Enwere *et al.*, 2006; Bassat *et al.*, 2009) and 2000–7500 cases per 100 000 in HIV-infected adults (van Oosterhout *et al.*, 2005; Gordon *et al.*, 2008; Reddy *et al.*, 2010). Additionally *Salmonella enterica* serovar Typhimurium ST313 is an invasive and phylogenetically distinct lineage present in sub-Saharan Africa and it was detected in patients in the Democratic Republic of Congo and Nigeria as well (Leekitcharoenphon *et al*., 2013). Although ST313 does appear to be causing invasive infections in compromised hosts, seemingly it also causes more typical gastrointestinal infections in otherwise healthy people (Wain, *et al*., 2013)

In the rural regions of Kenya, the estimated minimum incidence of bacteraemia was 505 cases per 100,000 person-years in the age group of <5 years old, of which 17% of the bacterial cases were NTS bacteraemia. In rural Mozambique, the incidence of childhood bacteraemia was 425 cases per 100,000 person-years among children aged <15 years, and within this category, NTS incidence accounted for 28% of bacteraemia cases (Sigauque *et al.*, 2009). In Uganda, NTS bacteraemia was observed to be rare among adults with high CD4+T-lymphocyte counts (CD4 cell counts) >500 cells/mm^3. However, the incidence increased in immunocompromised individuals with CD4 cell counts of 200-500 cells/mm^3 (approximately 500 cases per 100,000 person-years among adults with CD4 cell counts of 200–500 cells/mm^3 were reported. A further increase of NTS bacteraemia was observed among adults with CD4 cell counts <200 cells/mm^3 (7500 cases per 100,000) (Gilks, 1998).

Additionally, in many areas of sub-Saharan Africa, NTS is the most common cause of community-acquired invasive bacterial infection, especially in children; with an estimated overall incidence of 227 cases per 100,000 population annually (Kruger *et al*., 2004)

Several approaches have been implemented to monitor the emergence and spread of invasive non-typhoidal *Salmonella* (Sigauque *et al.*, 2009). For instance, a whole genome–based transmission study of iNTS isolates from sub-Saharan Africa was carried out. The results highlighted the power of these approaches to monitor the spread of clonal bacterial populations associated with epidemics locally or globally over time (Okoro *et al.*, 2012).

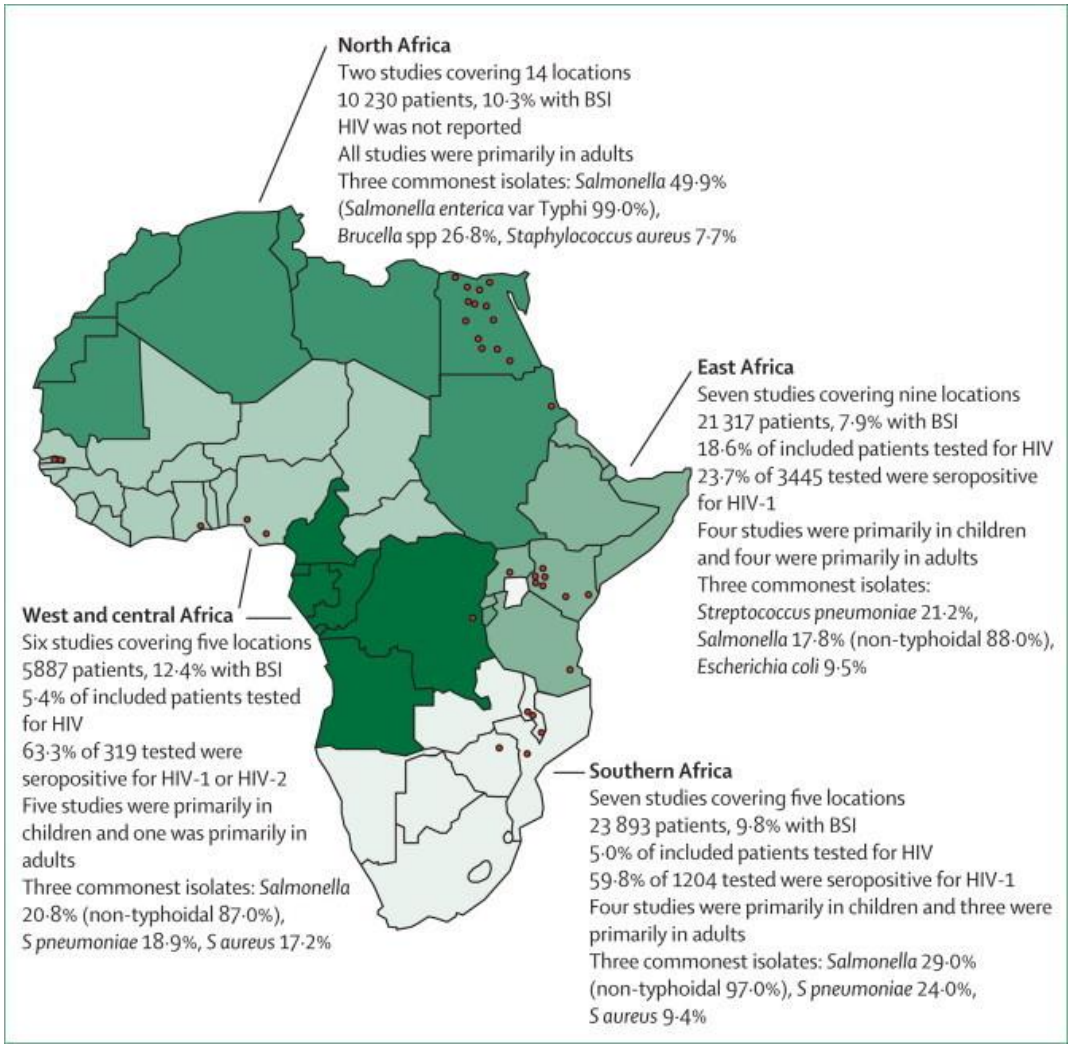

Figure 5 Map of Africa showing results of a meta-analysis of studies investigating the cause of bloodstream infection in febrile adults and children in Africa (Reddy *et al.*, 2010)

2.6.2 Water transmission of *S.* Typhimurium

Approximately one third of the global population live in regions where the water resourses are insufficient for its population (water stress). Water scarcity is expected to increase because of increased human population, and negative impacts of human activity on the environment (Asano *et al.*, 2007). Zoonotic salmonellae are well known as foodborne pathogens; however, drinking as well as natural waters are major routes of transmission of these enteric microorganisms (Leclerc *et al.*, 2002; Ashbolt, 2004).

Salmonellae are usually found in environmental samples. They occur in large numbers in raw sewage and can still be present in wastewater effluent after advanced secondary treatment such as coagulation, filtration and disinfection (Wery *et al.*, 2008). Soil and sediment were also found to harbour salmonellae (Abdel-Monem & Dowidar, 1990; Tobias & Heinemeyer, 1994; Gorski *et al.*, 2011). *Salmonellae* has been detected in various types of natural waters such as rivers, lakes, coastal waters, estuarine as well as contaminated ground water (Polo

et al., 1999; Martinez-Urtaza *et al.*, 2004; Haley *et al.*, 2009; Wilkes *et al.*, 2009; Levantesi *et al.*, 2010).

Contamination of water with water-borne pathogens is a major water quality concern throughout the world (Pandey *et al.*, 2014). Therefore, it is critical to examine the significance of easy, cheap, home water treatment methods such as SODIS in curbing the spread of waterborne diseases.

2.6.3 Effects of solar disinfection on *S.* Typhimurium

Solar disinfection has been evaluated by monitoring the inactivation of *E. coli* as a bacterial indicator. However, it has been shown that *E. coli* is one of the most susceptible microorganisms to solar disinfection (Berney *et al.*, 2006). Consequently, *E. coli* was deemed an unsuitable indicator for SODIS performance (Sciacca *et al.*, 2010). However, *Salmonella* Typhimurium was shown to be more resilient to SODIS treatment (Smith *et al.*, 2000; Winfield & Groisman, 2003; Berney *et al.*, 2006) resulting in the bacterial regrowth after being stored in the dark. Therefore the addition of photo-catalytic enhancers such as hydrogen peroxide was necessary to prevent its reactivation during storage in the dark (Sciacca *et al.*, 2010). Despite the subsequent regrowth of *S.* Typhimurium under dark storage conditions in previous studies, Bosshard *et al.* (2009) suggested that it is more favourable to store the treated water overnight before consumption because the injured cells are most likely to die from ATP exhaustion caused by the inactivation of the respiratory chain.

Despite the studies carried out on the effect of SODIS on *S.* Typhimurium, there is no reported research addressing the effects of solar disinfection on the microbial virulence factors such as bacterial replication in host cells and host-cell cytotoxicity. This study addresses these knowledge gaps. Both these factors have significant consequences on the innate and adaptive immune responses. Intracellular bacteria cause diseases by invading cells and promoting bacterial survival, replication, and spread. Pathogens that lose these virulence factors generally become avirulent and thus incapable of causing disease.

2.6.4 Salmonella pathogenesis and virulence

Rapid invasion of intestinal enterocytes by live *S.* Typhimurium occurs immediately after ingestion (Santos *et al.*, 2002), and the pathogen uses flagella to move closer to the intestinal epithelial cells and uses fimbriae and adhesins (*SiiE, BapA*) for cell attachment (Fig. 6). The **type III secretion system** encoded on pathogenicity island 1 (*T3SS-1*) (Muller *et al.*, 2012), influences Salmonella to insert effector proteins, such as SipA, SopA, SopB, SopD and SopE2 into host cells where they activate cytoskeletal rearrangement, bacterial engulfment and

formation of Salmonella-containing vacuole (SCV) (Fig. 6). The T3SS-1 effectors elicit the production of pro-inflammatory cytokines and antimicrobial peptides by epithelial cells (Fig. 6). Another type III secretion system is encoded on Salmonella pathogenicity island 2 (*T3SS-2*) and is expressed inside the SCV.

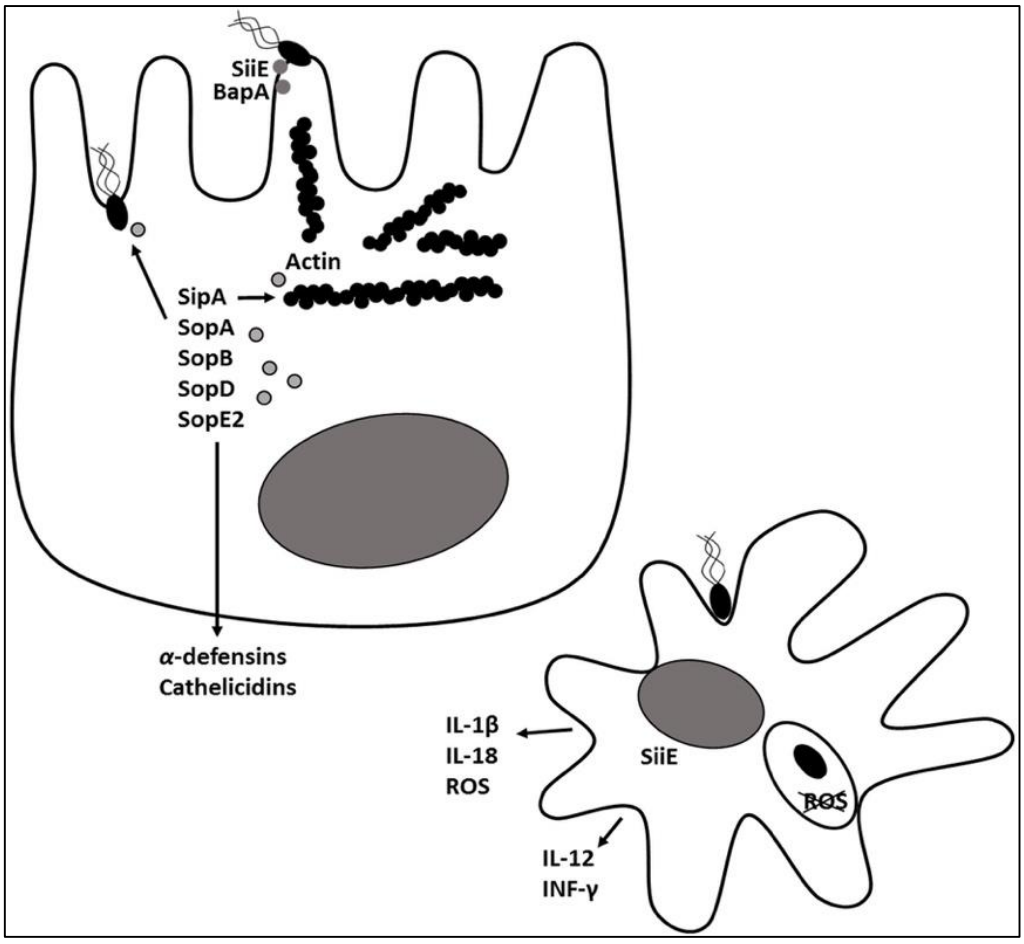

Figure 6 *Salmonella* pathogenesis and virulence (Gart *et al*., 2016)

Proteins such as the effector *VopL* secreted through T3SS-2 (de Souza Santos *et al.*, 2017) may prevent production of reactive oxygen species (ROS) and enable *Salmonella* to survive inside macrophages (Gart *et al.*, 2016).

2.6.5 Metabolic pathways of *S.* Typhimurium in the murine model

SODIS of water can inactivate *S.* Typhimurium (Smith *et al.*, 2000; Winfield & Groisman, 2003; Berney *et al.*, 2006; Bosshard *et al.*, 2010b), and adversely affects the metabolic activity of the bacteria; hence *S.* Typhimurium can lose its virulence after SODIS-treatment. For virulence to occur and for multiplication of the bacterium, *S.* Typhimurium must feed and be able to carry out metabolic processes inside the host cell. The ability of *S.* Typhimurium to

carry out metabolic functions is linked to its capacity to invade and evade host immune responses (Fig. 7).

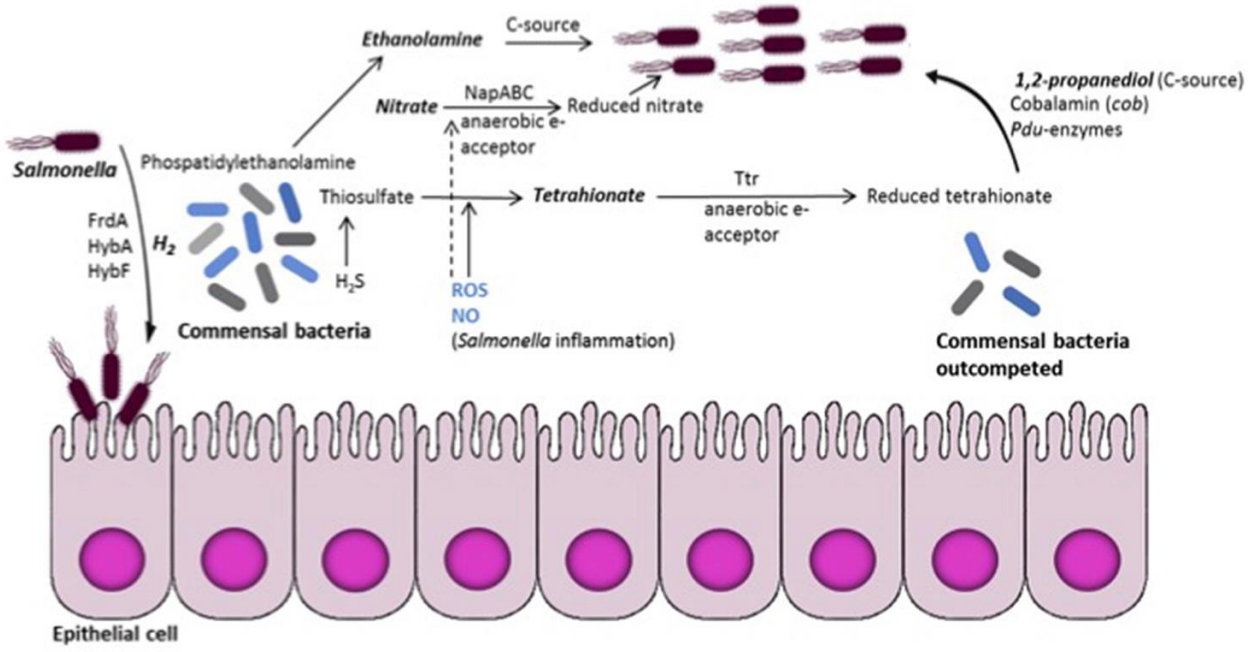

Figure 7 Metabolic pathways used by *S.* Typhimurium during growth in the gut of mice (Herrero-Fresno & Olsen, 2018).

Salmonella Typhimurium utilises hydrogen usually synthesized by the microbiota in the gut as an energy source during the initial colonisation of the gut in mice (Maier *et al.*, 2013). Inflammation of the gut usually ensues after *S.* Typhimurium invasion. The reactive nitric oxide and oxygen species produced by neutrophils leads to the conversion of thiosulfate to tetrathionate. This enables *S.* Typhimurium to degrade available substrates, such as 1,2 propandiol (Stecher *et al.*, 2007), ethanolamine (Thiennimitr *et al.*, 2011), fructose-asparagine (Ali *et al.*, 2014), as well as fucose and sialic acid (Ng *et al.*, 2013), by anaerobic respiration using tetrathionate as an electron acceptor (Stecher *et al.*, 2007). Nitrate derived from the host can also be used as an electron acceptor in the strict anaerobic environment (Lopez *et al.*, 2015). The use of anaerobic respiration, rather than fermentation, and a great ability to withstand anti-microbial factors gives *Salmonella* a growth advantage, when competing with the commensal microbiota in the inflamed gut (McNally *et al.*, 2016).

2.7 Campylobacter jejuni

*Campylobacter*s are primarily spiral-shaped, "S"-shaped, curved, or rod-shaped, gram-negative bacteria. It has microaerophilic and thermophilic characteristics. The optimal growth

temperature is 42°C, probably caused by adaptation to the avian gastrointestinal system (Park, 2002). The most common *Campylobacters*, *Camplybacter jejuni* and *Campylobacter coli* belong to the epsilon class of proteobacteria in the order *Campylobacteriales*. Most *C. jejuni* have small genomes (1.6–2.0 megabases) and can establish long-term associations with their hosts (Young *et al.*, 2007). The pathogen is 1 of 4 major global causes of diarrhoeal diseases and is the leading common cause of gastroenteritis in the world (World Health Organization, 2016).

Campylobacters are pervasive in the environment and they are usually an indicator of recent contamination of animal and avian faeces, agricultural run-off and sewage effluent. Although livestock, pets, poultry, wild birds and wild animals are commonly infected with campylobacters, contamination of the environment with the bacteria in faeces is widespread and varies seasonally due to factors such as stress and changes in diet (Jones, 2001).

2.7.1 Epidemiology of *Campylobacter* infection

Campylobacter jejuni is the causative agent of one of the most prevalent infectious diseases of the last century (Kaakoush *et al.*, 2015). Campylobacteriosis has increased in both developed and developing nations over the past decade. There has been a drastic increase of infections in North America, Europe and Australia (Fig. 8). Campylobacteriosis is endemic in parts of Africa, Asia, and the Middle East especially in children (Kaakoush *et al.*, 2015). The rate of *Campylobacter* infections worldwide often exceeds those of salmonellosis and shigellosis (Llarena *et al.*, 2017).

The most significant source of transmission of campylobacteriosis to humans is from poultry (Kaakoush *et al.*, 2015). Other risk factors include consumption of contaminated animal products and drinking contaminated water. Several biocontrol measures such as vaccination of poultry, practice of correct food hygiene throughout the food chain and health surveillance have been implemented to reduce the transmission of *Campylobacter* (Facciolà *et al.*, 2017). A global surveillance of Campylobacteriosis is desirable and should include data from all countries, including notifications of cases and the microbiological data typing of strains isolated from both human and animal cases (Facciolà *et al.*, 2017). Campylobacteriosis is still one of the most important infectious diseases that are likely to challenge global health in the future (Kaakoush *et al.*, 2015)

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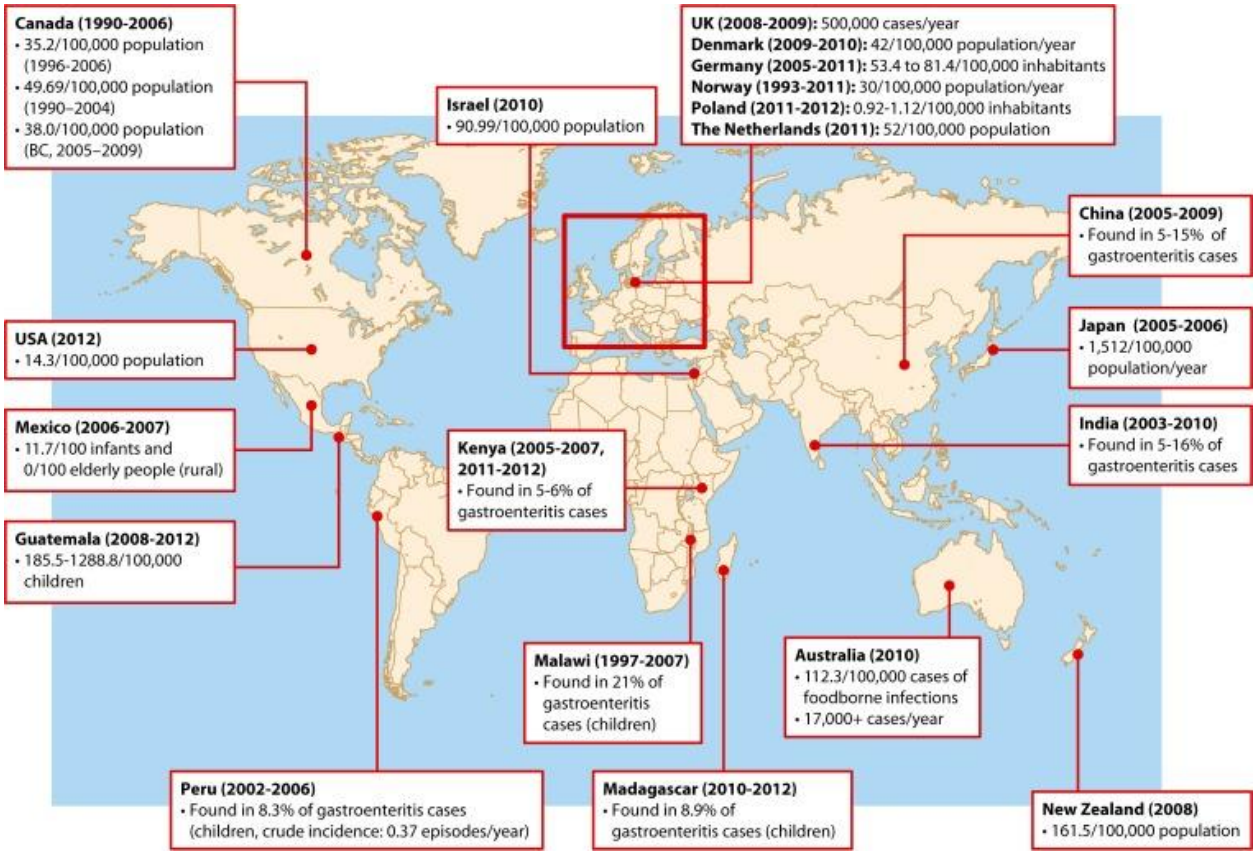


Figure 8 Incidence and prevalence of campylobacteriosis (*C. jejuni/C. coli*). The latest information on the global epidemiology of campylobacteriosis from the literature is shown (Kaakoush *et al.*, 2015)

2.7.2 Water transmission of *Campylobacter*

The consumption of contaminated water is a significant mode of transmission of *Campylobacter* species to humans and animals leading to several outbreaks of the disease in numerous countries (Taylor *et al.*, 1982; Rogol *et al.*, 1983; Hänninen *et al.*, 2003; Kapperud *et al.*, 2003; Richardson *et al.*, 2007) including several countries in Africa (Fig. 9). Proper farming practices such as emptying and cleaning water troughs on a regular basis have been shown to reduce the risk of *Campylobacter* infection in cattle (Ellis-Iversen *et al.*, 2009). The importance of clean water for animals was demonstrated when animals grazing outdoors in spring were observed to more likely to contract Campylobacteriosis because they drank water from the lake. The animals had a lower incidence of contracting Campylobacteriosis in the winter when they were confined indoors and drank municipal chlorinated tap water (Hanninen *et al.*, 1998). Other sources including faeces of wild bird and waste runoff from contaminated domesticated animals that can also contaminate water with *Campylobacter* (Carter *et al.*, 2009).

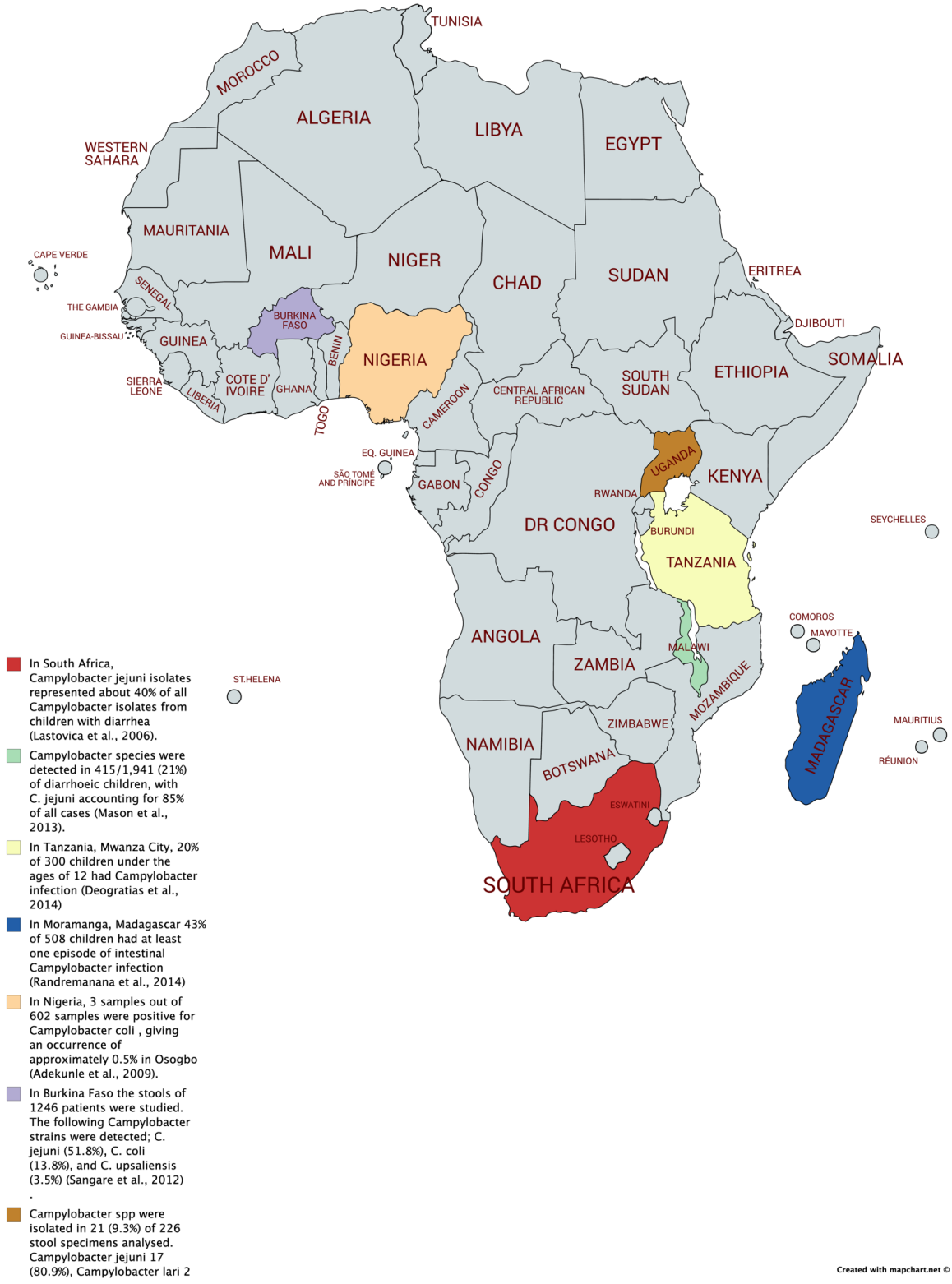


Figure 9 Incidence of Campylobacteriosis in Africa

2.7.3 Effects of SODIS on *Campylobacter jejuni*

Campylobacter jejuni, unlike *S.* Typhimurium, is very susceptible to solar irradiation. *Campylobacter jejuni* was reported to be one of the most susceptible microorganisms to SODIS treatment than any previously reported microbiological species (Boyle *et al.*, 2008). The susceptibility was illustrated by the detection of *C. jejuni* in saline waters in the morning and their elimination in the afternoon after half a day of sunlight exposure (Jones *et al.*, 1991). It was also noted that both seasonal distribution and diurnal variation in *C. jejuni* counts showed significant correlations with patterns of sunlight exposure (Jones *et al.*, 1991).

The great resistance of *S.* Typhimurium and susceptibility of *C. jejuni* make the bacteria ideal candidates in studying how solar irradiation of highly resistant and susceptible bacteria affect host immune cells such as macrophages.

2.7.4 *Campylobacter jejuni* pathogenesis and virulence

As an important human pathogen several investigations have been carried out to further understand the pathogenicity of *C. jejuni.* However, understanding the pathogenic processes of *C. jejuni* still lags behind those of other pathogens such as *Salmonella, Shigella, Vibrio* and *Listeria*. Campylobacter is distantly related to other entero-pathogens and therefore has evolved unique infection and virulence mechanisms that have not been fully elucidated.

There are several potential mechanisms by which *C. jejuni* interacts with the host. The complete genome sequencing of *pVir*, a plasmid that is found in several isolates of *C. jejuni*, has led to the identification of a **type IV secretion system (T4SS)** that was shown to play a crucial role in cell invasion and pathogenicity in ferrets (Bacon *et al.*, 2000; Bacon *et al.*, 2002). *Campylobacter jejuni* also exhibits large genetic variation which arise from intragenomic mechanisms as well as genetic exchange between strains. Genome sequencing of *C. jejuni* has revealed the presence of hypervariable sequences that consist of homopolymeric tracts (Parkhill *et al.*, 2000).

Campylobacter jejuni possesses several mechanisms to evade the immune response of the host. For instance, the lipooligosaccharide capsule (LOS) of *C. jejuni* is highly variable. Moreover, several *C. jejuni* LOS structures are similar to human neuronal gangliosides. This molecular imitation has been postulated to lead to autoimmune disorders, such as Guillain–barré syndrome (GBS). This condition is a paralytic neuropathy that occurs approximately 1 in every 1,000 cases of campylobacterosis (Nachamkin *et al.*, 1998; Nachamkin, 2002; Komagamine & Yuki, 2006; Yu *et al.*, 2006).

Flagella and flagellar motility are also vital to the pathogenicity of *C. jejuni.* The flagella is involved in host colonization, virulence, secretion and host-cell invasion (Jones *et al.*, 2004).

The **chemotaxis** of bacteria is governed by the environment. Usually bacteria swim towards favourable environments and away from unfavourable environments. The extracellular signals, often sugars or amino acids, are sensed by chemoreceptors that are called methyl-accepting chemotaxis proteins (MCPs). An alternative type of taxis is **energy taxis** when the bacteria responds to an intracellular signal, such as the proton motive force or the redox state of the electron-transport system (Young *et al.*, 2007).

Another factor that plays important roles in the pathogenicity of *C. jejuni* is the secretion of proteins such as *CiaB*. The *CiaB* protein is required for the invasion of cultured epithelial cells (Konkel *et al.*, 1999b; Konkel *et al.*, 1999a). The mechanism of *Ciab* secretion and its role in invasion has been compared to the model of **type III secretion systems**, in which effectors are injected directly into host cells (Konkel *et al.*, 1999a). However, *C. jejuni* does not encode a **type III secretion system** and evidence for the direct injection of *Ciab* is lacking. Rather, it has been hypothesised that *Ciab* and other secreted *Cia* proteins which are identical to those found in *Yersisnia* spp. require a functional flagellar export apparatus for their secretion (Konkel *et al.*, 2004). Additionally, the flagellar export apparatus of *C. jejuni* secretes *FlaC,* which is also involved in the invasion of host cells. FlaC shares limited homology with the major and minor flagellins (*FlaA* and *FlaB*).

Campylobacter jejuni also produces cytolethal distending toxin (CDT) which is also secreted by other bacterial species such as *E. coli*, *Actinobacillus actinomycetemcomitans*, *Haemophilus ducreyi* and *Helicobacter hepaticus*. The CDT causes cell cycle arrest at the G1/S or G2/M stages, depending on the cell type (Whitehouse *et al.*, 1998; Lara-Tejero & Galan, 2000).

2.7.5 Beneficial health effects of SODIS treated water

The consumption of SODIS treated water has several health benefits. For instance, a reduction in the incidence of diarrhoeal diseases has been reported in South Africa, Kenya and Zimbabwe (du Preez, 2010). There is compelling evidence that household water treatment methods can reduce pathogen levels in drinking water and lead to lower reported rates of diarrhoeal disease, even in the absence of sanitation or hygiene improvements (Fewtrell *et al.*, 2005; Clasen *et al.*, 2007). Clinical field trials conducted in Kenya show children consuming solar disinfected water showed a significant reduction (between 10 and 24%) in diarrheal incidences compared to control groups (Conroy *et al.*, 1999).

The success of SODIS is mostly due to the deleterious effects of UV-A and UV-B rays on bacteria (Bosshard *et al.*, 2009; Bosshard *et al.*, 2010; McGuigan *et al.*, 2012). However, very little work has been carried out to assess the impact of solar irradiated pathogens on immune cells, especially antigen presenting cells. Microorganisms that have been exposed to SODIS

treatment are killed by the SUVR and may result in bacterial cell death (Bosshard *et al.*, 2009; Bosshard *et al.*, 2010). Cellular death results in the release of intracellular and membrane components such lipopolysaccharides, glycopeptides, deoxyribonucleic acids and numerous antigenic materials (Bessler *et al.*, 1997). The antigenic material released by solar irradiated pathogens have been shown to elicit the maturation of dendritic cells *in vitro (Ssemakalu et al.*, 2015b). Dendritic cells (DCs) are considered the most effective antigen-presenting cell (APC) (Koski *et al.*, 2008) primarily responsible for sensitising naive T cells to specific antigens and play a crucial role in the establishment of immunologic memory (Mayordomo *et al.*, 1995; Ludewig *et al.*, 1999). Dendritic cells are also capable of using soluble protein antigens to sensitize naive T cells *in vitro* (Mehta-Damani *et al.*, 1994). Thus, the ability of solar treated bacteria to produce antigenic material capable of eliciting a Th1 type of response may enable the development of solar-treated DC-based vaccines that can be effectively used to sensitise T cells.

2.7.6 Antigen-presenting cells

Antigen-presenting cells (APCs) are a variety of immune cells that regulate the cellular immune response by processing and presenting antigens for recognition by specific lymphocytes such as T cells (Fig.10).

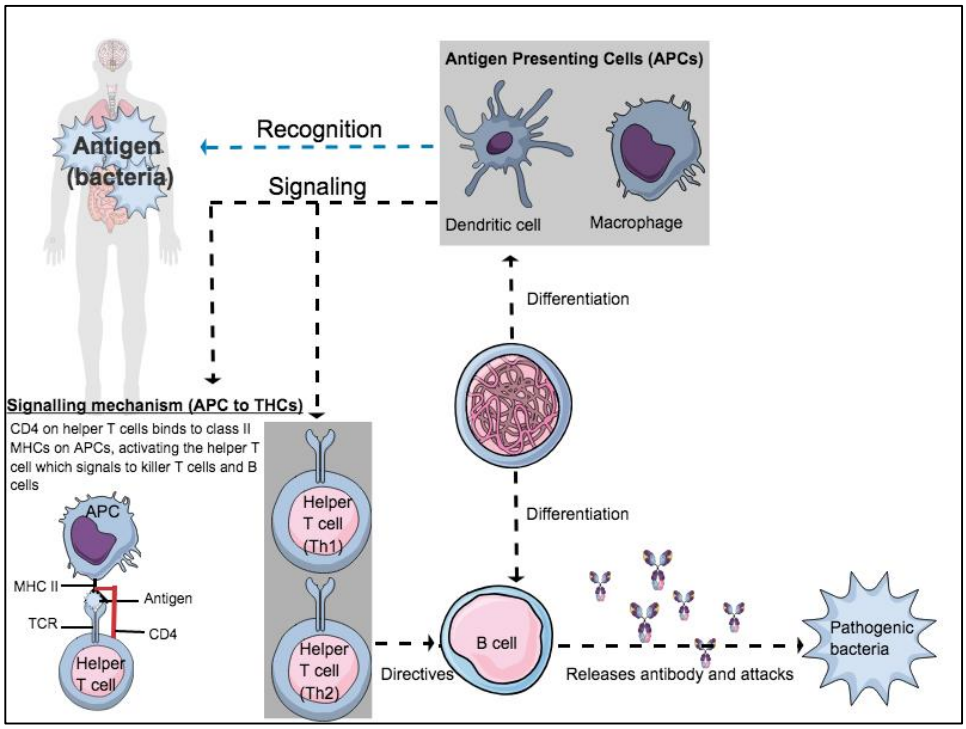


Figure 10 Pathogenic bacteria are sensed by antigen-presenting cells, e.g. dendritic cells or macrophages, and the signal is transmitted to helper T (Th) cells. Th1 cells direct killer T cells to attack virus-infected cells and cancerous cells, while Th2 cells direct B cells to produce

antibodies to attack pathogens. Most immune cells differentiate from hematopoietic stem cells in the bone marrow [adapted from (RIKEN, 2011)].

Classical APCs include dendritic cells, macrophages, Langerhans cells and B cells (Nature, 2017). Antigen presentation is the process whereby APCs take up an antigen and displays it as an immunogenic fragment to a T cell or B cell thus delivering an activation signal. Moreover, APCs may secrete co-stimulatory molecules such as chemokines and cytokines which are essential to the activation process. It is imperative to realize that antigen presentation is not entirely random since cytokines secreted by T cells and other inflammatory cells can significantly augment the antigen presenting capacity of specific cells (Hamilos, 1989).
A study to assess the ability of SODIS-treated *Vibrio cholerae* to induce the maturation of dendritic cells showed that solar-irradiated *V. cholerae* cultures were capable of causing maturation of the JAWS II dendritic cells *in vitro* (Ssemakalu *et al.*, 2015a). Such bacteria may be capable of eliciting a T-cell type 1 immune response (Ssemakalu *et al.*, 2015b). Since solar-irradiated pathogens induce dendritic cell maturation, it would be of interest to assess the activation of macrophages infected with solar-irradiated pathogens to further understand the immunological responses associated with consumption of SODIS treated water.

2.8 Macrophages

Macrophages are antigen presenting cells that play essential roles in host defence by recognising, engulfing, and killing microorganisms (Gordon, 2003). The direct bactericidal features of macrophages include the production of reactive oxygen species (ROS) and phagocytosis. Phagocytosis involves the engulfment of bacteria into phagosomes which fuse with late endosomes or lysosomes in the process of ‘‘maturation" leading to the eventual degradation of the bacteria (Karavitis & Kovacs, 2011). Alternatively, the secondary immune response involves inflammation, a process which results in increased production of inflammatory cytokines and chemokines which promote the recruitment of blood leukocytes to the site of infection and the activation of additional immune cells (Flannagan *et al.*, 2009). In some instances, the protective responses of the host are overcome by the invading pathogen triggering **the death of activated macrophages** (Gong *et al.*, 2012). Therefore, it is imperative to investigate how solar irradiated pathogens induce cell death in macrophages because cell death of immune cells determine the hosts immune response to the dying cells as described below.

2.9 Modes of cell death

In response to infection, macrophages are tightly regulated and undergo programmed cell death such as apoptosis, which may restrict bacterial growth and eliminate the replicating

intracellular pathogens (Ashida *et al.*, 2011). However, some pathogens are “sneaky” and can modulate macrophage cell death pathways for their own benefit. For instance, some pathogens can elicit uncontrolled cell death pathways such as necrosis which can be pathogenic and lead to tissue damage and increased bacterial infectivity (Chow *et al.*, 2016). Host cell death modes are hence important for tissue homeostasis and immune regulation. Cell death can be sub-divided into two primary groups, apoptotic and necrotic cell death. Apoptosis has come to be used synonymously with the phrase ‘programmed cell death' as it is a cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signalling pathways (Danial & Korsmeyer, 2004). Apoptosis is characterised by nuclear condensation and fragmentation, cleavage of chromosomal DNA into inter-nucleosomal fragments and packaging of the dead cell into apoptotic bodies without plasma membrane breakdown. Apoptotic bodies are recognised and engulfed by phagocytic cells, and thus apoptosis reduces inflammation around the dying cell (Edinger & Thompson, 2004). The morphological features of apoptosis are as a result of the activation of caspases (cysteine proteases) by either death receptor ligation or the release of apoptotic mediators from the mitochondria. Apoptotic cell death needs energy in the form of ATP; unlike necrosis which has been speculated to be a passive form of cell death. Necrosis is the result of ATP depletion to a level incompatible with cell survival and was thought to be initiated by toxic substances or physical damage (Edinger & Thompson, 2004). Necrosis is characterized morphologically by vacuolation of the cytoplasm, breakdown of the plasma membrane and induction of inflammation around the dying cell attributable to the release of cellular contents and pro-inflammatory molecules. Cells that die by necrosis exhibit changes in nuclear morphology but not the organized chromatin condensation and fragmentation of DNA into 200 bp fragments that is characteristic of apoptotic cell death (Edinger & Thompson, 2004).

Autophagy is characterised by the degradation of cellular components within the intact dying cell in autophagic vacuoles. The morphology of autophagic cells includes vacuolisation, degeneration of cytoplasmic contents, and slight chromatin condensation. Autophagic cells like apoptosis can also be taken up by phagocytosis. Pyroptosis is a programmed pathway to cell death mediated by the activation of caspase-1, a protease that activates the pro-inflammatory cytokines, IL-1β, and IL-18. This pathway is therefore inherently pro-inflammatory. Pyroptosis is also characterised by cell lysis and release of inflammatory cellular contents (Fink & Cookson, 2005) (Fig. 11).

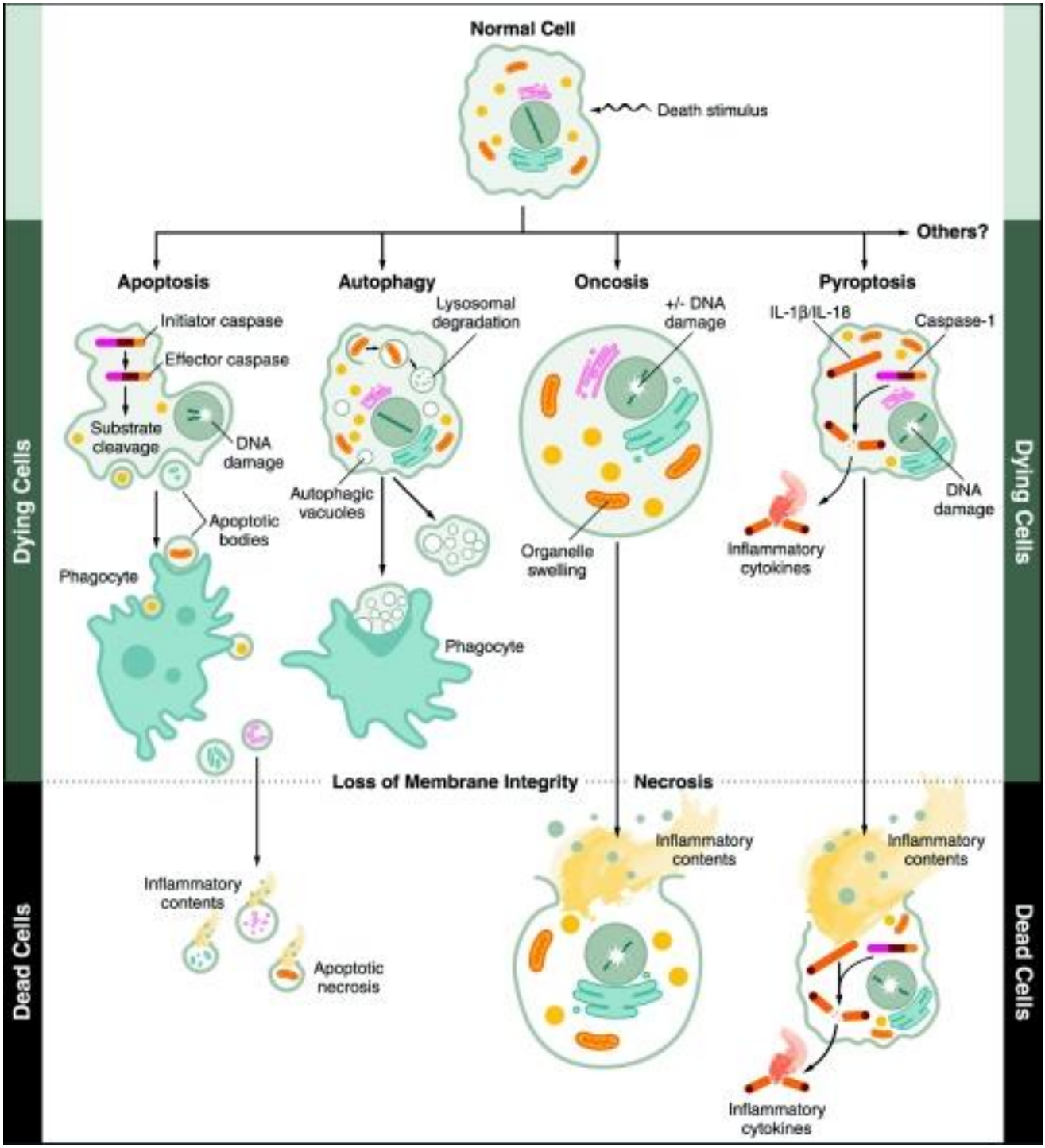


Figure 11 Modes of cell death (Fink & Cookson, 2005)

2.9.1 Effects of cell death on the immune system

Damaged cells can release danger signals that can alert the host to cell death (Fig.12). Some of these molecules are recognised by cellular receptors that stimulate the generation of pro-inflammatory mediators. Dead cells release signals that stimulate the production of mediators from extracellular sources. The mediators then lead to the inflammatory response which

releases signals that cause the maturation of dendritic cells and enhances the immune responses to antigens (Rock & Kono, 2008).

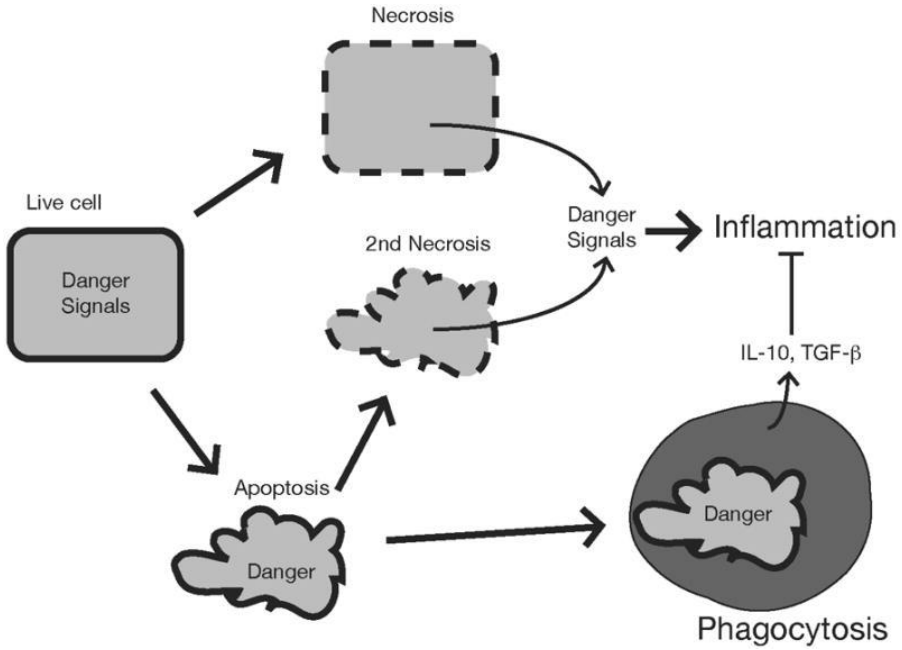


Figure 12 Responses to different forms of cell death (Rock & Kono, 2008)

Cells undergoing necrosis lose membrane integrity and leak their intracellular components some of which serve as danger signals that stimulate inflammation. Apoptotic cells may not stimulate inflammation if they are ingested by phagocytes before they release their intracellular contents. Moreover, during this process, apoptotic cells can stimulate phagocytes to produce anti-inflammatory cytokines. However, if the apoptotic cells are not cleared rapidly, they release danger signals when they proceed into secondary necrosis (Rock & Kono, 2008).

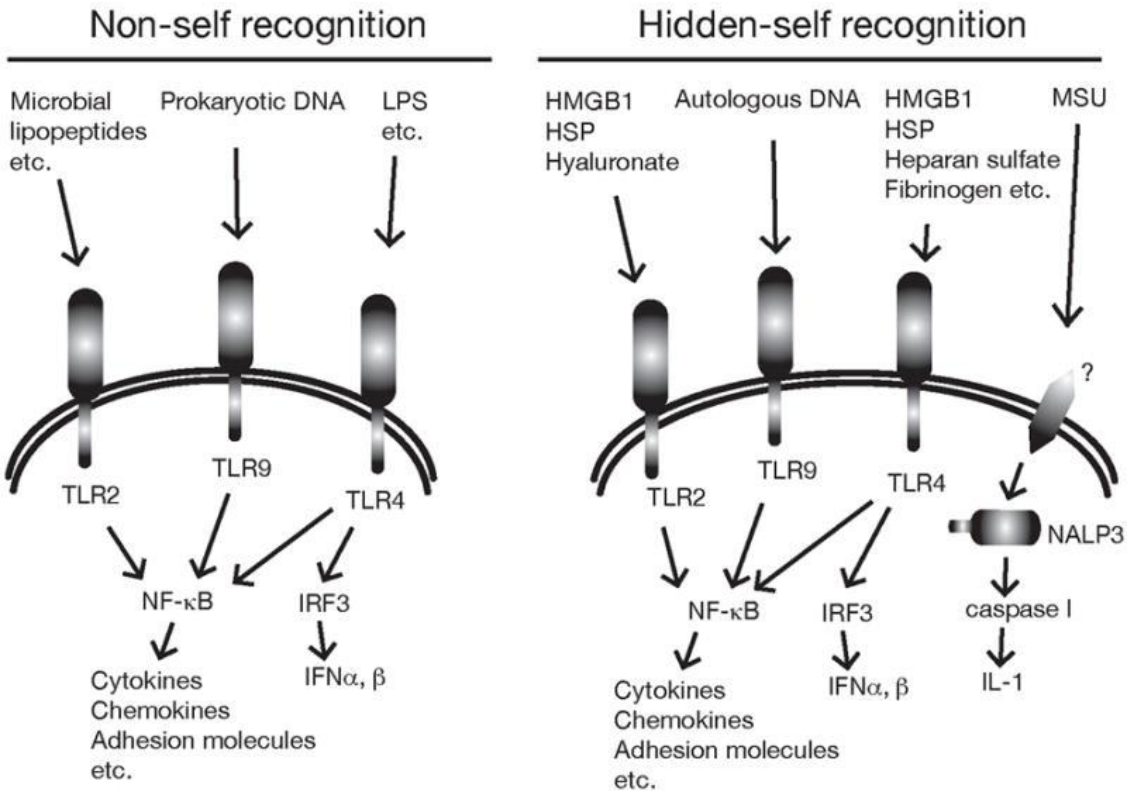


Figure 13 Examples of cellular receptors that sense infection and cell death (Rock & Kono, 2008).

Toll like-receptors (TLR) detect infections by recognising microbial molecules that are structurally different from mammalian ones (distinguishing self from non-self) (Fig.13). Also, there is emerging evidence that they can recognize specific autologous molecules that are usually hidden intracellularly ("hidden self") but are released when cells die. Once stimulated TLRs lead to the activation of the transcription factor NFkB and sometimes IRF3 which then turn on many of the critical components of the inflammatory response. The danger signal MSU stimulates a pathway that contains the intracellular Nod-like receptor NALP3 and results in the production of the proinflammatory cytokine IL-1 (Rock & Kono, 2008).

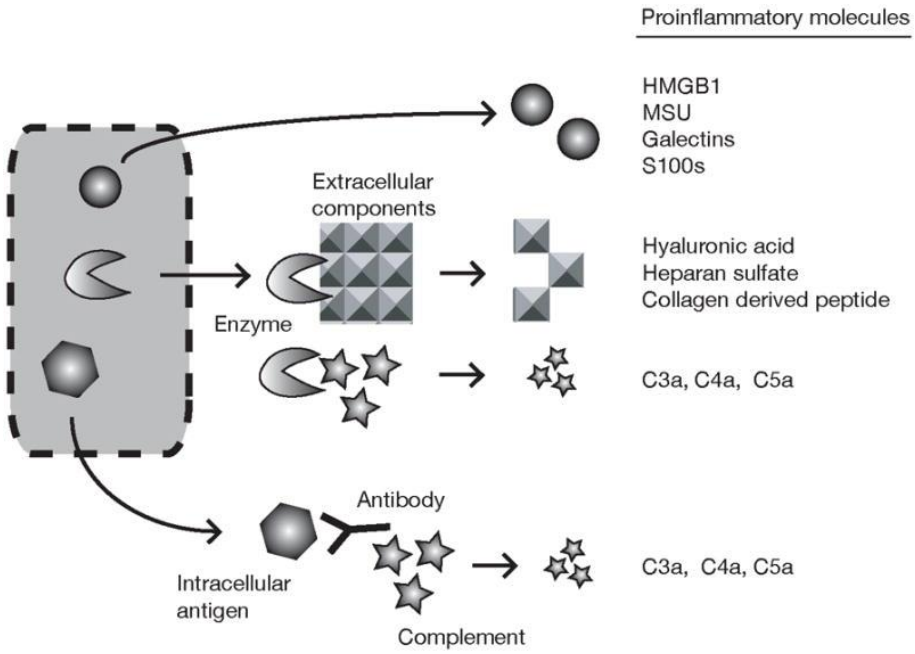


Figure 14 Pro-inflammatory molecules released from dying cells (Rock & Kono, 2008).

Cells contain some danger signals that can potentially stimulate inflammation through different mechanisms. Some of these signals are intracellular molecules that trigger inflammation by directly stimulating cells to make pro-inflammatory cytokines. Others work by generating pro-inflammatory mediators from extracellular components such as the extracellular matrix and complement (Fig.14) (Rock & Kono, 2008).

C*ampylobacter jejuni* and *S.* Typhimurium are also intracellular organisms and they do elicit particular changes within phagocytes, especially macrophages. For instance, *Salmonella* Typhimurium is an intracellular pathogen that has been reported to induce cytotoxic effects in both phagocytic and non-phagocytic cells (Mackaness *et al.*, 1966; Blaser & Newman, 1982; Alpuche-Aranda *et al.*, 1995; Schwan & Kopecko, 1997; Wijburg *et al.*, 2000; Fabrega & Vila, 2013). *Campylobacter jejuni*, on the other hand, was also found to induce inflammasome activation in both murine and human cells without apparent cytotoxicity (Bouwman *et al.*, 2014). However, some studies have shown that *C. jejuni* could induce cytopathic effects in mammalian cells (Yeen *et al.*, 1983). No information is available on highlighting the cytotoxicity of solar irradiated pathogens. Therefore, the study will focus on assessing the cytotoxicity and apoptotic abilities of solar irradiated *S.* Typhimurium and *C. jejuni.*

2.10 Macrophage activation

Macrophages carry out a variety of functions and have different effects on surrounding cells and tissues. There are two major classes of macrophages that have been extensively studied.

These are, the "classically" or M1 and "alternatively" or M2 activated macrophages (Stein *et al.*, 1992). The M1 macrophage phenotype (classically activated) produce high levels of pro-inflammatory cytokines, mediate resistance to pathogens, possess strong microbiocidal properties, provide high amounts of reactive nitrogen and oxygen intermediates, and promote T_H1 responses. In contrast, M2 macrophages (alternatively activated) are involved in parasite control, tissue remodelling, immune regulation, tumour promotion and efficient phagocytic activity (Bio-Rad, 2016) (Fig.15).

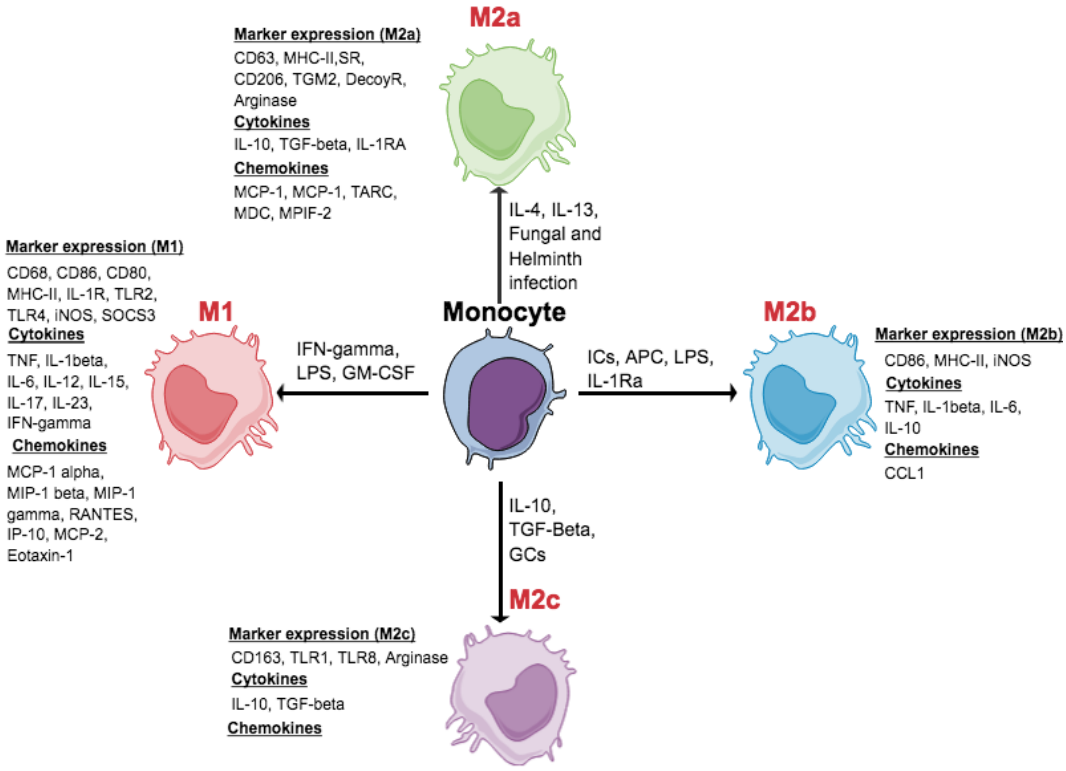


Figure 15 Macrophages are derived from monocytes which differentiate into a microbicidal (M1) phenotype, or immunosuppressive macrophages (M2). The phenotypes are highly dependent on the type of stimuli received for example; microbial substances and biochemical signals (e.g., cytokines) from the microenvironment of tissue. The different macrophage phenotypes release a variety of cytokines and chemokines that can either be pro-inflammatory or anti-inflammatory (Adapted from (Mantovani *et al.*, 2004).

Classically activated macrophages produce high levels of interleukin-12 (IL-12) and modest levels of IL-10 whereas, regulatory macrophages produce high levels of IL-10 and low levels of IL-12. Macrophages treated with IL-4 (wound-healing macrophages) produce low levels of these cytokines but express resistin-like molecule-α (RELMα) intracellularly, a marker that is not produced by the other macrophage populations. Treatment of IL-4-primed macrophages with lipopolysaccharide (LPS) results in a hybrid phenotype which expresses RELMα (similar

to wound-healing macrophages) but also produce high levels of IL-10 (identical to regulatory macrophages) (Fig.15) (Mosser & Edwards, 2008).

It has been shown that the M2 terminology has been used to describe a functionally diverse group of macrophages rather than activation phenotype. Alternatively activated (M2) macrophages can be further divided into the following categories, namely, M2a, M2b, M2c and M2d based on their distinct gene expression profiles (Fig.15) (Mantovani *et al.*, 2004). The M2a subtype is induced by IL-4, IL-13 or fungal and helminth infections. M2b is elicited by IL-1 receptor ligands, immune complexes, and LPS whereas M2c is elicited by IL-10, TGF-beta, and glucocorticoids. The fourth type, M2d, is obtained by IL-6 and adenosine. M2d macrophages have phenotypic and functional attributes similar to ovarian TAMs but are distinct from M2a-c. M1 and M2 macrophages have distinct chemokine and chemokine receptor roles, the M1 phenotype secretes the Th1 cell-attracting chemokines CXCL9 and CXCL10 and M2 secreting CCL17, CCL22, and CCL24. It has been shown that *in vitro*, macrophages are capable of complete repolarization from M2 to M1, and can reverse their polarization depending on the chemokine environment (Davis *et al.*, 2013).

Campylobacter jejuni circumvents the mucus layer in humans and interacts with the intestinal epithelial cells causing interleukin (IL)-8 production. *Campylobacter jejuni* binds to and is internalised by epithelial cells. The expression of IL-8 causes the recruitment of dendritic cells (DC), macrophages and neutrophils, which interact with *C. jejuni*. These interactions result in a pro-inflammatory response and increase in the corresponding cytokines (Fig. 16) (Young *et al.,* 2007).

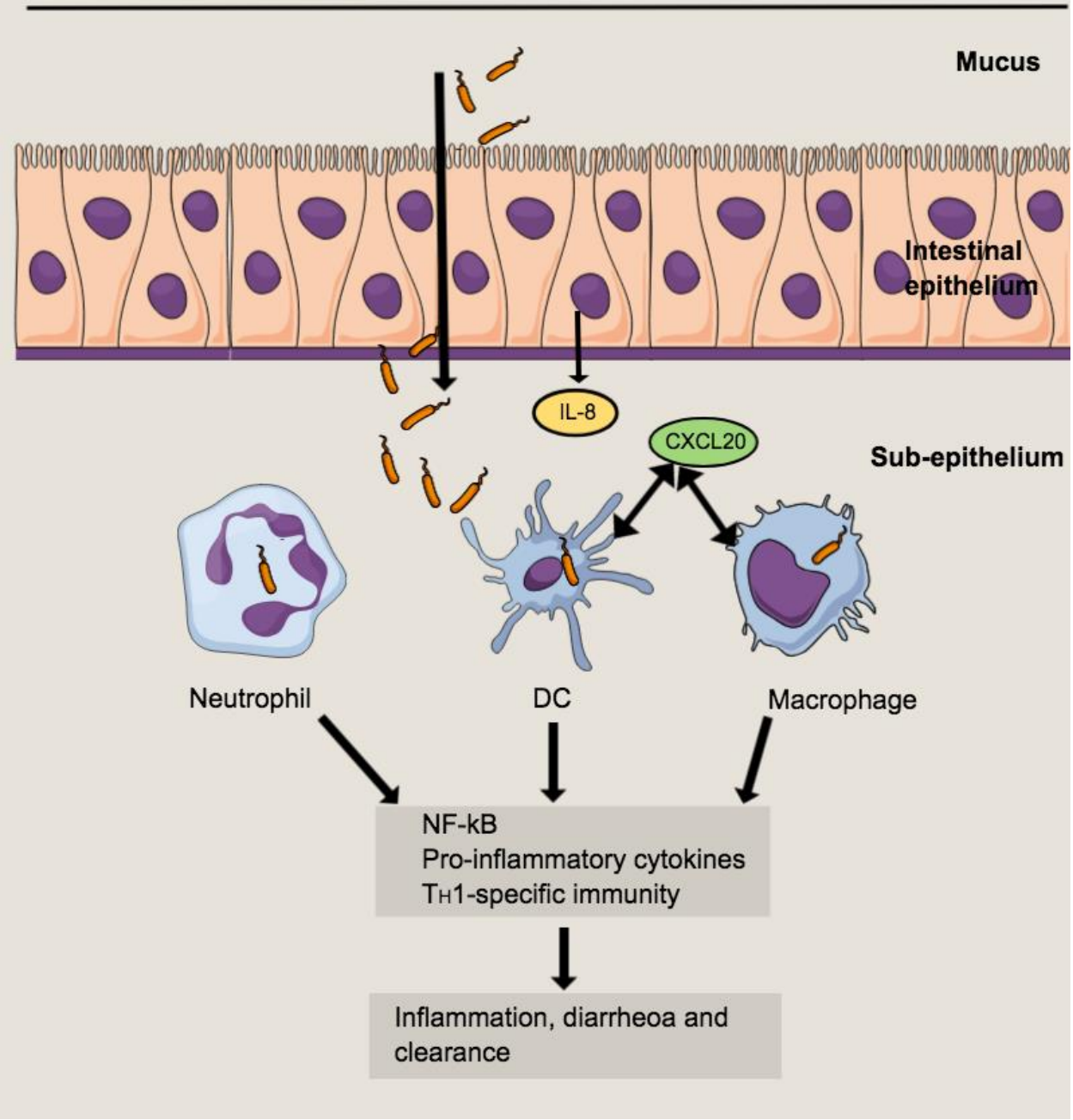


Figure 16 Molecular and cellular features of the innate immune response to *Campylobacter jejuni* in humans (adapted from Young *et al.*, 2007).

2.11 Cell death assays

Several experimental methods have been developed to ascertain modes of programmed cell death or apoptosis (Muppidi *et al.*, 2004). Cell death can be caused by specific death-inducing surface receptors such as Fas/CD95, or pathological conditions such as nutrient deprivation and hypoxia. When these conditions are worsened, the cell's internal mechanisms of programmed cell death are initiated, resulting in the programmed cell death. Caspases are a group of cysteine proteases which are crucial to most, but not all cell-death mechanisms (Muppidi *et al.*, 2004). Caspases are used as significant differentiators between necrosis and

apoptosis, because caspases are activated during programmed cell death mechanisms such as apoptosis and pyroptosis, and not in necrotic cells. Another feature distinguishing necrosis from apoptosis is the increase in membrane permeability due to the disintegration of the cellular membrane during necrosis. Therefore, **in this study**, flow cytometry was used to differentiate the different modes of cell death since this technique is regarded as one of the most accurate ways to quantitate cell death. During data acquisition, thousands of single-cell events can be rapidly acquired and analysed. However, the main disadvantage in using flow cytometry is that late-apoptotic cells may disintegrate into several smaller apoptotic bodies with variable size and dye-uptake properties and leave the gate used for the acquisition of events (Muppidi *et al.*, 2004).

The Guava MultiCaspase SR Kit for Flow Cytometry may be used to detect apoptosis through the covalent binding of a red fluorescent probe to active caspase enzymes in living cells. The sulforhodamine-labeled caspase inhibitor probe (SR-VAD-FMK; excitation at 550 nm and emission at 595 nm), comprising a sulforhodamine derivative of valylalanylaspartic acid fluoromethyl ketone (VAD-FMK), covalently binds to the reactive cysteine (Cys 285) on the large subunit of active caspases. This probe is both cell permeable and non-cytotoxic during the time of the labelling procedure and allows detection of apoptosis by flow cytometry. The kit also comprises of 7-AAD (7-amino-actinomycin D) dye which is used to identify necrotic cells. The 7-AAD has a high DNA binding constant and is effectively excluded by intact cells (such as apoptotic cells). It is useful for DNA analysis and dead cell discrimination during flow cytometric analysis. When excited by 488 nm laser light, 7-AAD fluorescence is detected in the far red range of the spectrum (650 nm long-pass filter) (BioLegend, 2018). To differentiate the necrotic cells and the pyroptotic cells a qualitative analysis approach may be used e.g. an inverted light microscope can be used to observe the morphologies of the dying cells. Pyroptotic cells appear large, swollen and rounded, and the cellular membrane seems to be intact (DiPeso *et al.*, 2017).

2.12 Chemokine and cytokines assays

The study of cytokines and other analytes is becoming increasingly crucial in the study and management of many diseases. Thus, there is a greater demand for rapid, precise, and cost-effective measurement of such analytes in both clinical and research laboratories (Elshal & McCoy, 2006). Multiplex bead array assays (such as the Luminex kits) provide a quantitative assessment of large numbers of analytes using an automated 96-well plate format. ELISAs (enzyme-linked immunosorbent assay) has been the standard for quantitative analysis of cytokines and other biomarkers but are not suitable for high throughput multiplex analyses. However, prior to replacement of ELISA assays with multiplex bead array assays, there is a

need to know how comparable these two methods are for quantitative analyses. Some published studies have compared these two methods, and it is apparent that some aspects of these assays, such as the clones of monoclonal antibodies used for detection and reporting, are pivotal in obtaining similar results from both tests. By careful consideration of these variables, it was possible to utilize multiplex bead array assays instead of ELISAs for studies requiring high throughput analysis of numerous analytes.

2.13 Proteomic analysis

The term proteome is used to represent the whole set of cellular proteins. This term was first was first used by Marc Wilkins in 1996 when he carried out the analysis of 20 *E. coli* proteins using two-dimensional (2-D) gel electrophoresis and mass spectrometry (MS) (Wilkins *et al.*, 1996).

Proteomic interactions of pathogenic bacteria with host cells during infection states could reveal several changes that can occur in the proteome or in post-translational modification (PTM) of proteins. High-resolution mass spectrometry-based proteomics can bridge the gap between genomics and the physiological mechanisms of behaviour (Dove, 1999). Therefore, a quantitative analysis of the host-pathogen proteome was carried out in this study, because proteomics provides a greater elucidation of the molecular events that occur during the onset of infection. Intricate changes occurring at the proteomic level and quantitative post-translational modification analyses are some of the applications of the shotgun proteomics technology. The high-throughput "omics" technology also enables identification of macromolecular components essential for specific host-cell interactions (Tracz *et al.*, 2013; Alvarez Hayes *et al.*, 2015). Currently, there is no literature on the effects of solar irradiated pathogens on the host on a macromolecular level. This study seeks to elucidate and compare interactions of *S.* Typhimurium and *C. jejuni* in the host cell (macrophages) (Kelleher, 2004; Chait, 2006; Zhang & Ge, 2011; Ansong *et al.*, 2013; Tracz *et al.*, 2013; Alvarez Hayes *et al.*, 2015).

The functional interface between solar irradiated pathogens and their host cells is still relatively unknown and the exact molecular mechanisms governing different stages of infection and activation of immune cells are still poorly understood. This lack of knowledge in understanding the role of solar irradiated pathogens in the immune system impedes the development of new solar-based therapeutic strategies. Thus, this study aimed to assess the impact of solar irradiated *C. jejuni* and *S. Typhimirium* on macrophages by using a global proteomic approach. The interaction between host cells and pathogens involves several hundred to thousands of proteins from both sides (Hartlova *et al.*, 2011; Zhang & Ge, 2011). Elucidation of molecular mechanisms in host-pathogen interactions is crucial for the control and treatment of infectious

diseases. Within the last decade, mass spectrometry (MS)- based proteomics has become a practical approach to better elucidate complex and dynamic host-pathogen interactions at the protein level (Yang *et al.*, 2015). Most of the studies have previously focused on the characterization of individual bacterial virulence factors and their interacting host targets by using traditional methods such as genetic and biochemical ways. These methods have contributed tremendously to the current body of knowledge with regards to infection biology. However, such studies alone are insufficient to explain the complex nature of host-pathogen interactions (Walduck *et al.*, 2004).

In this study, sample clean-up and digestion of macrophages infected with *S.* Typhimurium and *C. jejuni* was carried out using multi-mode hydrophilic interaction chromatography magnetic microparticles for solid phase extraction (HILIC SPE)-based sample clean-up and digestion (Stoychev *et al.*, 2012). The HILIC-SPE was developed to improve reproducibility of sample preparation. This method is a robust automatable sample preparation workflow that integrates sample clean-up and digestion using multi-mode hydrophilic interaction chromatography magnetic micro-particles for solid phase extraction (HILIC SPE), followed by on-bead tryptic digestion, and direct LC-MS/MS analysis (Fig. 17). Automation of the workflow provides processing capability of up to 96 samples (inclusive of digestion) without time-consuming offline steps such as centrifugation. The performance of the magnetic HILIC-SPE workflow was compared to commonly used universal methods for pre-MS sample clean-up, including Filter Aided Sample Preparation and it was shown that approximately a 2-fold increase in peptide recovery, translating to over 30% increase in identified peptide spectrum matches (PSMs), peptides, and ultimately unique proteins identified.

After HILIC clean up samples preparation, LC-MS/MS acquisition was carried out The spectral library was constructed using Data Dependant Acquisition DDA LC-MS/MS. Spectral libraries play a crucial role in the analysis of data independent acquisition (DIA) (Ammar *et al.*, 2018). DIA experiments require spectral libraries, as most current methods cannot apply traditional peptide identification via database searching on DIA data. Then sequential window acquisition of all theoretical mass spectra (SWATH-MS) (Gillet *et al.*, 2012) was carried out.

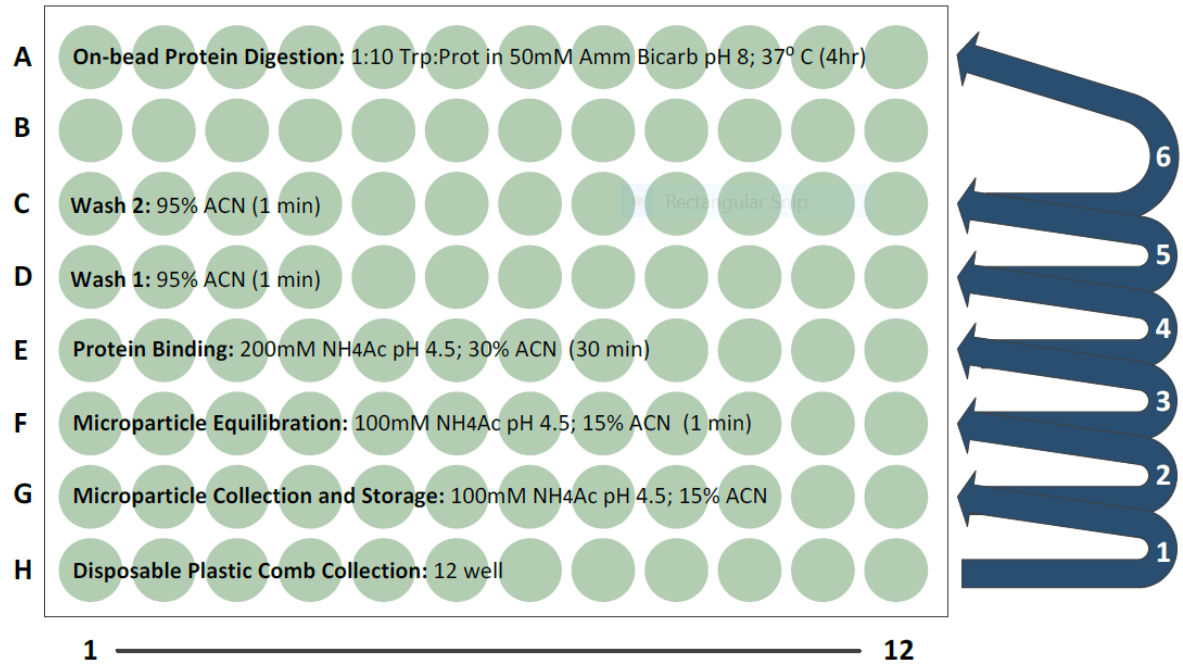


Figure 17 A KingFisher™ Duo magnetic handling station was configured for HILIC based clean-up and protein digestion. MagReSyn® HILIC micro-particles are placed in row G and protein samples, pre-mixed with Binding Buffer, in row E. The micro-particles are collected from row G, transferred to row F for equilibration after that transferred to row E for protein binding. Contaminants are removed by two successive washes in rows D and C. Digestion proceeds for 4 h in row A (peltier system). Micro-particles are then removed from the digested protein mixture back to the storage position in row G. Peptide – containing supernatant from row A is recovered and dried in 0.5 ml microcentrifuge tubes (Stoychev *et al.*, 2012)].

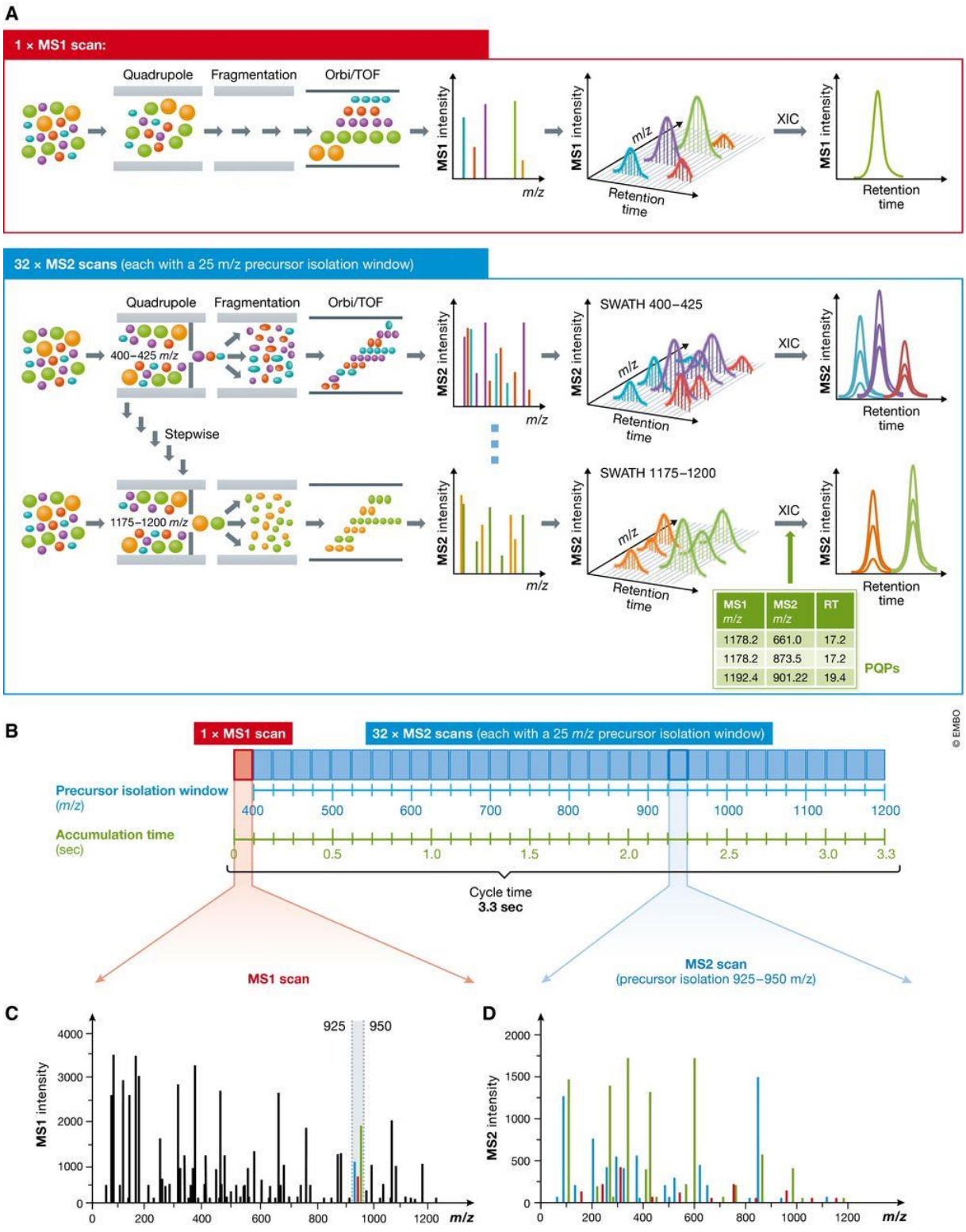


Figure 18 Principle of sequentially windowed data-independent acquisition in SWATH-MS (Ludwig *et al.,* 2018)

(A) SWATH-MS measurements are carried out on fast scanning hybrid mass spectrometers, usually employing a quadrupole as a first mass analyser and a TOF or Orbitrap as a second

mass analyser. In SWATH-MS mode, a single precursor ion (MS1) spectrum is recorded, followed by a series of fragment ion (MS2) spectra with wide precursor isolation windows (for example 25 m/z) (Fig.18). Through repeated cycles of consecutive precursor isolation windows over a defined mass range, a detailed data set is recorded, because continuous information is obtained on all detectable fragment and precursor ions. Therefore, extracted ion chromatograms can be constructed on MS2 as well as MS1 level. For SWATH-MS data analysis, a peptide-centric scoring strategy can be done, which requires specific knowledge about the chromatographic and mass spectrometric behaviour of all queried peptides in form of peptide query parameters (PQPs). **(B)** The SWATH-MS (Gillet et al 2012) for a Q-TOF mass spectrometer uses 32 MS2 scans with defined increments of 25 m/z, starting at 400 m/z and ending at 1,200 m/z. One full MS1 scan is recorded at the beginning. **(C)** The MS1 full scan detects all peptide precursors eluting at a given time point. For example, in the mass range from 925 to 950 m/z, three co-eluting peptide species are detected (green, red and blue). **(D)** The corresponding MS2 scan with a precursor isolation window of 925–950 m/z represents a mixed MS2 spectrum with fragments of all three peptides (Ludwig *et al.*, 2018). SWATH-MS is being used in this study because it supports quantitative analyses of peptides covering a large number of proteins with high quantitative consistency and accuracy. It is ideal for projects that require a large number of samples and that require accurate and reproducible quantification for the significant fraction of the expressed proteome or peptidome in each sample e.g. biomarker studies (Liu *et al.*, 2014; Muntel *et al.*, 2015; Kulkarni *et al.*, 2016).

2.14 Thesis Summary: The effect of solar irradiated pathogens on the activation of macrophages

The research presented in this thesis aimed to investigate the effects of solar irradiated *S.* Typhimurium and *C. jejuni* on the activation of murine macrophages. Based on the current health benefits that have been experienced by SODIS users, it is apparent that there is a need to explore the immunogenic responses to solar irradiated waterborne pathogens. From the above review of the literature, it is evident that the interaction between resistant (*S.* Typhimurium) and susceptible (*C. jejuni*) pathogens to SODIS and the host macrophage is essential in determining the immune response to the solar irradiated pathogens. However, the manner in which solar irradiated bacteria may elicit an immune response of the macrophages is still not completely understood. There are gaps in the knowledge, including how the solar irradiated bacteria induce cell death, and how they interact with the macrophages at the molecular level. The main objectives of this work were to assess the links between cell death and immune response and the proteome (protein analysis using Luminex and SWATH-MS) of the macrophages in response solar irradiated *S.* Typhimurium and *C. jejuni.* This thesis

presents evidence on how solar irradiated *S.* Typhimurium prevents necrotic cell death in macrophages, and solar irradiated *C. jejuni* reduces apoptotic cell death in macrophages. Thus, the immune function in macrophages was further investigated, due to the known connections between cell death and the immune response. This study showed that SODIS treatment of pathogens does have a significant impact on the innate immune response of macrophages as shown by the chemokine and cytokine profiles, as well as upregulated and downregulated proteins in macrophages. Thus, this work established a new experimental platform for investigating possible host immune responses to solar irradiated pathogens, and this may be used for future vaccine-development. Solar derived vaccines may offer an alternative, sustainable, and strategic therapeutic interventions in infectious diseases.

Chapter 3 Assessing the cytotoxicity and apoptosis-inducing ability of solar irradiated *Salmonella* Typhimurium in RAW264.7 cell line in vitro

Abstract

The consumption of solar disinfected (SODIS) water has reduced diarrhoeal incidences in resource-poor regions of the world. Thus, this study assessed the effects of solar radiation on the viability and on three virulence characteristics (invasion, cytotoxicity, and apoptosis) of *S.* Typhimurium on macrophages*.* Samples of *S.* Typhimurium were treated in three ways: (i) suspended in water and exposed to solar ultra-violet radiation (SUVR), (ii) exposed to SUVR but in a non- transparent flask, and (iii) heat and chemically attenuated by treating the bacteria with 0.5% phenol at 65°C for one hour. *Salmonella* Typhimurium samples were pipetted out from the three treatments after 4 and 8 h and used to infect macrophage RAW264.7 cells. The intracellular growth of *S.* Typhimurium was assessed by using the gentamicin protection assay. Cytotoxicity assays were carried out using the Lactate Dehydrogenase Assay (LDH). Apoptosis of infected macrophages was analysed using flow cytometry. The results showed that *S*. Typhimurium was inactivated after 4 and 8 h of solar irradiation. The solar irradiated *S*. Typhimurium exhibited no intracellular growth, and produced lower cytotoxicity and fewer necrotic cells in RAW 264.7 cells. The non-irradiated *S.* Typhimurium survived within the macrophages, were highly toxic to the cells and caused a significant increase ($p<0.01$) in necrotic cells when compared to the solar irradiated samples. In conclusion, solar irradiated *S.* Typhimurium impacts the viability and virulence properties especially the survival of RAW264.7 cells and also reduces their ability to induce cytotoxicity and necrotic cell death in the macrophages.

Keywords: SODIS, *Salmonella* Typhimurium*,* cytotoxicity, apoptosis, macrophages

3.1 Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an enteric zoonotic pathogen that infects both humans and animals. The ingestion of *S.* Typhimurium through the consumption of contaminated food or water could produce an invasive form of gastroenteritis (Fabrega & Vila, 2013; Das *et al.*, 2014). Globally, *Salmonella* infections (typhoid and non-typhoidal) are a leading cause of morbidity, with high rates of mortality, in resource-limited settings particularly in sub-Saharan Africa and parts of the Asian sub-continent (Reddy *et al.*, 2010). Solar disinfection of water has been shown to be an effective means of reducing water-related infections such as gastrointestinal diseases in developing countries (Conroy *et al.*, 1999; du Preez, 2010). During solar disinfection, the UV-A rays and visible light lead to oxidative stress by generating reactive oxygen species (ROS) that interact with DNA, proteins, and lipids (Sinha & Hader, 2002; Friedberg, 2003). The success of SODIS has been attributed to solar ultra-violet inactivation of the disease-causing microbes in water. However, the influence of the solar inactivated microorganisms on the cells of the host's immune system is not well understood.

Salmonella Typhimurium is an intracellular pathogen that has been reported to induce cytotoxic effects in both phagocytic and non-phagocytic cells (Mackaness *et al.*, 1966; Blaser & Newman, 1982; Alpuche-Aranda *et al.*, 1995; Schwan & Kopecko, 1997; Wijburg *et al.*, 2000; Fabrega & Vila, 2013). Once *S.* Typhimurium invades a phagocytic cell such as a macrophage, it replicates and produces cytotoxins within the cell inducing an apoptotic response (Chen *et al.*, 1996; Monack *et al.*, 1996). Studies have shown that the proteins encoded within the *Salmonella* pathogenicity islands (SPIs) found on the chromosome of *S.* Typhimurium are important for both invasion and intracellular survival (Hueck, 1998; Wood *et al.*, 1998). The SPIs encode several proteins which lead mainly to the invasion of epithelial cells by mediating actin cytoskeletal rearrangements and internalisation of bacteria (Cookson & Brennan, 2001). The live form of *S.* Typhimurium has been reported to induce a caspase-1 dependent form of cell death known as pyroptosis (Cookson & Brennan, 2001). Pyroptosis is dependent on the inflammasome, a multiprotein complex that regulates the activation of caspase-1, leading to proteolytic activation of the cytokines IL-1β and IL-8. These cytokines play crucial roles in acute and chronic inflammation and stimulate recruitment of immune cells, which cause an inflammatory response (Fink & Cookson, 2007).

Microbial virulence factors such as bacterial replication and host cell cytotoxicity have significant consequences on the innate and adaptive responses. Intracellular bacteria cause diseases by invading cells and promoting bacterial survival, replication, and spread. Pathogens that lose these virulence factors generally become avirulent, and thus incapable of causing diseases. Thus, the three main objectives of this study was to evaluate the effectiveness of SODIS method for the inactivation of *S.* Typhimurium*.* Secondly*,* to assess

the possibility or resuscitation of *S.* Typhimurium in host cells (macrophages) during infection, and thirdly to investigate the cytotoxic and apoptotic effects of solar irradiated *S.* Typhimurium on macrophages.

3.2 Methodology

3.2.1 Culture of *S.* Typhimurium

Salmonella enterica serovar Typhimurium (ATCC® 29629) was grown on Luria-Bertani (LB) agar. A single colony of *S.* Typhimurium was then inoculated in 33% LB broth and incubated at 37ºC with shaking at 150 rpm for 24 h until an exponential growth phase was reached. The culture was then diluted to an OD_{548} of 0.002 in pre-warmed LB broth and incubated at 37ºC with shaking at 150 rpm overnight or for 18 h until the stationary phase was reached (Bosshard *et al.*, 2009).

3.2.2 Preparation of solar and non-solar irradiated *S.* Typhimurium

Salmonella Typhimurium that reached the stationary phase were harvested by centrifugation at 4000xg for 15 min and washed thrice with autoclaved still bottled mineral water (Oasis, Vanderbijlpark, South Africa). The bacteria were diluted with the still bottled mineral water to an OD_{546} of 0.1 (approximately $1x10^7$ CFU/ml) in a total volume of 15 ml in 25 cm^3 transparent tissue culture flasks. The flasks were then placed on aluminium foil and exposed to the sun for 0, 4 and 8 h. The experiment was conducted on the rooftop of a building at the Vaal University of Technology (26°42'39.1"S 27°51'46.2"E -26.710858, 27.862820) where there was no shade during the spring of 2017. The amount of solar ultra violet irradiation (UVA +UVB radiation) was measured at 2 h intervals and captured by the Lutron 340A UV Light Meter (Lutron Electronics Company, Coopersburg, PA). For each experiment, the control samples were exposed to similar atmospheric conditions, except for SUVR, by enclosing the samples in an opaque ventilated box. All solar irradiation experiments were carried out on sunny, cloudless days from 8.00 am - 4.00 pm. The viability of the solar and non-solar irradiated bacteria was evaluated using the Miles and Misra drop method (Miles *et al.*, 1938).

3.2.3 Preparation of heat-chemical inactivated *S.* Typhimurium

Salmonella Typhimurium was harvested as above and washed thrice with autoclaved 1X PBS to remove all traces of LB broth. The bacteria were then diluted to an OD_{546} of 0.1 (approximately $1x10^7$ CFU/ml) in 1X PBS and were then inactivated using a combination of heat and chemical conditions (0.5% phenol at 65ºC for 1 h) (Salisbury *et al.*, 2006). The heat-inactivated culture was incubated at room temperature at 150 rpm for 48 h. The viability of the

bacteria was assessed on LB agar plates using the Miles and Misra drop counting technique (Miles *et al.*, 1938).

3.2.4 Testing of bacterial viability

The viability of the heat/chemically attenuated, solar irradiated and non-irradiated controls was assessed as follows. In brief, 100 µl of each sample were serially diluted in 900 µl of sterile PBS up to a dilution factor of 10^{-6}. Then 20 µl was spotted on LB agar plates in numbered sectors, air dried for 30 min and incubated at 37°C for 24 h. The number of CFU were enumerated in drop areas containing the largest number of colonies without signs of confluence or gross diminution in colony size due to overcrowding. The CFUs were expressed as Log_{10}CFU/ml.

3.2.5 Establishment of the macrophage cell line

A murine macrophage cell line RAW 264.7 was obtained from Cellonex (Separations, Randburg, South Africa). The cell line was grown in complete growth medium consisting of Dulbecco's Modified Eagles Medium (DMEM), with 10% foetal bovine serum (FBS) and 1% Penicillin /Streptomycin at 37ºC inside a humidified 5% CO_2 incubator.

3.2.6 Invasion and intracellular growth analysis of *S.* Typhimurium in RAW264.7 cells

The intracellular growth of *S.* Typhimurium in the macrophage cell line was assessed using the three types of treated *S.* Typhimurium (heat/chemically inactivated, non-solar irradiated and, solar irradiated for 4 and 8 h) after 3, 24 and 48 h of infection with the Gentamicin exclusion assay (Jolly *et al.*, 2015). Before infection, the RAW 264.7 macrophage cells were seeded in 6 well plates at a density of $1x10^5$ cells/ml and incubated for 24 h at 37ºC in a humidified CO_2 incubator. On the day of infection, the pre-seeded macrophage monolayers were washed once with 1x DPBS buffer, and 1000 µl of infection media (DMEM-I) containing *Salmonella* (MOI 10:1) was added to the plates that were incubated in humidified incubator at 37ºC with a 5% CO_2 atmosphere for 4 h. Thereafter, the unbound bacteria were washed off using DPBS. Then DMEM with 10% FBS and 20 µg gentamicin/ml was added to the cells followed by incubation at 3, 24 and 48 h at 37ºC in a 5% CO_2 atmosphere.

3.2.7 Harvest of infected macrophage lysate for CFU plating

At time-points of 3, 24 and 48 h, the media was aspirated from infected macrophage wells, and 1000 µl of sterile lysis buffer (0.5% Triton X-100) was added to each well. The plates were incubated at room temperature for 5 min (Crane *et al.*, 1999). After mixing, the lysates were transferred to sterile 96 well plates for serial dilutions. Serial dilutions (1:10) of lysates were

prepared in the 96 well plate by pipetting 25 µl into 225 µl of LB broth. Then 20 µl was spotted on LB agar plates.

3.2.8 Cytopathic and cytotoxic effects of *S.* Typhimurium in RAW264.7 cells

The potential for the i) heat and chemically inactivated, ii) non-solar irradiated, and iii) solar irradiated for 4 and 8 h *S.* Typhimurium cells to induce cytopathic effects in the macrophages after 3, 24 and 48 h of infection was investigated. The macrophages for each treatment were monitored for cell detachment, rounding and floating using an inverted bright field light microscope at a 20x magnification (Agnihothram *et al.*, 2015).

Cytotoxicity of the macrophages induced by *S.* Typhimurium was assessed after 3 and 24 h after infection using the lactate dehydrogenase (LDH) assay using the LDH detection kit from Thermofischer (Waltham, MA) according to the manufacturer's instructions. Cell death was expressed as the percentage of LDH release, which was calculated using the formula shown in equation (1).

Equation (1)

$$\%\ LDH\ release = \frac{Experimental\ value - Effector\ cells\ spontaneous\ control - Target\ Cells\ spontaneous\ control}{Target\ Cell\ Maximum\ Control - Target\ Cells\ Spontaneous\ Control}\ x\ 100$$

Experimental value stands for LDH released by macrophages infected with *S.* Typhimurium; effector cells spontaneous control is the LDH control for *S.* Typhimurium cells; Target cells Spontaneous control represents the LDH released by non-infected macrophages and the Target Cell Maximum Control represents the LDH maximum release by lysis of macrophages using 10% (vol/vol) of 10X lysis buffer.

3.2.9 Apoptosis assay of treated RAW264.7 cells

Apoptosis of treated RAW264.7 macrophages was assessed after 3 and 24 h after infection. Mid and late apoptosis was monitored by flow cytometry using the Guava EasyCyte 8HT instrument (Merck/Millipore, Molsheim, France) using a MultiCaspase SR kit (Merck/Millipore) according to the manufacturer's instructions. In brief, the macrophages were incubated for 1 h at 37°C in MultiCaspase SR solution containing an SR-Peptide flourophore. The cells were washed twice with apoptosis wash buffer, marked with 7-AAD, incubated at room temperature for 10 min, and analysed by flow cytometry. Apoptotic positive control for each experiment was treated with 50 µg/ml of Malphalan.

3.2.10 Statistical analysis

All experiments were replicated biologically three times. Where comparison in the mean was made a student's t-test was performed using Graph Pad Prism 7.0d Software. A two-tailed p value of <0.05 was taken to indicate statistical significance.

3.3 Results

3.3.1 Viability assessment of *S.* Typhimurium

The sensitivity of *S.* Typhimurium to natural sunlight is shown in Fig. 19. The lowest solar irradiance was observed at the beginning of the experiment at 8.00 (15.7 ± 0.3 W/m^2) whereas the highest solar irradiance of 47.4 ± 0.8 W/m^2 was reached at midday.

Salmonella Typhimurium was completely inactivated within four hours of exposure to sunlight. However, the non-solar irradiated samples remained viable at 0, 4 and 8 h. There was a highly significant *p<0.001* difference in the mean viability between the solar- and non-solar treatments. The heat/chemical attenuated *S.* Typhimurium were not viable (results not shown on graph).

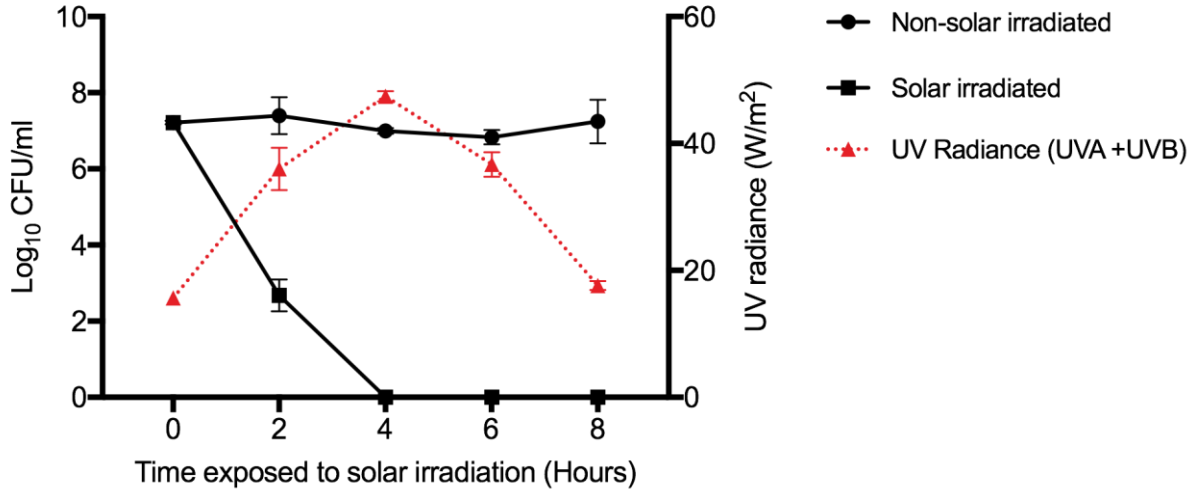


Figure 19 Viability and inactivation curve of solar irradiated and non-solar irradiated *S.* Typhimurium in water. The bacterial populations are expressed in Log_{10}CFU/ ml. Error bars indicate standard errors of triplicate measurements.

On the basis of the above results the effect of solar irradiation on the virulence properties of *S.* Typhimurium was done at the mid-point of 4 h solar irradiation (which in this case, the point where S. Typhimurium ceased to be viable) and the highest solar irradiation exposure period of 8 h. This represents the usual time for solar disinfection of contaminated water.

3.3.2 Invasion and Intracellular growth assessment of *S.* Typhimurium

This study used the gentamicin protection assay to assess the potential for the i) heat/chemical attenuated (HA) ii) non-solar irradiated (NS) and (iii) solar irradiated (SI4 and SI8) bacteria to recover and grow in the macrophages. Co-cultures of macrophages and *S.* Typhimurium that were solar irradiated for 4 and 8 h showed no bacterial growth after 3, 24 and 48 h of culture (Fig. 20).

Similarly, *S.* Typhimurium that were heat and chemically inactivated were unable to grown in the macrophages. However, the non-solar irradiated *S.* Typhimurium were observed to grow inside the macrophages after 3, 24 and 48 h. The highest level of intracellular growth of *S.* Typhimurium occurred after 3 h of stimulation with the non-solar irradiated samples (Fig. 20). However, at 24 h a slight reduction of growth was observed. An increase in intracellular bacterial growth was observed after 48 h of macrophage infection with non-solar irradiated *S.* Typhimurium (NS) (Fig. 20).

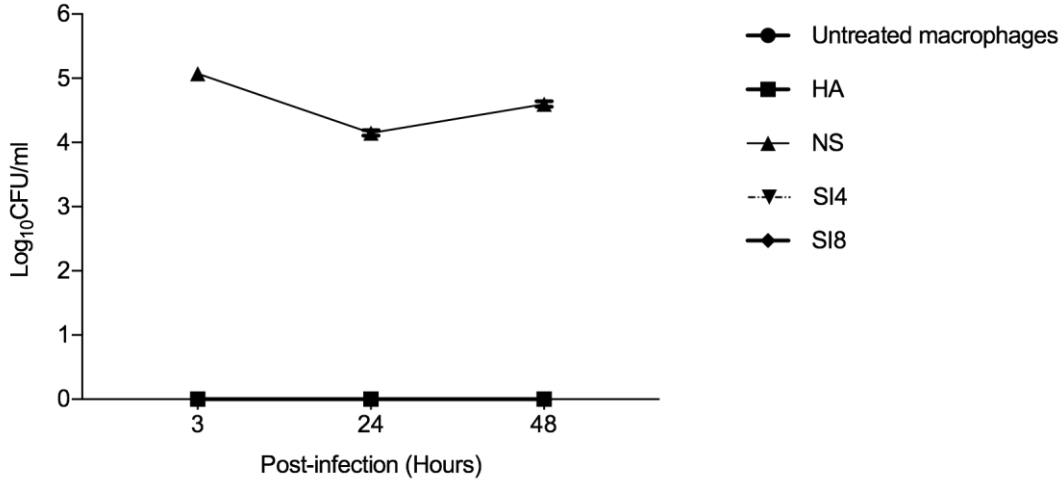


Figure 20 Intracellular growth analysis of macrophages that were (i) untreated (ii) treated with heat and chemical attenuated *S.* Typhimurium (HA), (iii) treated with non-solar irradiated *S.* Typhimurium (NS), (iv) treated with 4h- and 8h-solar irradiated *S.* Typhimurium, (SI4 and SI8, respectively). The intracellular growth analysis was conducted after 3, 24 and 48 h post-infection. Error bars indicate standard errors of triplicate experiments.

3.3.3 Cytopathic and cytotoxic effects of *S.* Typhimurium in RAW264.7 cells

At 3 h p.i, the untreated macrophages (Fig 21A), the macrophages treated with heat/chemically attenuated (21B), non-solar irradiated (21C) and solar irradiated *S.* Typhimurium (21D, 21E) showed no cytopathic effects (Fig. 21B). At 24 h p.i. and 48 h p.i., the morphology of the untreated macrophages remained the same (Fig. 22A, 22A) and they appear to have proliferated. The macrophages treated with heat and chemically inactivated *S.* Typhimurium (Fig. 22B, 23B) showed an increase in size and cellular elongation was

observed in some of the macrophages. However, the macrophages treated with solar irradiated *S.* Typhimurium showed shrinkage and disintegration of cellular membrane resulting in the accumulation of cellular debris (Fig. 22C, 23C). On the other hand the macrophages treated with solar irradiated *S.* Typhimurium resulted in cell swelling and cell rounding (Fig. 22D, 22E) at 24 h p.i. and at 48 h p.i. (23D, 23E).

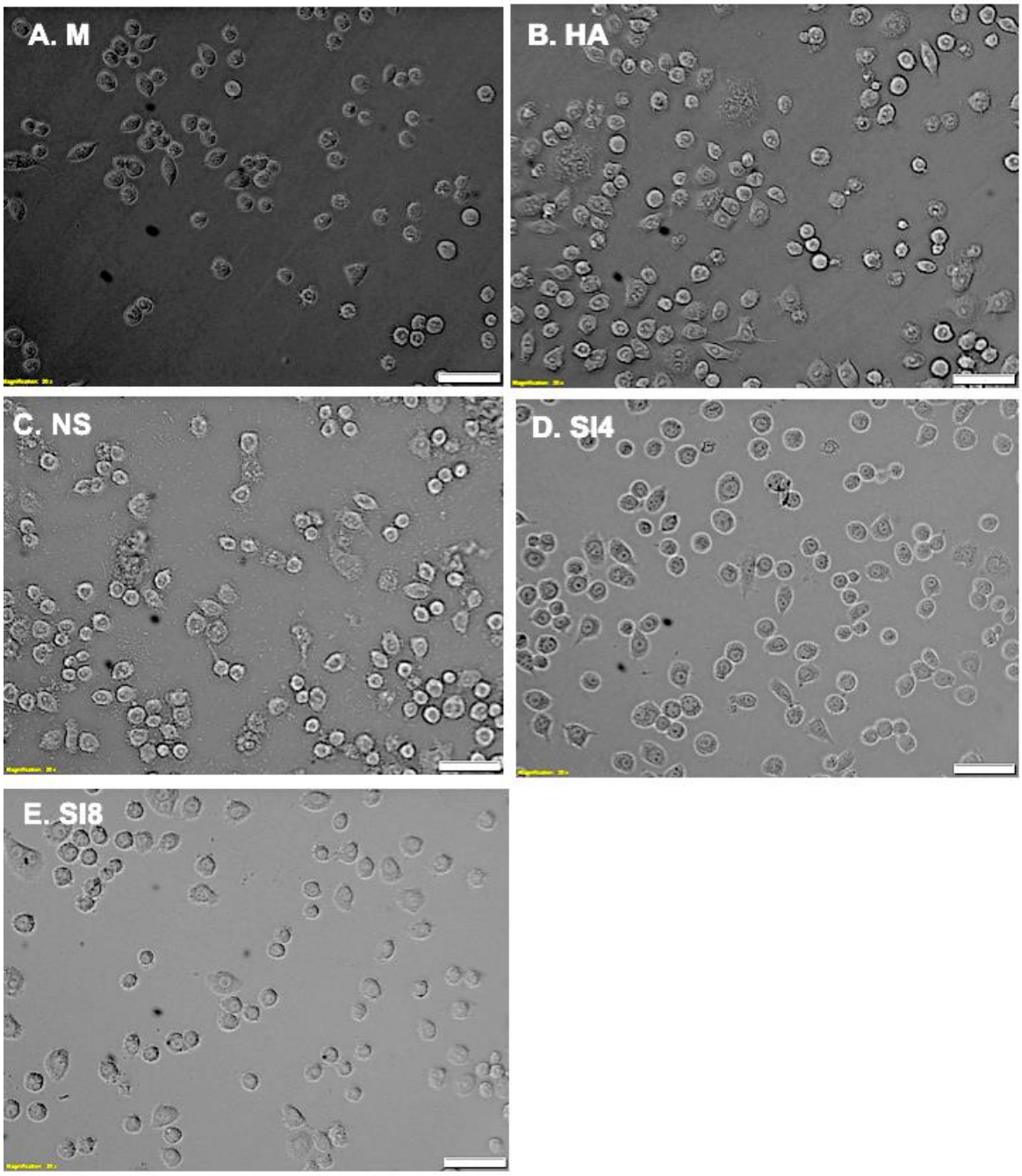


Figure 21 Cytopathic effects of RAW264.7 macrophages at 3 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *S.* Typhimurium (HA), (C) treated with non-solar irradiated *S.* Typhimurium (NS), (D) treated with 4h-solar irradiated *S.* Typhimurium (SI4) (E) treated with 8h-solar irradiated *S.* Typhimurium (SI8). (Scale bar = 50 µm).

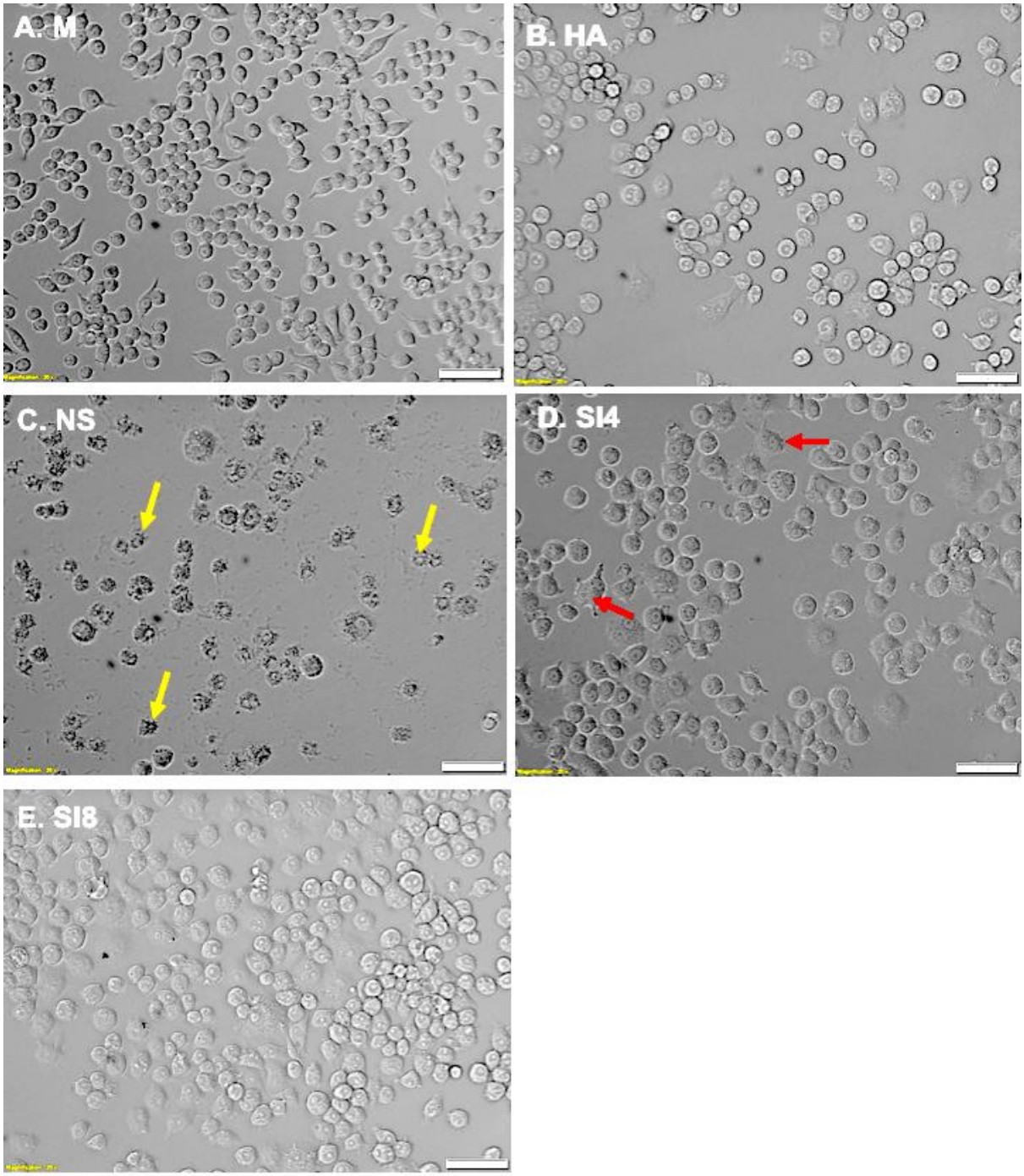


Figure 22 Cytopathic effects of RAW264.7 macrophages at 24 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *S.* Typhimurium (HA), (C) treated with non-solar irradiated *S.* Typhimurium (NS), (D) treated with 4h-solar irradiated *S.* Typhimurium (SI4) (E) treated with 8h-solar irradiated *S.* Typhimurium (SI8). Note: Macrophages infected with solar irradiated *S.* Typhimurium exhibit reduced cytopathic effects. KEY; Red arrows show macrophages with pseudopods; Yellow arrows show shrunken macrophages (Scale bar = 50 µm).

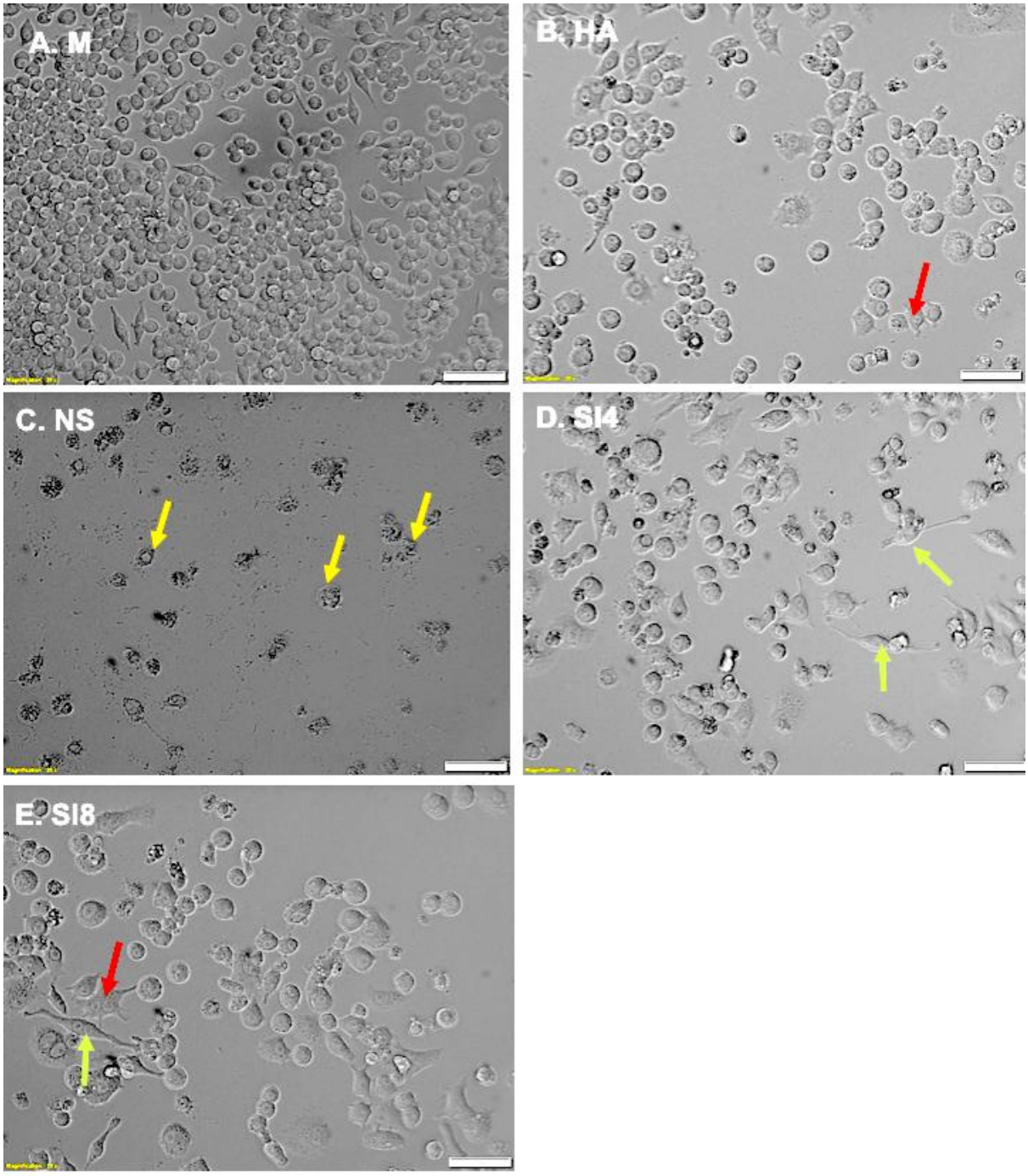


Figure 23 Cytopathic effects of RAW264.7 macrophages at 48 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *S.* Typhimurium (HA), (C) treated with non-solar irradiated *S.* Typhimurium (NS), (D) treated with 4h-solar irradiated *S.* Typhimurium (SI4) (E) treated with 8h-solar irradiated *S.* Typhimurium (SI8). Note: Macrophages infected with irradiated *S.* Typhimurium exhibit reduced cytopathic effects. KEY; Green arrows show elongated macrophages; Red arrows show macrophages with pseudopods; Yellow arrows show shrunken macrophages. (Scale bar = 50 µm).

For cytotoxicity analysis, the macrophages co-cultured with both the heat and chemical attenuated and solar irradiated *S.* Typhimurium did not release any LDH in the first three hours (Fig. 24). However, after 24 h the macrophages treated with heat and chemical attenuated and solar irradiated *S.* Typhimurium for 4 and 8 h produced LDH at the rate of 53% ± 3%, 32% ± 7% and 7% ± 5%, respectively. The highest level of LDH was produced in macrophages stimulated with non-solar irradiated samples of *S.* Typhimurium after 3 h p.i. (55% ± 6%) and 24 h p.i. (75% ± 0.1%) (Fig. 24). The non-solar irradiated sample of S. Typhimurium produced 40% ± 5% and 71% ± 3% of LDH after 3 and 24 hours p.i., respectively (Fig 24). Macrophages stimulated with the heat and chemically inactivated and the solar irradiated *S.* Typhimurium produced significantly lower levels of LDH in comparison to non-solar irradiated controls (Fig. 24).

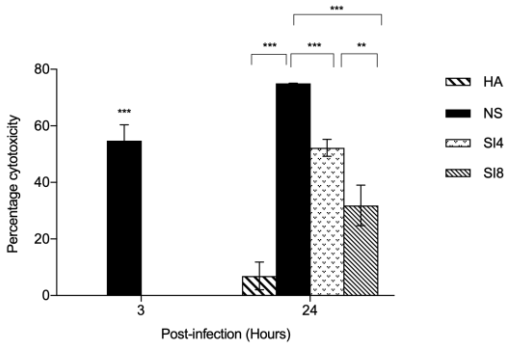


Figure 24 LDH Cytotoxicity Assay. Cytotoxicity analysis of macrophage following 3 and 24 h of infection with *S.* Typhimurium that had been (i) heat and chemical attenuation (HA), (ii) non-solar irradiated *S.* Typhimurium (NS), and (iii) solar irradiated samples after 4 (SI4) and 8 (SI8) hours. The data are means ± standard deviations of a representative experiment that was repeated twice. Significant differences between irradiated and the non-irradiated controls are indicated on the graph as; ***p<0.001; **p<0.01; and *p< 0.05 was considered significant.

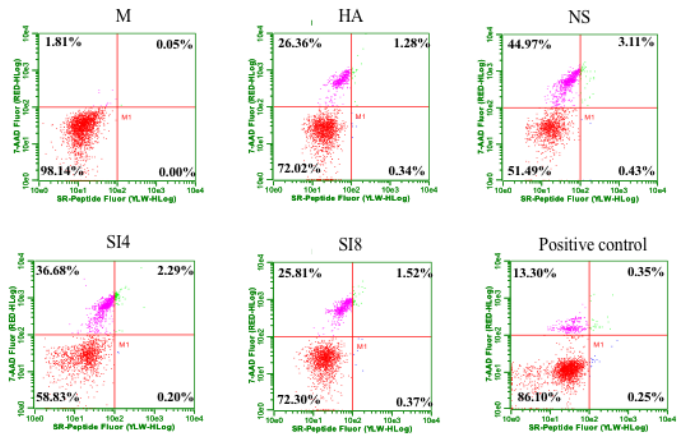
3.3.4 Apoptotic assays for macrophages infected with *S.* Typhimurium

The potential for the i) heat and chemically inactivated, and ii) non-solar irradiated for 0 hours, and solar irradiated for 4 and 8 h and iii) *S.* Typhimurium to induce apoptosis in the macrophages after 3 and 24 h of stimulation was assessed. After 3 h of stimulation, a decrease in the number of live macrophages in all treatment groups was observed in comparison to the untreated macrophages (Fig. 25A, B). After 3 h post-infection, the highest proportion of necrotic cells was found in macrophages treated with the non-irradiated controls and the lowest was noted for macrophages treated with heat/chemical attenuated *S.* Typhimurium.

Salmonella Typhimurium also induced necrosis in macrophages in a time-dependent manner as shown in Figs 25A, B and 26A, B, respectively. No statistical differences were detected between the proportion of necrotic cells present in the heat attenuated samples and the non-

infected control. However, macrophages stimulated with the non-solar irradiated (NS) and those irradiated for 4 and 8 h (SI4 and SI8) exhibited a statistically significant ($p<0.001$) increase in necrotic cells (47%±4%, 37±1% and 22±3%, respectively) in comparison to the untreated group (1%±1%) (Fig. 25). No significant difference in was detected in the perecntage necrotic cells between the macrophages treated with heat attenuated (HA) and the 8-h solar irradiated samples (SI8) (Fig. 25A, B). However, a significant difference ($p = 0.04$) in the number of necrotic cells between the macrophages treated with *S.* Typhimurium that was solar-irradiated for 4 h (37%±1%) and 8 h (22%±3%), respectively.

A.



B.

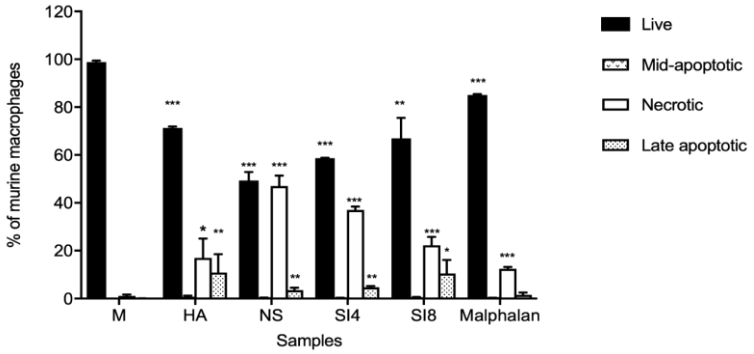
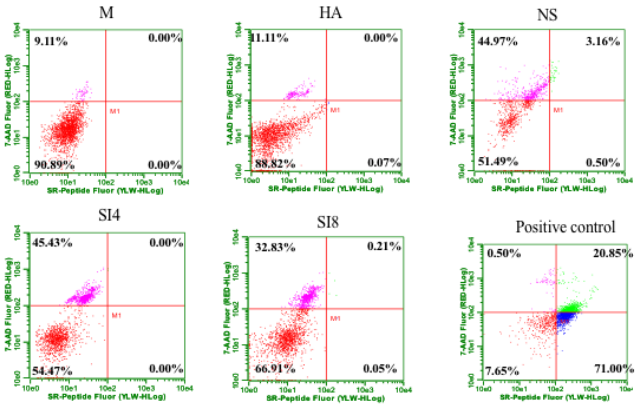


Figure 25 Flow cytometry analysis (A) Representative fluorescence plot RAW264.7 of macrophages following 3 h of stimulation with *S.* Typhimurium that were (i) untreated (M) (ii) treated with heat and chemical attenuated *S.* Typhimurium (HA), (iii) treated with non-solar irradiated *S.* Typhimurium (NS), (iv) treated with 4h- and 8h-solar irradiated *S.* Typhimurium (SI4 and SI8, respectively) and (v) treated with 50 μg/ml of Malphalan (positive control). (B) Bar graph representing the apoptosis analysis of samples. Each bar represents the mean ±SEM of three independent experiments. Significant differences of necrotic cells between infected and the non-infected controls are indicated on the graph; *$p < 0.05$; **$p < 0.01$; ***$p<0.001$.

After 24 h p.i., the heat and chemical attenuated, non-solar irradiated and solar-irradiated *S.* Typhimurium there was a greater increase in necrotic cells compared to those treated for 3 h

p.i. (Fig. 26). The heat/ chemical treated *S.* Typhimurium exhibited the lowest proportion of necrosis (9%). There was a significant (*p<0.001*) increase in the number of necrotic macrophages that were infected with the non-irradiated *S.* Typhimurium (49%±1%).The macrophages treated with *S.* Typhimurium that was solar irradiated for 8 h (SI8) had fewer necrotic cells compared to the macrophages treated with non-irradiated *S.* Typhimurium and the differences in the percentage necrotic cells were highly significant (*p<0.001*). However, there were non-significant differences of necrotic cells between the macrophages infected with *S.* Typhimurium irradiated for 4 and 8 h (SI4 and S8) (43%±2% and 35%±0.3%, respectively). There were also no significant differences of necrotic cells between NS and SI4 samples (49%±1% and 43%±2%, respectively) (Fig. 26)

A.



B.

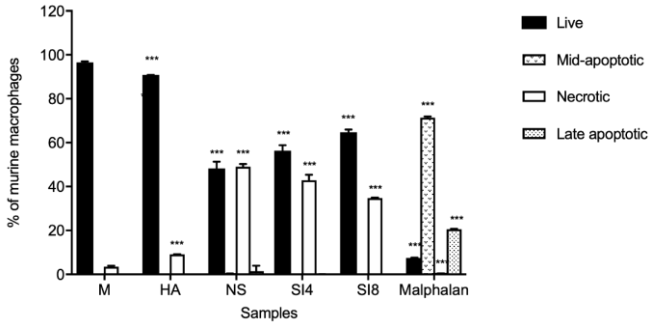


Figure 26 Flow cytometry analysis (A) Representative fluorescence plot RAW264.7 of macrophages following 24 h of stimulation with *S.* Typhimurium that were (i) untreated (M) (ii) treated with heat and chemical attenuated *S.* Typhimurium (HA), (iii) treated with non-solar irradiated *S.* Typhimurium (NS), (iv) treated with 4h- and 8h-solar irradiated *S.* Typhimurium (SI4 and SI8, respectively) and (v) treated with 50 μg/ml of Malphalan (positive control). (B) Bar graph representing the apoptosis analysis of samples. Each bar represents the mean ±SEM of three independent experiments. Significant differences of necrotic cells between infected and the non-infected controls are indicated on the graph; *p < 0.05; **p < 0.01; ***p<0.001; ****p<0.0001.

At 48 h post-infection, there no cells that were detected by flow cytometry for the non-solar irradiated control; thus the results at the 48 h p.i. were not considered.

3.4 Discussion

3.4.1 Viability of *S.* Typhimurium

One of the main findings of this study is that the viability of *S.* Typhimurium was significantly ($p<0.001$) reduced when exposed to chemical and heat treatment (positive control) and solar radiation for either 4 or 8h (Fig. 19). This suggests that SODIS of water contaminated with *S.* Typhimurium represents a potential method to make water potable. These results concur with previous studies that assessed the effect of SODIS on the culturability of microorganisms in natural or experimentally contaminated water (McCambridge & McMeekin, 1981; Deller *et al.*, 2006; Ssemakalu, 2010a). One of the reasons proposed for the complete loss of viability of micro-organisms in SODIS treated water is due to the effects of reactive oxygen species (ROS) generated by the UV-A radiation (Goodsell, 2001; McGuigan *et al.*, 2012), loss of membrane potential (Acharya *et al.*, 1987) as well as protein damage (Chatgilialoglu *et al.*, 2011). It is possible that one or more of these factors could be responsible for the reduced viability of the solar irradiated samples of *S.* Typhimurium in this study.

3.4.2 Intracellular growth assessment of *S.* Typhimurium

The intracellular survival and replication of the non-solar irradiated samples of *S.* Typhimurium observed in this study are shown in Fig. 20. No growth was observed in the HA-treated samples suggesting that the bacteria were completely denatured in the process. There was a slight decrease in CFUs of the bacteria 3 to 24 h whereas the numbers increased from 24 to 48 h. The initial decrease in the CFUs in the macrophages is probably due to the phagocytic and bactericidal activity of the macrophages that are known to ingest and destroy invading pathogens (Martin *et al.*, 2014).

The viable non-solar irradiated samples may be responsible for the observed increase in the number of *S.* Typhimurium CFU/ml after 24 h due to lysis of the infected macrophages (Fig. 20). Various studies have shown that *Salmonella* species are invasive pathogens capable of replicating within host cells as well as enhancing host cell survival to prolong infection (Knodler & Finlay, 2001; Knodler *et al.*, 2005). It is this persistence and growth of the *Salmonella*-related bacterial species inside the host cells that are most notably the main cause of the severity of gastroenteritis (Buchmeier & Heffron, 1989).

3.4.3 Cytopathic and cytotoxic effects of *S.* Typhimurium in RAW264.7 cells

Cytopathic analysis of the macrophages during early infection (3 h p.i.) for all treatments generally showed that the cells exhibited less pronounced cytopathic effects (Fig. 21).

However, further infection for 24 and 48 h (Fig. 22, 23) produced variable results depending on the type of treatment. The untreated macrophages showed no signs of cytopathic effects. However, macrophages infected with heat/chemical attenuated *S.* Typhimurium (HA) (Fig. 4B, 5B) and solar-irradiated *S.* Typhimurium (Fig. 22D-E; 23D-E) were larger, and some appeared more swollen and rounder than the untreated macrophages (Fig. 22A, 23A). The swollen, rounded morphology is characteristic of cells undergoing pyroptosis (DiPeso *et al.*, 2017). Pyroptotic cell death in the absence of cell lysis could occur without releasing large cytosolic components into surroundings (DiPeso *et al.*, 2017). In this particular study, the macrophages treated with heat attenuated and solar irradiated *S.* Typhimurium did not undergo cell lysis, and these findings are congruent with previous studies by DiPeso *et al.* (2017). Pyroptosis entraps intracellular pathogens within the cellular remains, facilitating uptake by immune cells and pathogen clearance (Jorgensen *et al.*, 2017).

In some instances, some of the cells treated with solar irradiated *S.* Typhimurium developed pseudopods and were elongated (Fig. 22D-E; 23D-E). The elongation and enlargement could be a result of macrophage activation (Han *et al.*, 2002; Agnihothram *et al.*, 2015). Macrophages can also be activated through Toll-like receptor signalling pathways resulting in M1 polarized macrophages (Weigert *et al.*, 2006). Toll-like receptor signalling is the most probable way through which the macrophages stimulated with the solar irradiated as well as the heat/chemically treated *S.* Typhimurium could have been activated.

The non-solar treated bacteria (positive controls) showed severe cytopathic effects on macrophages. The macrophages appeared fragmented, detached and were floating in the media at 24 h (Fig. 22C) and 48 h post-infection (Fig. 23C). *Salmonella* Typhimurium has been shown to cause cell death in RAW 264.7 cells (Monack *et al.*, 1996; Weigert *et al.*, 2006), which is accompanied by rapid cytopathic effects as shown by the cellular debris, detachment, and floating of the cell fragments at 24 and 48 h post infection.

The LDH cytotoxicity assay showed greater macrophage cell death in the samples that were treated for 24 h compared to the 3 h treatment p.i. After 3 h p.i., no cytotoxicity was recorded for the HA, and solar irradiated samples whereas the non-solar irradiated *S.* Typhimurium showed a significant increase ($p<0.001$) in cytotoxicity (Fig. 24). The absence of cytotoxicity on macrophages during the 3 h p.i. treatment with HA and solar-irradiated *S.* Typhimurium was due to the inactivation of bacteria. After 24 h p.i slight toxicity was observed for the HA-treated samples while toxicity increased in the solar-irradiated samples (Fig. 24). This study demonstrated that macrophages infected with solar irradiated *S.* Typhimurium showed some cytotoxicity although the cells were not viable. Cytotoxicity of non-replicating *S.* Typhimurium

has been shown to be cytotoxic to macrophages (Monack *et al.*, 1996; Schwan *et al.*, 2000). The SI4 sample induced higher cytotoxicity (52% ± 3%) on macrophages in comparison to the 8 h exposed sample (32% ± 7%). The difference in cytotoxicity between the 4 h and 8 h solar irradiated samples could have been due to the difference in the level of protein damage caused by prolonged solar irradiation (Bosshard *et al.*, 2010).

Since the macrophages treated with heat attenuated (HA) and solar irradiated *S.* Typhimurium (SI4 and SI8) are possibly undergoing pyroptosis without lysis, small cytosolic molecules such as LDH can diffuse through gasdermin D (GSDMD) pores (DiPeso *et al.*, 2017) thus increasing percentage cytotoxicity readings. Additionally, a rise in LDH content could have been due to secondary necrosis of activated macrophages (Matta & Kumar, 2015). Whereas, the high level of cytotoxicity in macrophages infected with non-solar irradiated *S.* Typhimurium during early and late infection (Fig. 24) is probably the result of the higher intracellular survival which resulted in cell lysis. Once viable salmonellae invade macrophage cells, they replicate and produce cytotoxins within these host cells (Ashkenazi *et al.*, 1988). The results of this study concur with previous studies which have demonstrated that *Salmonella* spp. are capable of causing cytotoxic effects in macrophage cells of murine origin (Baloda *et al.*, 1983; Chen *et al.*, 1996; Monack *et al.*, 1996).

3.4.4 Cell death assays for macrophages infected with S. Typhimurium

Upon bacterial infection, macrophages can undergo cell death through a variety of pathways such as apoptosis, necrosis, autophagic cell death, necroptosis, oncosis, pyronecrosis, and pyroptosis (Lai *et al.*, 2015). Therefore, flow cytometric analysis was done to ascertain the type of cell death induced by the various treatments. The heat/chemical attenuated *S.* Typhimurium showed the lowest necrotic effects in the macrophages after 3 h (Fig. 25). This result may be due to the harsh treatment that killed most of the *S.* Typhimurium. There was a further decrease in necrosis in the cells that were treated for 24 h (Fig. 26). *Salmonella* Typhimurium solar irradiated for 4 hours induces higher pyroptotic cell death than heat attenuated and 8 h-solar irradiated *S.* Typhimurium probabaly because there was less protein damage induced by the short irradiation exposure (Bosshard *et al.*, 2010b).

The macrophages stimulated with non-solar irradiated *S.* Typhimurium for 3h and 24 h showed a significant increase ($p<0.01$) in necrotic cells (Fig. 25 and 26). This was also shown by the greater proportion of cellular debris in Fig. 23. The solar irradiated samples of *S.* Typhimurium showed signs of pyroptotic cell death, which has similar characteristics as necrotic cell death such as increased membrane permeabilization which allows the 7-AAD dye to permeate and intercalate in the DNA (Brennan & Cookson, 2000; Edgeworth *et al.*, 2002). Pyroptosis arises from the caspase-1 dependent inflammasome activation and or recognition by the macrophage NLRP3 and NLRC4 receptors. Several studies have proposed that pyroptosis

may benefit the host during infection because it is an inflammatory form of cell death which results in the recruitment of neutrophils *in vivo* (Miao *et al.*, 2011). Caspase-1 activation also influences the development of adaptive immune responses by inducing the production of IL-18 which plays a significant role in stimulating the differentiation of T helper cells (Fantuzzi & Dinarello, 1999). The heat/chemical inactivated and solar-irradiated *S.* Typhimurium also showed signs of pyroptotic cell death but to a lesser extent (Fig. 22 and 23). The increase in necrotic cells in the samples could also be due efferocytosis. When macrophages die, they are engulfed and digested by other macrophages via the process of efferocytosis (Martin *et al.*, 2014). Efferocytosis can, therefore, aid in eliminating inflammatory conditions. However, in the current study, efferocytosis was most likely not taking place as rapidly as it should, and the macrophages released danger signals which probably led to secondary necrosis (Rock & Kono, 2008).

Pyroptosis and necrosis are processes associated with the release of cellular contents from the dying cells, which act as damage-associated molecular patterns (DAMPs) to stimulate pro-inflammatory processes, including the recruitment and activation of neutrophils, macrophages and other immune cells (Nagata & Tanaka, 2017). Thus, although these necrotic systems combat pathogens, they also lead to strong tissue-damaging inflammation. Prolonged exposure of DAMPs to the immune system may lead to autoimmunity (Nagata & Tanaka, 2017). Therefore, solar irradiation for 8 hours dramatically reduces the necrotic cell death of macrophages. These findings show that consumption of SODIS treated water may illicit a mild pro-inflammatory response decreasing the chances of tissue damage in SODIS users.

3.5 Conclusion

This study showed that solar irradiation of *S.* Typhimurium reduces its viability and affects the virulence properties of the organism especially the intracellular survival of the pathogen. Therefore, SODIS of water contaminated with *S.* Typhimurium may be safe for drinking. Solar radiation of *S.* Typhimurium also reduced the cytotoxicity, infectivity and necrosis of RAW 264.7 macrophages compared to the non-solar radiated control. Further research is necessary to determine the exact mechanisms of cellular death as well as the cytokine profiles of the macrophages.

Chapter 4 Investigating the intracellular growth, cytotoxicity and apoptotic effects of solar irradiated *Campylobacter jejuni* in a murine macrophage cell line (RAW 264.7)

Abstract

Campylobacter jejuni is a leading cause of gastroenteritis worldwide. Solar disinfected (SODIS) water can reduce diarrhoeal incidences in communities where potable water is inaccessible. This study assessed the effects of solar irradiation on the viability, metabolic activity and three virulence characteristics (invasion, cytotoxicity, and apoptosis) of *C. jejuni* on RAW 264.7 cells. Samples of *C. jejuni* were suspended in water and treated in the following ways (i) heat and chemically attenuated (1% formalin at 60°C for one hour), (ii)) exposure to solar ultraviolet radiation (SUVR) for 0, 4 and 8 and, (iii) non-exposure to SUVR. The *C. jejuni* samples were used to infect macrophage RAW264.7 cells and its intracellular growth was assessed by using the gentamicin protection assay. Cytotoxicity was assessed by using the Lactate Dehydrogenase Assay (LDH). Apoptosis of the treated macrophages was analysed by Flow Cytometry. The results showed that all the *C. jejuni* were not culturable. However, the non-solar irradiated *Campylobacter jejuni* retained its metabolic activity (40.2%±1.1%) whereas no metabolic activity was observed in the heat and chemically attenuated and solar irradiated bacteria. Intracellular growth of the bacteria in the RAW264.7 cells was not detected in all the treated samples. The non-irradiated *C. jejuni* showed higher cytotoxic and apoptotic effects on macrophages than the heat attenuated and solar irradiated samples. In conclusion, solar irradiation of *C. jejuni* eliminates its metabolic activity and also reduces its ability to induce cytotoxicity and apoptosis in the macrophages.

Keywords: apoptosis, *Campylobacter jejuni*, macrophages, solar disinfection,

4.1 Introduction

Campylobacter species are the most common cause of bacterial gastroenteritis in humans (Nic Fhogartaigh & Dance, 2013). *Campylobacter* spp. are present in contaminated food products such as raw meat, and milk as well as water (Wieczorek & Osek, 2013). As a waterborne pathogen, solar disinfection (SODIS) treatment of water could curb the spread of this pathogen since consumption of solar irradiated water has been associated with reduced diarrhoeal cases (Conroy *et al.*, 1999). Previous studies demonstrated the effectiveness of natural sunlight to inactivate *C. jejuni* (Boyle *et al.*, 2008). This microorganism is highly susceptible to SODIS treatment because of its microaerophilic nature. Hypothetically, microaerophilic organisms are more sensitive to oxidative stresses due to the lack of essential enzymes such as oxidase. Thus, SODIS of contaminated water is one of the recommended methods for reducing gastroenteritis caused by *C. jejuni* infection (Boyle *et al.*, 2008).

Ingested *C. jejuni* colonises the intestinal tract of the colon where it replicates and ruptures the epithelial cells. In turn, this causes acute inflammation accompanied by strong neutrophil recruitment and activation of T- and B-cell (Black *et al.*, 1988; Rathinam *et al.*, 2008). The pathogenicity of *C. jejuni* is due to the presence of virulence traits. The virulence characteristics include, apoptosis-inducing proteins (cytolethal distending toxin, *FspA2*), and bacterial adhesion and invasion promoting factors (*FlaC, PEB1, JlpA, CapA,* and *CadF)* (Young *et al.*, 2007; van Putten *et al.*, 2009; Bouwman & van Putten, 2012). In addition, when *C. jejuni* is engulfed by monocytes and macrophages it activates the nucleotide-binding oligomerization domain 1 (NOD1) (Kiehlbauch *et al.*, 1985; Wassenaar *et al.*, 1997; Hickey *et al.*, 2005; Zilbauer *et al.*, 2007). NOD1 encodes an intracellular multi-domain scaffolding protein which consists of caspase activation and a recruitment domain (CARD) (Ogura *et al.*, 2001). Cellular infection is accompanied by the secretion of numerous pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and IL-1β (Jones *et al.*, 2003; Siegesmund *et al.*, 2004; Sun *et al.*, 2012).

Campylobacter jejuni was also found to induce inflammasome activation in both murine and human cells without apparent cytotoxicity in primary cells (cells obtained from living tissue) without a need for priming (Bouwman *et al.*, 2014). However, some studies have shown that *C. jejuni* can induce cytopathic effects in mammalian cells. A study by Yeen *et al*. (1983) demonstrated that a culture filtrate of *C. jejuni* had cytopathic effects on three human cell lines, namely, *HeLa*, *MRC-5*, and *Hep-2*. These cell lines exhibited cytopathic effects such as cell rounding, loss of adherence and cell death after 24 to 48 h of incubation. It was concluded that the cytopathic effects were due to toxic factors in the culture filtrate of *C. jejuni* (Yeen *et al.*, 1983). In another study, Epoke and Coker (2001) used culture filtrates from five clinical isolates of *C. jejuni* to assess their cytotoxic activity on BHK cells and found that only two isolates induced cytotoxicity (Epoke & Coker, 2001).

Host cell death has conservatively been divided into two distinct morphological and biochemical processes known as apoptosis and necrosis (Majno & Joris, 1995). Apoptosis is an energy-dependent process that plays a primary role in the elimination of cells during development and homeostasis (Ren & Savill, 1998). It plays a role in the pathogenesis of several enteric micro-organisms (Fiorentini *et al.*, 1998; Kim *et al.*, 1998b; Chin *et al.*, 2002). Morphologically, apoptotic cells shrink and at least initially maintain the integrity of their plasma membrane (Rock & Kono, 2008). In contrast, necrotic cell death occurs in response to many kinds of stimuli, such as trauma, infarction, and toxins. As a result, necrosis is typically the outcome of a pathological process. Morphologically, it is associated with cell swelling and the rapid loss of membrane integrity (Rock & Kono, 2008).

Microbial virulence factors such as bacterial replication and host cell cytotoxicity have significant consequences on the innate and adaptive responses of the host. Thus, the three main objectives of this study were: (i) to evaluate the effectiveness of SODIS in inactivating *C. jejuni,* (ii) to assess whether *C. jejuni* is resuscitated in host cells (macrophages) during infection, and (iii) to investigate the cytotoxic and apoptotic effects of solar irradiated *C. jejuni* on macrophages.

4.2 Methodology

4.2.1 Bacterial Culture Preparation

Campylobacter jejuni ATCC® 33560™ were inoculated on Chocolate Blood Agar Plates and incubated at 43°C for 48 h under microaerophilic conditions in an anaerobic jar using an Oxoid™ Campygen gas generation kit (Thermo Fischer Scientific, Waltham, MA). A single colony from the incubated plate was transferred to Mueller Hinton broth and incubated at 42°C for 48 h without shaking. The *C. jejuni* was harvested by centrifugation at 4 000xg for 15 min and washed thrice with autoclaved still mineral water. The pellet was suspended in sterile mineral water up to an optical density (OD) of 0.2 at 546 nm (OD_{546}), approximately 10^7 cells/ml.

4.2.2 Sample preparation for solar irradiation and enumeration of *C. jejuni*

The cultured cells were harvested by centrifugation at 4 000xg for 15 min and washed thrice with autoclaved still mineral water. The pellet was suspended in sterile mineral water up to an OD_{546} of 0.2 (approximately 10^7 cells/ml). Aliquots of 15 ml of the cell suspension were shaken for 15 seconds and exposed to solar irradiation in 25 cm^3 tissue culture flasks under atmospheric conditions. Previous studies have shown that Ultraviolet-A rays (wavelengths, 315 to 400 nm) causes indirect damage to DNA, proteins, and lipids through reactive oxygen intermediates (ROS) (Gelover *et al.*, 2006) and ultraviolet-B rays (wavelengths, 290 to 315

nm) causes direct damage to the DNA by inducing the formation of DNA photoproducts (Douki, 2013). Control flasks with the same mixture were exposed to similar atmospheric conditions except to SUVR by enclosing the samples in an opaque black ventilated box (Ssemakalu, 2010a). The flasks were then placed on aluminium foil and exposed to the sun for 0, 4 and 8 h. The SODIS experiments were performed on the roof of the laboratory at the Vaal University of Technology in South Africa in October 2017 (26°42'39.1"S 27°51'46.2"E -26.710858, 27.862820) from 8.00am-4.00pm. The amount of solar ultra-violet irradiation (UVA+UVB radiation) was measured at 30 min intervals and captured by the Lutron 340A UV Light Meter (Lutron Electronics Company, Coopersburg, PA). The non-solar and solar irradiated samples were enumerated at time-points 0, 4 and 8 h using the Miles and Misra drop method (Miles *et al.*, 1938) under microaerophilic conditions on Brilliance Campycount plates at 42°C for 48 h.

4.2.3 Preparation of inactivated bacterial samples

The heat/chemical attenuated *C. jejuni* was prepared by diluting *C. jejuni* in Mueller Hinton broth to an OD_{546} of 0.2 and then heating the mixture at 60°C for 1 h in 1% formalin.

4.2.4 Metabolic activity of *C. jejuni*

Both the solar and non-solar irradiated samples were not culturable on Brilliance Campycount Plates. Therefore, their metabolic activity was assayed using the Alamar Blue Assay Kit (Promega, Madison, WI) that incorporates a colorimetric growth indicator for the detection of metabolic activity. Specifically, the system includes an oxidation-reduction indicator that changes from blue to red in response to the chemical reduction of the growth medium resulting from bacterial metabolic activity. Briefly, the 1 ml aliquot of the heat-chemically attenuated, non-solar irradiated and solar irradiated samples were centrifuged at 13000 xg at ambient temperature for 5 min. The supernatant was discarded, and the pellet was re-suspended in fresh 1 ml Mueller Hinton Broth. Precisely 100 µl of the bacterial suspension was then aliquoted in 96 well plates followed by the addition of 10 µl of Alamar Blue. A negative control containing media only was also prepared. The 96 well plate was incubated in an anaerobic jar containing Oxoid™ Campygen gas generation kit (Thermo Fischer Scientific) at 42°C for 3 h and then the absorbance was monitored at 570 nm (reduced) and 600 nm (oxidized). The percent reduction (equivalent to the metabolic activity) was determined by subtracting the absorbance at 600-nm from that of 570-nm and multiplying that value by 100. The replicative ability of heat/chemical treated *C. jejuni* was also performed with 1-ml aliquots of *C. jejuni* in 1.5 ml microcentrifuge tubes (Magnani *et al.*, 2009).

4.2.5 RAW 264.7 culture and infection with treated *C. jejuni*

RAW 264.7 macrophages were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% foetal bovine serum (FBS) and 1% pen/strep antibiotics (Gibco, London, UK). The cells were incubated for 48 h at 37°C in 5% CO_2 humidified incubator before co-incubation with the heat-chemically treated, solar irradiated and non-solar irradiated *C. jejuni*.

4.2.6 Intracellular growth assays

Twenty-four well tissue culture plates were seeded with 1 x 10^5 macrophages per ml and incubated for 24 h. Thereafter, the pre-seeded macrophage monolayers were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco) and then co-incubated with *C. jejuni* that was: a) heat-chemically attenuated, b) non-irradiated, and c) solar irradiated for 4, and 8 h at a multiplicity of infection of 1:10 (macrophage: *C. jejuni*). The plates were incubated for 12 h to allow for adhesion and invasion of the bacteria. Then the monolayers were washed with infection media, (media containing 5% FBS, without antibiotics) to remove the unbound bacteria. Tissue culture media containing antibiotics (40 µg of gentamicin/ml) was then added to the cells and incubated further for 3, 24 and 48 h (Siegesmund *et al.*, 2004a). At time-points 3, 24 and 48 h, the media was removed from infected macrophage wells and 1 ml of sterile lysis buffer (0.5% Triton X-100) was added to each well. The plates were incubated at room temperature for 5 min. After mixing, the lysates were transferred to sterile 96 well plates for serial dilutions. Serial dilutions (1:10) of lysates were prepared in the 96 well plate by pipetting 25 µl into 225 µl of LB broth. Then 20 µl was spotted onto Brilliance Campycount plates (Miles *et al.*, 1938).

4.2.7 Cytopathic analysis of infected RAW 264.7 cells

After 3, 24 and 48 h of post-infection, the macrophages were assessed for cytopathic effects using an Olympus inverted microscope (Olympus, Tokyo, Japan). Cell detachment, cell rounding and floating in the medium were recorded according to the methods described in Agnihothram *et al.* (2015).

4.2.8 Cytotoxicity assay

After infection of the macrophages with: a) heat-chemically attenuated (HA), b) non-irradiated (NS), and c) solar irradiated *C. jejuni* for 4 and 8 h (hereinafter referred to as SI4 and SI8, respectively), the cytotoxicity of the treated macrophages was assayed with the LDH Cytotoxicity Assay kit (Pierce, Thermo Fischer Scientific, Waltham, MA) according to the manufacturers' protocol. In summary, 50 µl of the supernatants from the untreated and treated

macrophages were transferred to a 96-well plate. LDH cell lysis buffer was added to each well, followed by addition of 50 µl of the reaction mixture and incubation at room temperature for 30 min. The reaction was stopped by adding 50 µL of Stop Solution to each sample and the absorbance was measured at 490 and 680 nm using an Epoch 2 plate reader (Biotek, Winooski, VT). The cytotoxicity was calculated as shown in equation 1.

$$\%\ LDH\ release = \frac{Experimental\ value - Effector\ cells\ Spontaneous\ control - Target\ Cells\ spontaneous\ control}{Target\ Cell\ Maximum\ Control - Target\ Cells\ Spontaneous\ Control}\ x\ 100$$

[1]

4.2.9 Apoptosis assay

An apoptotic assay was carried out after 3 and 24 h post-infection (p.i.) for macrophages treated with HA, NS, SI4 and SI8. The live, necrotic, mid and late apoptosis RAW264.7 cells was captured by flow cytometry (Guava EasyCyte 8HT (Merck/Millipore), Molsheim, France) using the MultiCaspase Sulforhodamine (SR) kit (Merck/Millipore) according to the manufacturer's instructions. In brief, the macrophages were incubated for 1 h at 37°C in MultiCaspase SR solution containing an SR-Peptide fluorophore. They were then washed twice with 1X apoptosis wash buffer, marked with 7-aminoactinomycin (7-AAD) and incubated at room temperature for 10 min, and analysed by flow cytometry. The positive control for apoptosis in each experiment was represented by treating the macrophages with 50 µg/ml of Melphalan.

4.2.10 Statistical analysis

All the experiments were carried out in triplicate. Means were compared using the student's t-test in the Graph Pad Prism 7.0d Software. A two-tailed p value of <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 UV Radiance, viability and metabolic activity of *C. jejuni*

The solar UV-A irradiance increased from 15.8 ± 0.3 W/m^2 (t = 0 min; 8.00am) to 47.4 ± 0.8 W/m^2 (t = 240 min; 12.00pm) and then decreased to 17.6 ± 0.7 W/m^2 at the end of the experiment (t = 480; 4.00pm) (Fig 27).

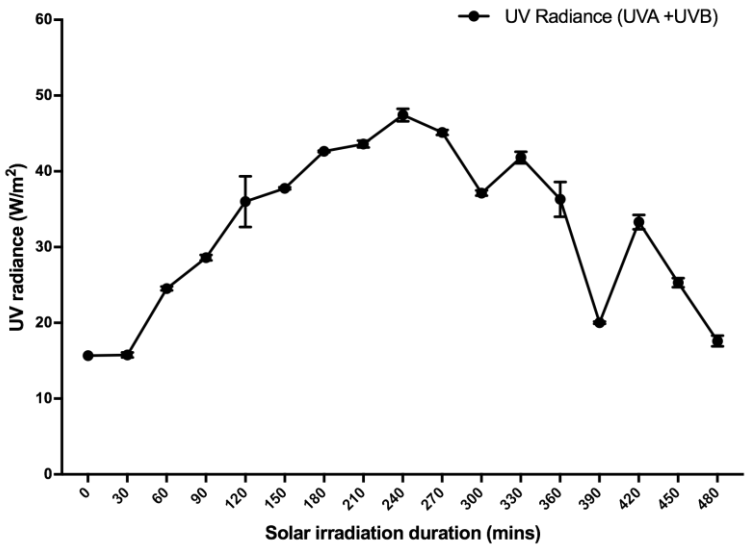


Figure 27 UV-A irradiance during solar irradiation. Standard error bars for three readings at each time point are indicated.

No viable counts were observed in all the samples of *C. jejuni* that were heat/chemical attenuated (HA), non-solar irradiated (NS) and solar irradiated for 4hr (SI4) and 8hr (SI8). Metabolic assays showed that there was activity in the heat/chemical attenuated (HA) and the SI4 and SI8 solar-irradiated treatments (Fig. 28). However, the non-irradiated controls (NS) retained their metabolic activity (40.2%±1.1%) although they did not grow on the agar plates. There was a highly significant difference (p=0.0004) between the means of the metabolic activity of the non- and solar-irradiated *C. jejuni* samples (Fig. 28).

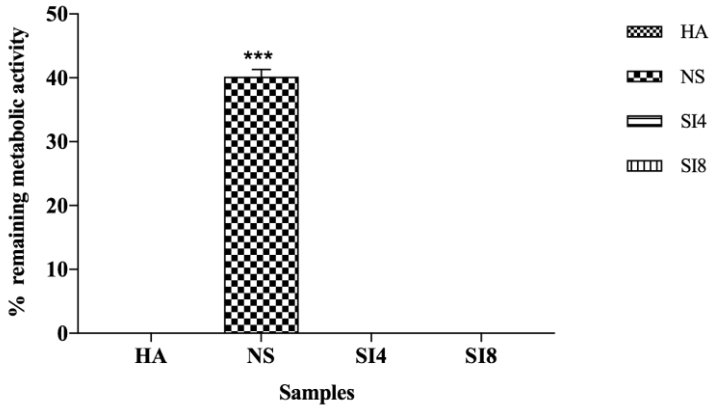


Figure 28 Metabolic activity of *C. jejuni* that were treated according to the following conditions: (A) heat and chemical treatment (HA), (B) Non-solar irradiated (NS), (C) solar irradiated for a duration of 4 h (SI4) and 8 h (SI8). Standard error bars are indicated. Significant differences between the treatments are indicated on the graph where a p-value < 0.05 was considered significant.

4.3.2 Intracellular growth assays

No intracellular growth was observed for macrophages treated with the heat-chemical attenuated, non-irradiated, and solar irradiated *C. jejuni*.

4.3.3 Morphological analysis of macrophages infected with *C. jejuni*

The murine macrophages were later evaluated for evidence of cytopathic effects following 3, 24 and 48 hr of co-incubation with the solar and non-solar irradiated *C. jejuni*.

After 3 h p.i. (early infection) the cell morphology of the macrophages treated with HA, SI4, and SI8 were similar to those of the non-infected macrophages (M). However, at 24 h p.i. and 48 h p.i. the macrophages infected with solar irradiated *C. jejuni* cells appeared round and there was slight detachment of the monolayers. Macrophages infected with solar-irradiated *C. jejuni* proliferated (Figs. 29-31) whereas macrophages treated with non-solar irradiated bacteria appeared much larger and they did not proliferate at 24 h p.i. At 48 h p.i. the cells with non-solar irradiated *C. jejuni* were disintegrating and the cells appeared shrunken in size (Fig. 31).

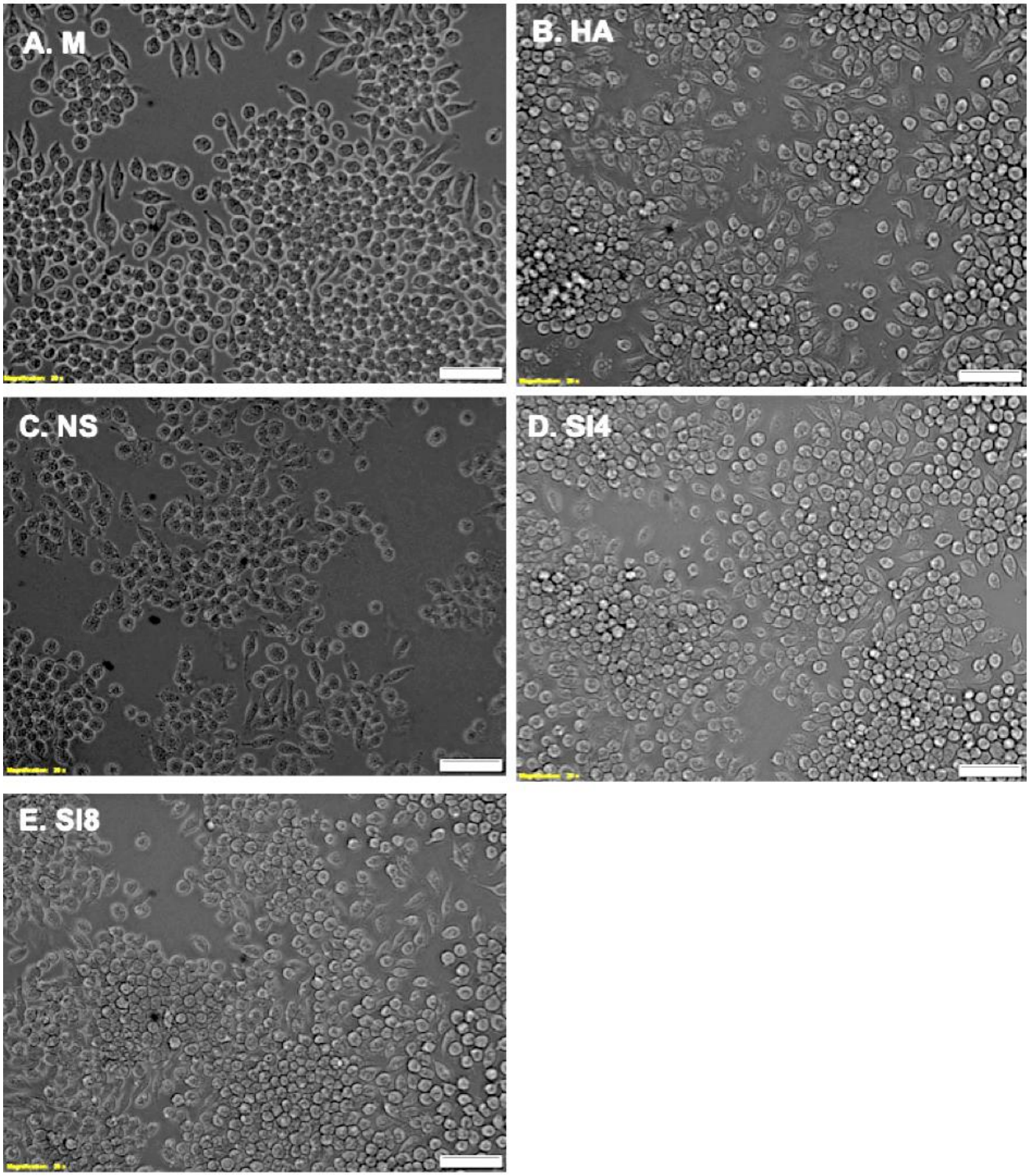


Figure 29 Cytopathic effects of RAW264.7 macrophages at 3 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *C. jejuni* (HA), (C) treated with non-solar irradiated *C. jejuni* (NS), (D) treated with 4h-solar irradiated *C. jejuni* (SI4) (E) treated with 8h-solar irradiated *C. jejuni* (SI8). (Scale bar = 50 µm).

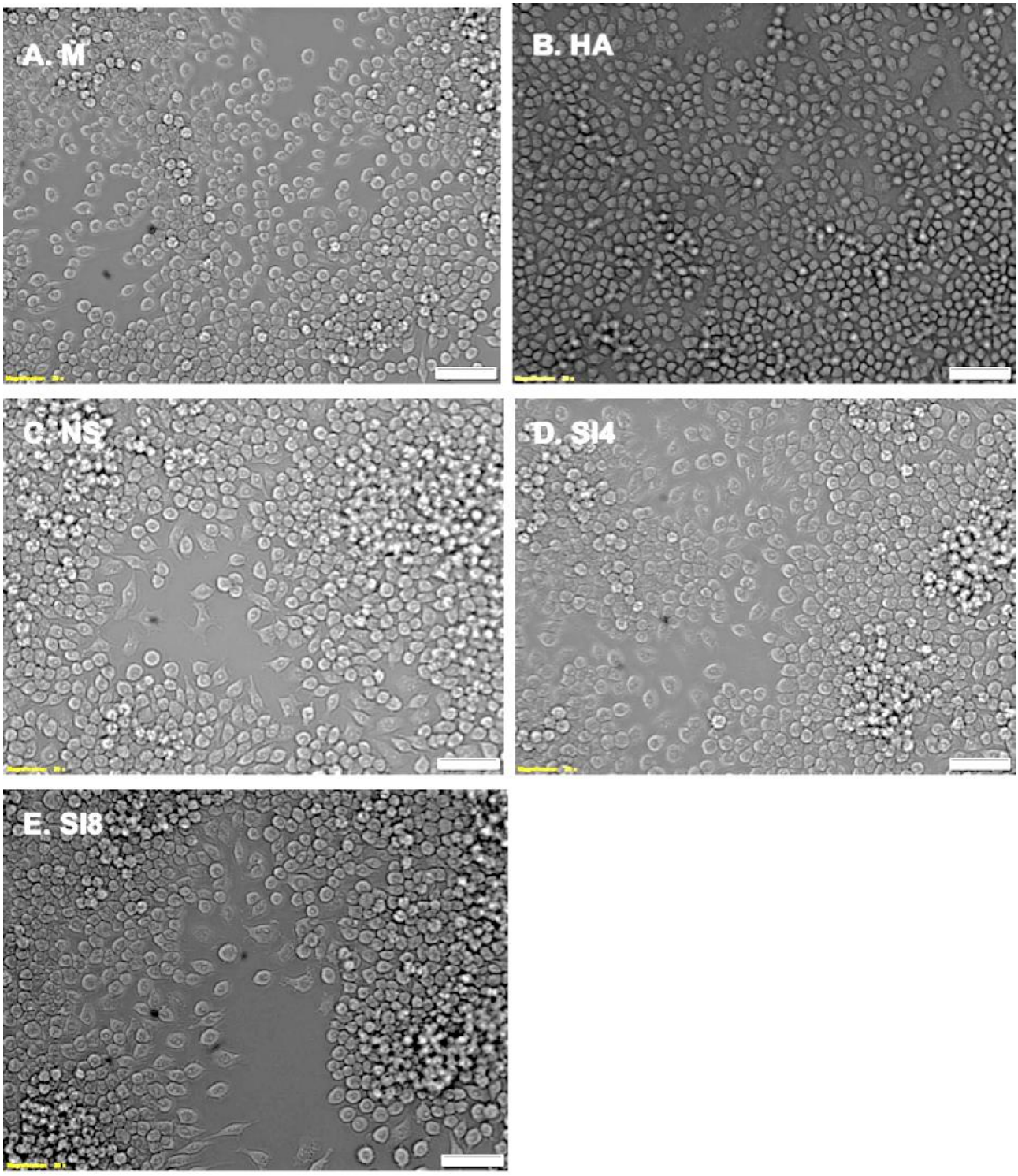


Figure 30 Cytopathic effects of RAW264.7 macrophages at 24 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *C. jejuni* (HA), (C) treated with non-solar irradiated *C. jejuni* (NS), (D) treated with 4h-solar irradiated *C. jejuni* (SI4) (E) treated with 8h-solar irradiated *C. jejuni* (SI8). (Scale bar = 50 µm).

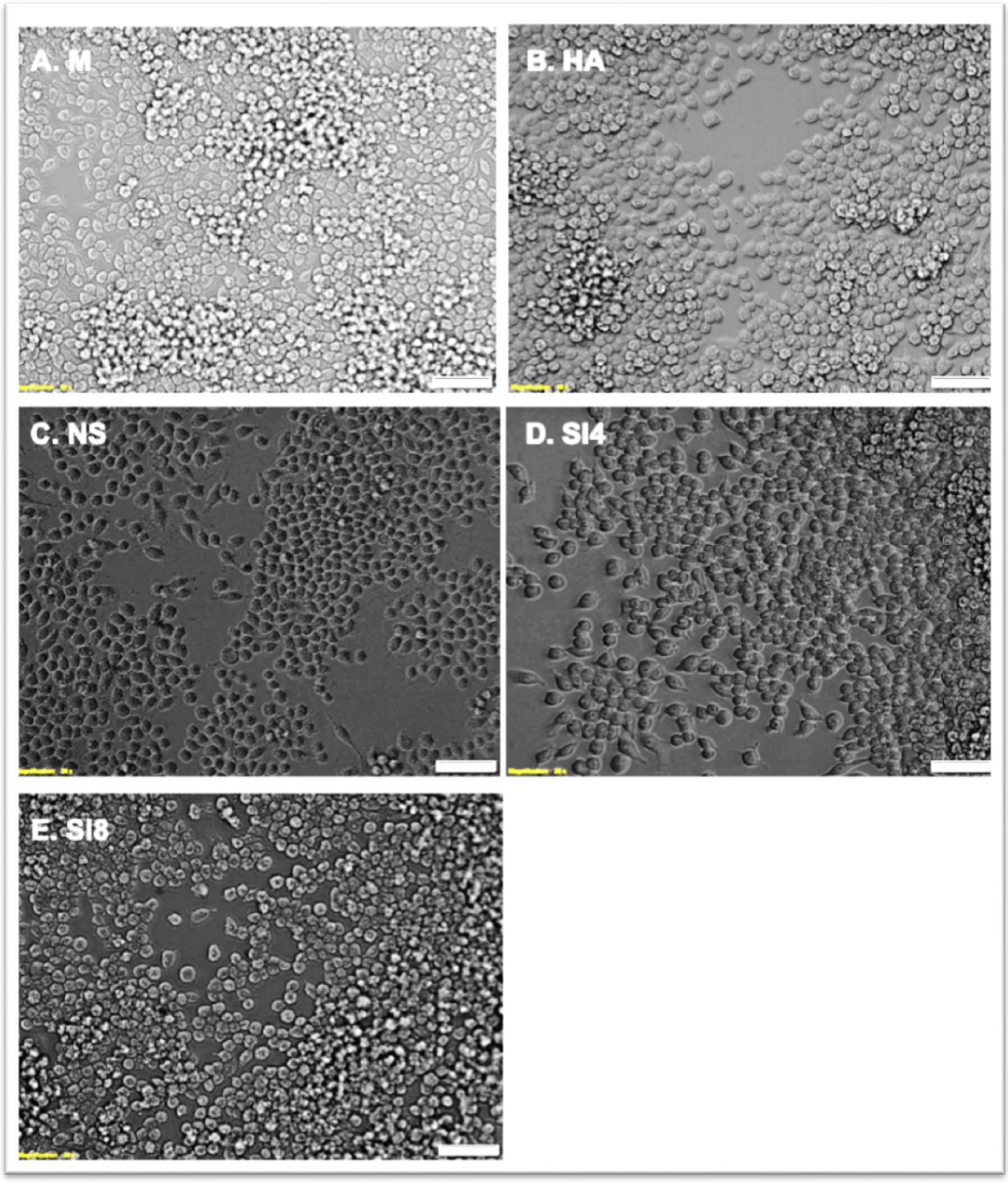


Figure 31 Cytopathic effects of RAW264.7 macrophages at 48 h p.i. that were (A) untreated (B) treated with heat and chemical attenuated *C. jejuni* (HA), (C) treated with non-solar irradiated *C. jejuni* (NS), (D) treated with 4h-solar irradiated *C. jejuni* (SI4) (E) treated with 8h-solar irradiated *C. jejuni* (SI8). (Scale bar = 50 µm).

4.3.4 Cytotoxicity analysis of macrophages infected with *C. jejuni*

At 3 h post-infection (p.i.), cytotoxicity levels of the macrophages treated with heat attenuated, non-solar irradiated and solar irradiated *C. jejuni* were very low (<10%). The highest percentage cytotoxicity was observed in macrophages treated with non-solar irradiated *C. jejuni* (9.75% ± 0.03%) while the least cytotoxicity occurred in samples of *C. jejuni* that were solar irradiated for 8 h (SI8) (4.98% ± 0.85%) (Fig. 32). After 24 h p.i. a significant ($p<0.001$) increase in cytotoxic activity was noted in all the samples (Fig. 32). The highest cytotoxic level (30.28% ± 0.05%) was observed in macrophages treated with non-solar irradiated *C. jejuni* (NS). A significant decrease in cytotoxicity ($p<0.001$) was noted in macrophages treated with heat/chemical attenuated *C. jejuni* (HA) (1.41% ± 0 .00%). The least cytotoxicity (2.57% ± 0.32%) occurred in the 8h-solar irradiated samples whereas the 4h-solar irradiated samples had cytotoxicity levels of 14.18% ± 9.14%.

After 48 h post-infection, an increase in cytotoxicity levels was observed in all the macrophages except for those that were co-cultured with heat-chemically treated *C. jejuni.* The highest cytotoxicity level of the macrophages was observed with the non-solar irradiated controls (NS) with a cytotoxicity level of 39.66% ± 0.87% and the lowest occurred in the heat-chemically treated *C. jejuni* (HA) (0.45% ± 0.037%). Between the solar irradiated samples, the macrophages treated with SI4 exhibited significantly ($p=0.01$) higher cytotoxicity levels compared to those irradiated for 8 hr (SI8). Moreover, the macrophages treated with the solar irradiated as well as the heat-chemical inactivated samples produced significantly lower levels of LDH in comparison to their controls (Fig. 32)

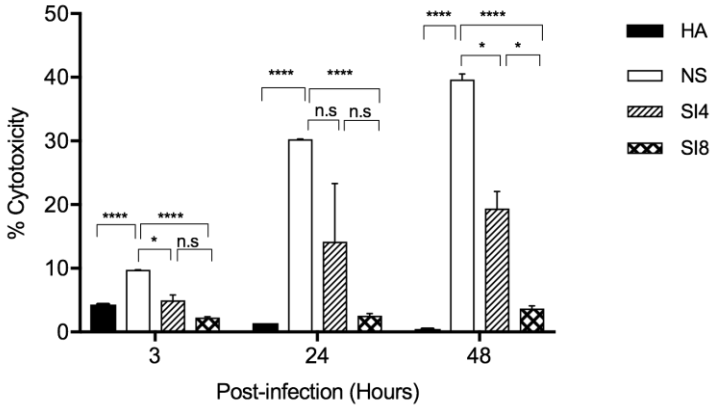


Figure 32 Host cytotoxicity assay (LDH) after infection with a) heat/ chemical attenuated, b) solar irradiated, and c) solar irradiated *C. jejuni*. LDH in the supernatants of infected and uninfected macrophages was sampled and measured at 3, 24, and 48 hr after infection at various MOI 1: 10. The values are expressed as percent host cell cytotoxicity, relative to the uninfected cell control obtained by lysing uninfected macrophages. Error bars indicate standard errors of experiments that were done in triplicate and **** represents $p<0.0001$; ***$p<0.001$; **$p<0.01$; *$p<0.05$ and n.s represents non-significance.

4.3.5 Apoptotic assays

The potential for the i) heat/chemical, ii) non-solar irradiated, and iii) solar irradiated *C. jejuni* to induce apoptosis in the macrophages during early (3 h) and late (24 h) post-infection was determined. During early infection, there was a statistically significant decrease ($p<0.001$) in the number of live macrophages for all treatment groups in comparison to the untreated macrophages (Fig. 33). The highest percentage of live cells was observed in macrophages treated with *C. jejuni* that was solar irradiated for 4 h (84.70% ± 2.05%). The lowest viability was noted in macrophages treated with non-solar irradiated *C. jejuni* (NS) and was 71.80% ± 1.70% (Fig. 33). A significant ($p<0.001$) increase in necrotic cells was noted in macrophages treated with HA, NS, SI4, and SI8 samples of *C. jejuni* with macrophages treated with NS exhibiting the highest portion of necrotic cells (25.80% ± 1.60%). Macrophages treated SI8 showed the lowest percentage of necrotic cells (12.29% ± 1.73%). There was no significant difference between the live, necrotic and late–apoptotic proportion of macrophages treated with heat attenuated (HA) and the 8- hr irradiated samples (SI8) (Fig. 33). However, a significant increase in mid-apoptotic cells ($p=0.031$) was observed in macrophages treated with SI8.

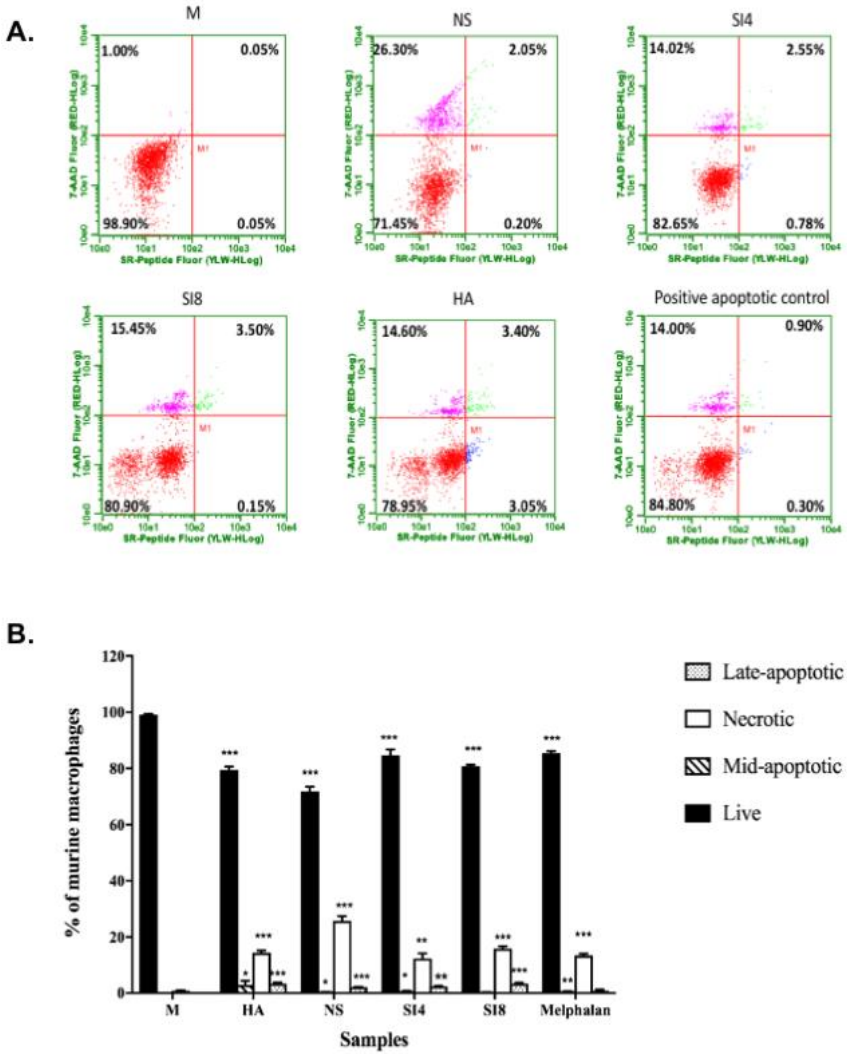


Figure 33 (A) Representative fluorescence plot of RAW264.7 cells at 3 h p.i. *C. jejuni* that were (i) untreated macrophages (ii) heat and chemical attenuation (HA), (iii) non-solar irradiated at 0 h (NS), (iv) solar irradiated samples after 4 (SI4) and 8 (SI8) h and (v) positive control macrophages treated with 50 μg/ml of Melphalan. (B) Apoptosis analysis of samples. Each bar represents the mean ±SEM of three independent experiments. Significant differences of necrotic cells between infected and the non-infected controls are indicated on the graph. *p < 0.05; **p < 0.01; ***p<0.001; ****p<0.0001

During late infection (24 h p.i.) the heat attenuated, non-solar irradiated and solar irradiated *C. jejuni* showed a significant increase (p<0.001) in the percentage of apoptotic cell death and a drastic decrease in necrotic cell death. Macrophages treated with heat/chemical treated C. *jejuni* had the highest viability (39.42% ± 3.00%). The macrophages treated with non-solar irradiated (NS) *C. jejuni* had the highest proportion of necrotic cells (2.27% ± 0.17%) and lowest viability (19.98% ± 1.56%). The macrophages treated with the 8-h solar irradiated *C. jejuni* (SI8) had higher rates of viability when compared to the 4h-irradiated sample (SI4) (Fig. 34).

The highest portion of mid-apoptotic cells was noted in macrophages treated with SI4 (52.82% ± 0.55%) and the lowest percentage was noted in macrophages treated with HA (37% ± 1.64%). Significant increases ($p<0.001$) in late-apoptotic cells were also observed in all the samples; with macrophages stimulated with NS showing the highest fraction of late-apoptotic cells (24.5% ± 0.74%) whereas the lowest portion of late apoptotic cells was noted in SI8 (21.87% ± 0.50%). A significant difference was also noted between HA and SI8 samples with live ($p=0.002$) and mid-apoptotic cells ($p<0.001$) (Fig. 34).

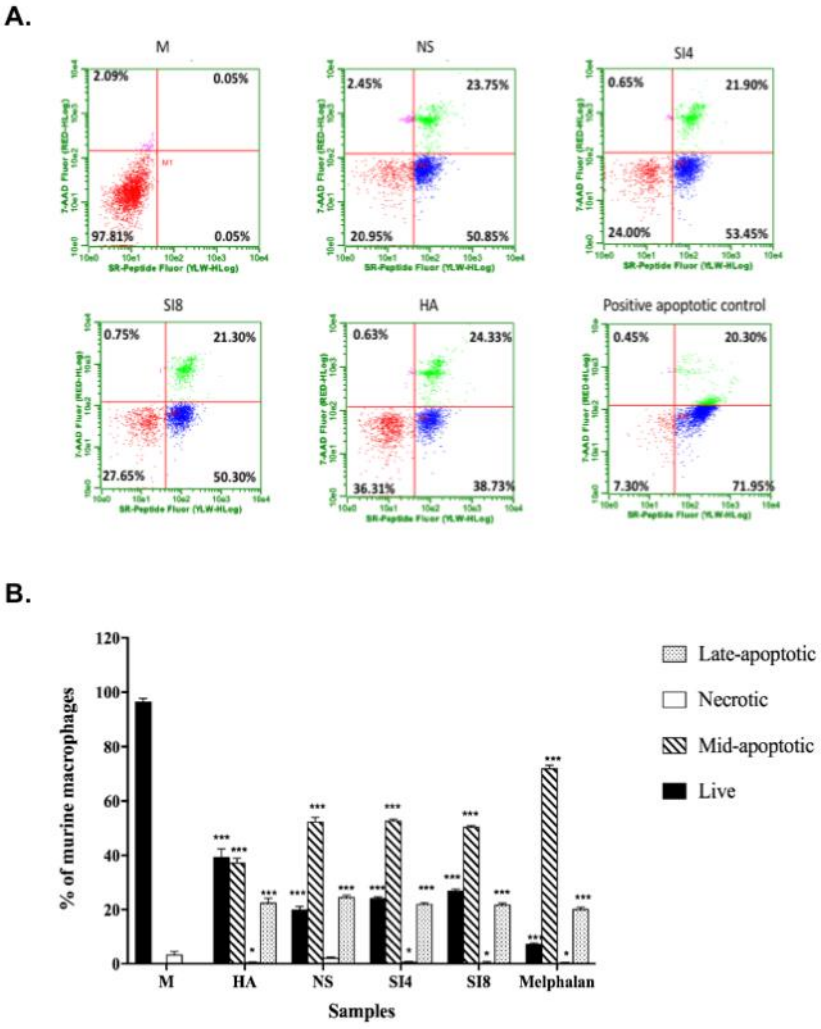


Figure 34 Flow cytometry analysis (A) Representative fluorescence plot RAW264.7 of macrophages following 24 h of stimulation with *C. jejuni* that had been (i) media alone (M) (ii) heat and chemical attenuation (HA), (iii) non-solar irradiated at 0 h (NS), (iv) solar irradiated samples after 4 (SI4) and 8 (SI8) h and (v) positive control macrophages treated with 50 μg/ml of Melphalan. (B) Apoptosis analysis of samples. Each bar represents the mean ±SEM of three independent experiments. Significant differences of necrotic cells between infected and the non-infected controls are indicated on the graph; *$p < 0.05$; **$p < 0.01$; ***$p<0.001$; ****$p<0.0001$

In the 48 h p.i. samples no cell counts were observed for the apoptotic positive control (Malphalan) and some of the samples treated with non-solar irradiated *C, jejuni*, therefore those results were not included for comparative reasons.

4.4 Discussion

4.4.1 UV radiance, viability and metabolic activity of *C. jejuni*

The high solar irradiation with a UV-radiance of 47.4 ± 0.8 W/m^2 (t = 240 min) (Fig. 27) led to enhanced cellular damage and consequent inactivation of the bacteria. The SODIS efficiency was probably enhanced by the aluminium foil through the return of UVA and short-wavelength visible radiation through the containers (Mani *et al.*, 2006).

It was interesting to find that no detectable viable counts of *C. jejuni* occurred in both the solar and non-solar-irradiated samples. The expectation was that *C. jejuni* in the non-solar-irradiated samples remain viable since they were not exposed to UV radiation. One possible explanation for this phenomenon is that the bacteria had entered into a viable-but-non-culturable (VBNC) state. The VBNC state is a unique survival strategy adopted by many species of bacteria in response to adverse environmental conditions (Pinto *et al.*, 2015). Other factors including temperature (Medema *et al.*, 1992), reactive oxygen species (ROS) and growth conditions could have also influenced the VBNC state of *C. jejuni* (Moore *et al.*, 2005). The SODIS process involves exposure of samples to atmospheric temperatures that are below the optimum growth temperature of *C. jejuni* which is 42°C. This could have also affected their viability. The SODIS procedure involves shaking the samples to increase the amount of dissolved oxygen in the water and this could have increased the amount of reactive oxygen species (ROS) in the system. This reaction may have affected the survival of *C. jejuni* since the organism is microaerophilic and grows best in 5% O_2 and 10% CO_2 (Moore *et al.*, 2005). It is also known that when *C. jejuni* is exposed to high levels of oxygen it expresses iron superoxide dismutase (*FeSOD*) that eliminates superoxide anions and seemingly lacks general and oxidative stress-specific vital regulators and defence proteins such as *SoxRS, OxyR,* and *RpoA* (Garenaux *et al.*, 2007). It is possible that the above factors played a role in the viability of *C. jejuni.* Standard culture methods cannot detect the viability of VBNC cells efficiently, although the cells remain potentially pathogenic under favourable conditions (Ravel *et al.*, 1995; Rahman *et al.*, 2001). Therefore, the viability of the cells was determined by assessing the metabolic activity of heat/chemical, non-solar irradiated and solar irradiated *C. jejuni* using Alamar Blue. The non-solar irradiated *C. jejuni* retained its metabolic activity whereas heat/chemical attenuated and solar irradiated *C. jejuni* showed no metabolic activity (Fig. 28).

This study showed that while non-solar irradiated *C. jejuni* bacteria are metabolically active they were non-culturable. It appears that they acquired a metabolically active-but-non-culturable (ABNC) state (Ramamurthy *et al.*, 2014). The ABNC state seems to support the long-term survival of bacteria under unfavourable conditions. This state can be thought of as an inactive form of life waiting for revival under suitable conditions. The ABNC state of bacteria appears to occur when bacteria are present in unfavourable environments conditions not conducive for growth. Although the conditions that trigger VBNC have not been well investigated, it has been established that bacteria in this state can resuscitate under "appropriate" conditions (Ramamurthy *et al.*, 2014).

The loss of metabolic activity in the solar irradiated *C. jejuni* is perhaps due to the denaturation of the proteins and nucleic acids. A previous study showed that prolonged solar irradiation might denature bacterial proteins by carbonylation and aggregation of proteins and this could negatively affect the structural and enzymatic proteins within the cells (Chatgilialoglu *et al.*, 2011). Vital cellular functions such as transcription and translation, respiration, ATP synthesis, catalase, molecular chaperone functions, amino acid synthesis and degradation are all affected by UVA irradiation. With the loss of catalase activity, the cells lose their defence against ROS making them more susceptible to oxidative stress. Additionally, the damage to translational proteins decreases the cells' ability to self-repair (Bosshard *et al.*, 2010).

The loss of metabolic activity in the solar irradiated *C. jejuni* could be beneficial to the SODIS user since it has been hypothesized that loss of metabolic activity of bacteria has been associated with reduced virulence and lack of toxin production (Rahman *et al.*, 2001; Maalej *et al.*, 2004).

4.4.2 Intracellular growth assays of *C. jejuni* in the macrophages

As expected heat/chemically attenuated and solar irradiated *C. jejuni* did not multiply in the macrophages suggesting that the bacteria were denatured in both treatments. However, the non-solar irradiated *C. jejuni* also did not exhibit any intracellular growth in the macrophages even after 3, 24 and 48 hr of post-infection. This may due to their VBNC state. However, a previous study showed that metabolically active *C. jejuni* in its VBNC state remained potentially virulent and was shown to resuscitate in host cells (Chaisowwong *et al.*, 2012). Considering this there is the likelihood that VBNC non-solar irradiated *C. jejuni* may be potentially virulent and pose a threat to the health of individuals.

Several studies have shown that *C. jejuni* (which are not VBNC) can survive within phagocytic cells (Siegesmund *et al.*, 2004; Šikić Pogačar *et al.*, 2009). The survival of intracellular bacteria within phagocytic cells presents a way to evade the host immune defences and allows the proliferation and dissemination of bacteria throughout the host (Kaufmann, 1993). However,

once the bacteria are inside the macrophages they must be able to survive the unfavourable conditions present in the host's cell, such as oxidative products (e.g., ROS), minimal nutrients, and unfavourable pH conditions (Mitchell *et al.*, 2016).

Campylobacter jejuni samples in this study appear to have been adversely affected by the increased oxygen levels brought about by shaking the flasks and lower temperatures during sample preparation (below optimal growth temperature of 42°C for *C. jejuni*). These factors are possible reasons why C*. jejuni* still retained their VBNC state even during co-infection with macrophages.

4.4.3 Cytopathic effects

The cytopathic effects of heat/chemical treated bacteria, non-solar and solar irradiated *C. jejuni* were not apparent during the 3 hr and 24 hr p.i. period (Fig. 29 and 30). However, after 48-hr p.i. non-solar irradiated *C. jejuni* produced cytopathic effects on RAW 264.7 cells (Fig. 30). These effects included cell rounding and shrinkage, loss of adherence and cell death (Fig. 31). The cytopathic effects may be due to cytolethal distending toxin (CDT) production (Méndez-Olvera *et al.*, 2016) since the non-solar irradiated *C. jejuni* retained its metabolic activity. The absence of morphological changes in the macrophages that were co-cultured with heat/chemical treated (HA) and solar irradiated (SI4 and SI8) *C. jejuni* is probably due their metabolic inactivity.

4.4.4 Cytotoxicity analysis of macrophages infected with *C. jejuni*

Figure 6 shows that there was a general progressive increase in cytotoxicity of macrophages infected with *C. jejuni* that were solar-and non- solar irradiated for 3, 24 and 48 hr post-infection (Fig. 32). The greater cytotoxic effects exhibited by the non-solar irradiated *C. jejuni* may be the result of the presence of the cytolethal distending toxin (CDT) since the *C. jejuni* were metabolically active as described above. Several investigations have established that bacterial toxins function as virulence factors (Hickey *et al.*, 2000; Hickey *et al.*, 2005). These toxins have specific effects on different processes in eukaryotic cells; for instance, some toxins interfere with intracellular signalling by interacting with particular proteins in various signalling cascades and others such as the cytolethal distending toxin (CDT), interfere with the cell cycle (Oswald *et al.*, 2005).

Solar irradiation of *C. jejuni* resulted in the loss of metabolic activity. Therefore, it is highly likely that CDT was no longer being produced and thus reducing the ability of *C. jejuni* to induce cytotoxicity. However, the 4 h-solar irradiated *C. jejuni* induced higher levels of cytotoxicity than the 8 h-solar irradiated sample (Fig. 31); this may perhaps be due to the higher level of protein damage caused by prolonged solar irradiation (Bosshard *et al.*, 2010b).

This finding suggests that solar irradiation does have the potential of denaturing cytotoxic proteins in *C. jejuni*.

The heat/chemical attenuated *C. jejuni* showed the lowest cytotoxic effects on the macrophages probably because this extreme treatment resulted in complete inactivation of bacteria, loss of metabolic activity and extensive protein damage.

The macrophages treated with heat/chemical attenuated (HA), solar irradiated (SI4 and SI8) and non-solar irradiated (NS) *C. jejuni* produced low levels of LDH (Fig. 32). This is an indication that that the macrophages were undergoing some form of necrotic cell death as described by Chan *et al.* (2013).

4.4.5 Apoptotic assays for macrophages infected with *C. jejuni*

Macrophages can undergo several types of cell death upon bacterial infection, namely, apoptosis, necrosis, autophagic cell death, necroptosis, pyronecrosis, and pyroptosis. This study showed that macrophages stimulated with heat/chemical attenuated (HA), non-solar irradiated (NS) and solar irradiated (SI4 and SI8) *C. jejuni* showed a significant increase ($p<0.001$) in necrotic cells during early infection (3 h p.i.) (Fig. 33A, B). Macrophages treated with non-solar irradiated *C. jejuni* had the highest proportion of necrotic cells (Fig. 33A, B) whereas treatments involving solar irradiated samples of *C. jejuni* showed lower levels of necrotic cell death. The reduced necrotic values could have been associated with the loss of metabolic activity of both the heat/chemical inactivated and solar irradiated *C. jejuni*. Necrosis is characterised by increased membrane permeability which allows the 7-AAD dye to permeate and intercalate to DNA (Brennan & Cookson, 2000; Edgeworth *et al.*, 2002).

Infection of macrophages for 24 h showed a decrease in the proportion of necrotic cells (Fig 34A, B). This may be due to efferocytosis. When macrophages die they are engulfed and digested by other macrophages via the process of efferocytosis (Martin *et al.*, 2014). Once a macrophage has engulfed a damaged macrophage through efferocytosis it undergoes programmed cell death (apoptosis) thus eliminating inflammatory conditions in the body (Rock & Kono, 2008). The highest and lowest portion of apoptotic cells was observed in non-solar irradiated and heat-chemical attenuated *C. jejuni*, respectively. Apoptosis is essential in the immune system and plays significant roles in the control of the immune response, the deletion of immune cell recognising self-antigens, and cytotoxic killing (Ekert & Vaux, 1997). Since apoptotic cells initially maintain their membrane integrity they do not release their intracellular contents rapidly, thus pro-inflammatory signals are not released (immunosuppressive).

4.5 Conclusion

This study has demonstrated that non-solar irradiated *C. jejuni* reaches a VBNC state. Bacteria that enter the VBNC state pose a significant threat to public health mainly due to the difficulty in detecting the bacteria and their potential to resuscitate in the host's body. However, solar irradiated *C. jejuni* becomes non-viable and metabolic inactive and has reduced virulence properties especially the cytotoxic and apoptotic-inducing ability. Thus, SODIS-treatment of water containing *C. jejuni* may be safe to drink because the organism may become avirulent. Further research is necessary to determine the cytokine profiles of the macrophages since they play a role in the regulation of cell death.

Chapter 5 Effects of solar irradiated S. Typhimurium on cytokine production by RAW264.7 macrophage cells

Abstract

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the leading bacterial causes of gastroenteritis worldwide. It is a waterborne pathogen which is quickly inactivated by solar disinfection (SODIS). However, the immunological effects of consuming SODIS water has not been thoroughly investigated. This study aimed at ascertaining the effect of *S.* Typhimurium spiked SODIS water to induce the production of nitric oxide (NO), cytokines, chemokines and growth factors in RAW264.7 cells *in vitro*. Heat and chemically attenuated samples were prepared by using a combination of heat and chemical conditions (0.5% phenol at 60°C for one hour). *Salmonella* Typhimurium was suspended in mineral water and exposed to solar ultraviolet radiation (SUVR) for 0, 4 and 8 hours and the non-solar irradiated control was covered with a box. The macrophage RAW264.7 cell line was treated with LPS, heat/chemical attenuated, non-solar and solar irradiated *S.* Typhimurium. After 3 and 24 hours post-infection the supernatants from each treatment were used to measure the nitric oxide levels using the Greiss reagent. The levels of chemokines, cytokines and growth factors were quantified using the Luminex 200. The results showed that both solar and non-solar irradiated *S.* Typhimurium inhibited the production of NO_2^- in the RAW264.7 cells. The heat/chemically attenuated *S.* Typhimurium induced a significant increase ($p<0.0.5$) in the production of NO_2^- in the macrophages when compared to the unstimulated RAW264.7. The chemokine and cytokine levels produced by the macrophages were similar in the solar inactivated and the live untreated *S.* Typhimurium.

Keywords: chemokines, cytokines, growth factors, macrophages, SODIS, *S.* Typhimurium

5.1 Introduction

The drinking of water contaminated with *S.* Typhimurium poses a health risk, especially to young children and immune-compromised individuals, since it is the causative agent of salmonellosis that is characterised by severe gastroenteritis. Invasive non-typhoidal *Salmonella* (NTS) is now recognised as a causative agent for meningitis and septicaemia particularly in children below the age of 5 years in sub-Saharan Africa (Feasey *et al.*, 2012). Immune compromised individuals and young children who suffer from severe anaemia have a higher risk of contracting diseases caused by invasive non-typhoidal *Salmonella* (Brent *et al.*, 2006; Gordon & Graham, 2008). The introduction of home water treatment methods such as solar disinfection (SODIS) has reduced the spread of diarrhoeal diseases (Conroy *et al.*, 1999; du Preez, 2010). For instance, a reduction in the incidence of diarrhoeal diseases due to SODIS has been reported in South Africa, Kenya and Zimbabwe (du Preez, 2010). However, in spite of the known health benefits associated with SODIS consumption, very little is known about the effect of solar-irradiated pathogens on the immune system.

Solar disinfection of water inactivates microorganisms by exposure to ultraviolet-A and B rays. Ultraviolet-A rays (wavelengths 315 to 400 nm) causes indirect damage to bacterial DNA, proteins, and lipids through reactive oxygen intermediates (ROS) (Gelover *et al.*, 2006). UV-B causes **direct** damage to the DNA by inducing the formation of DNA photoproducts such as cyclobutane pyrimidine dimers (CPDs) (Douki, 2013). The accumulation of DNA photoproducts in the cells stops DNA replication and RNA transcription resulting in cellular death (Britt, 1996; Rincón & Pulgarin, 2004). Bacterial cell death leads to the release of antigenic microbial intracellular and cellular membranes components such as glycopeptides, lipopolysaccharides, and deoxyribonucleic acids (Bessler *et al.*, 1997). SODIS-treated *V. cholerae* induced the maturation of the JAWS II dendritic cells *in vitro* (Ssemakalu *et al.*, 2015a). Ssemakalu *et al.* (2015b) also suggested that solar-irradiated *Vibrio* may be capable of eliciting a T-cell type 1 immune response. This suggests that solar irradiated pathogens may have the potential to elicit either an innate or adaptive immune response in consumers of SODIS water. The innate immune system is the host's first line of defence against invading pathogens. The adaptive immune system provides additional protection enhancing immunological memory which enables a faster response upon repeated exposure to the same pathogen or antigen (Hurley *et al.*, 2014). In addition to phagocytic cells, there are humoral elements such as the complement system that makes up the innate immune system. The interactions between the innate and adaptive immune systems, including different types of cells, cytokines, and antibodies form the most important components of the host immunity (Hurley *et al.*, 2014).

Cytokines are small secreted proteins released by cells that have a specific effect on the interactions and communications between cells (Zhang & An, 2007). Whereas, chemokines

are chemotactic cytokines which regulate cell trafficking (Wu *et al.*, 1977). Cytokines play a significant role on the host cells, especially macrophages during infection. Macrophages are antigen presenting cells that play an essential role in the innate defence of the host by recognising, engulfing, and killing microorganisms (Gordon, 2003). There are two significant phenotypes that have been characterised, namely, the M1 and M2 or "classically" and "alternatively" activated macrophages, respectively (Stein *et al.*, 1992). Macrophages of the M1 phenotype (classically activated) express numerous pro-inflammatory mediators including tumour necrosis factor (TNF-α), interleukins IL-1 and IL-6, reactive nitrogen and oxygen intermediates that possess microbicidal and tumouricidal activity (Gordon & Martinez, 2010). The nitrogen intermediates such as nitric oxide are a significant weapon of the immune system, and they possess strong antimicrobial properties. However, excess production of nitric oxide can also harm host cells (MacMicking *et al.*, 1997).

The M2 phenotype (alternatively activated) expresses molecules such as resistin-like-α (alternatively known as Fizz1), Arginase1 (Arg1), chitinase 3-like protein 1 (also known as Ym1), IL-10 and Mrc1 (also known as CD206), which are involved in parasite infestation, tissue remodelling, immune regulation, tumour promotion and efficient phagocytic activity (Gordon & Martinez, 2010). Alternative activation is also marked by the secretion of IL-4, IL-10, and IL-13. These cytokines lead to the formation of polyamines and prolines which induce proliferation and collagen production (Van Dyken & Locksley, 2013).

Cytokines regulate both the innate and adaptive host immune responses. The equilibrium between pro- and anti-inflammatory cytokines controls the level of infection preventing damage to the host from prolonged inflammation. *In vitro* cell cultures of bone marrow-derived macrophages and primary cell lines have shown that *Salmonella* promotes chemokine and cytokine expression in both dendritic and epithelial cells as well as macrophages (Jung *et al.*, 1995; Svensson *et al.*, 2001; Pietila *et al.*, 2005).

Salmonellosis elicits the production of pro-inflammatory cytokines, which include the following: interleukins (IL-1β and IL-6), interferons (IFN-γ), and tumour necrosis factor (TNF-α). These pro-inflammatory cytokines promote systemic inflammation (Butler *et al.*, 1993; Monack *et al.*, 2004; Thompson *et al.*, 2009; Gal-Mor *et al.*, 2012). Interferon-γ, also known as a macrophage activating factor (MAF), plays a pivotal role in continuous infection as it influences the duration of macrophage activation. Secretion of IFN-γ is dependent on IL-18, which is essential for establishing an early host immune response to *Salmonella* (Mastroeni *et al.*, 1999; Monack *et al.*, 2004).

The presence of *Salmonella* within macrophages leads to programmed cell death through apoptosis (Rosenberger *et al.*, 2001; Svensson *et al.*, 2001). However, pyroptosis has normally been associated with pro-inflammatory responses (Bergsbaken *et al.*, 2009), unlike

apoptotic cell death which has been shown to be immunosuppressive (Voll *et al.*, 1997; Zhang & Zheng, 2005).

It is well established that infection of macrophages with *S.* Typhimurium stimulates the cytokine production in the cells. However, the effect of solar irradiated pathogens on the chemokine and cytokine profiles of macrophages is not well established. Therefore, this study aimed to investigate the activation of macrophages by solar irradiated *S.* Typhimurium. The aim of this study was to assess the effect of SODIS of *S.* Typhimurium spiked water on the immune response of macrophages. The production of NO_2^-, chemokines, cytokines and growth factors in RAW264.7 cells were assessed. The controls included lipopolysaccharide, heat/chemical inactivated and non-solar irradiated *S.* Typhimurium. Macrophages respond to pathogens through the engagement of pattern recognition receptors (PRRs) of which the most studied are Toll-like receptors (TLRs) (Medzhitov *et al.*, 1997; Janeway & Medzhitov, 2002). Innate activation through TLR4 by LPS (Beutler & Rietschel, 2003) is well characterized as being responsible for most of the activation programs induced by gram-negative bacteria (Nau *et al.*, 2002). Therefore, LPS was used as a positive control.

5.2 Methodology

5.2.1 Culturing of *S.* Typhimurium

Salmomella Typhimurium (ATCC® 29629™) was streaked onto Luria Bertani (LB) agar plates to obtain single colonies. For batch cultivation, LB broth was autoclaved and diluted to 33% of its original strength. Erlenmeyer flasks containing 30 ml of diluted LB were inoculated with a single colony and incubated at 37°C with vigorous shaking until the cells reached exponential growth (OD_{546} between 0.1 and 0.2). The cultures were diluted to an OD_{546} of 0.002 into 150 ml of pre-warmed diluted LB in a 1000 ml Erlenmeyer flask and shaken overnight for 18 h until the stationary phase was reached. The stationary phase was determined by taking five consecutive OD_{546} readings (Bosshard *et al.*, 2010).

5.2.2 Preparation of heat-chemical inactivated *S.* Typhimurium

Salmonella Typhimurium that was cultured as above was harvested by centrifugation at 4000xg for 15 min. The pellet was washed 3 times with autoclaved 1X PBS to remove all traces of LB broth. The bacterial cells were then suspended in sterile mineral water, and the optical density of the suspension was adjusted to OD_{546} of 0.1 (approximately $1x10^7$ CFU/ml). The bacteria were heated and chemically inactivated by treating them at 65ºC for one hour in 0.5% phenol (Salisbury *et al.*, 2006). The heat-chemical inactivated culture was incubated at room temperature at 150 rpm for 48 h. The viability of the bacteria was assessed on LB agar plates using the Miles and Misra drop counting technique (Miles *et al.*, 1938).

5.2.3 Preparation of *S.* Typhimurium samples and exposure to SUVR

The cultured *S.* Typhimurium was harvested as explained above and washed 3 times with autoclaved still mineral water. The pellet was re-suspended in sterile mineral water up to an OD_{546} of 0.1 ($1x10^7$ CFU/ml) and incubated for 1 h at 37°C to allow the bacteria to adapt to the mineral water. Aliquots of 15 ml of bacterial suspension were transferred to 25 cm^3 tissue culture flasks and exposed to SUVR light for a period of 0, 4 and 8 h on the rooftop of a building at the Vaal University of Technology (26°42'39.1"S 27°51'46.2"E -26.710858, 27.862820) under sunny conditions from 8.00am-4.00pm. For each time point (0, 4, 8h) controls were exposed to similar atmospheric conditions except to SUVR by enclosing the samples in an opaque black ventilated box (Ssemakalu, 2010b). The samples were enumerated by the Miles and Misra method (Miles *et al.*, 1938).

5.2.4 Establishment of the macrophage cell line (RAW 264.7)

A RAW 264.7 cell line obtained from Cellonex (Separations, Randburg, South Africa) was grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37ºC in a humidified 5% CO_2 incubator. The macrophages were seeded in 48 well plates at a density of $1x10^5$ cells/ml and incubated for 24 h at 37°C in a humidified CO_2 incubator. Thereafter the macrophages were co-cultured with a) LPS, b) heat-chemical attenuated, c) non-solar and, d) solar irradiated S. Typhimurium for 3 and 24 hours in DMEM media and 10% foetal bovine serum without antibiotics. Macrophages co-cultured with *E. coli* LPS (Sigma, St. Louis, MO) were used as the positive controls, and untreated macrophage cultures were used as the negative controls.

5.2.5 Spectrophotometric analysis of nitric oxide production (iNOS expression)

iNOS activity was determined by quantifying NO_2^- in the supernatants of the macrophage-*S.* Typhimurium co-cultures using a commercial Nitric oxide detection kit (ThermoFisher Scientific, Germiston, South Africa). Briefly, 150 µl of supernatant, 20 µl Griess reagent and 130 µl of double deionized water was mixed in a 96-well plate and incubated at room temperature for 30 min. The absorbance of the mixture was measured at 540 nm in a microplate reader (Biotek, Winooski, VT). The amount of nitrite in the supernatants was calculated from a sodium nitrite ($NaNO_2$) standard curve (APPENDIX A).

5.2.6 Chemokine, cytokine and growth factor production

Supernatants of the treated and untreated macrophages that were collected at 3 and 24 h p.i. were assessed for the expression of chemokines, cytokines and growth factors (Table 1) using the Milliplex MAP Mouse Cytokine/ Chemokine Kit (Millipore, Burlington, MA). The assays

were performed in a Luminex 200 System (Luminex, Austin, TX) and the results were analysed using the Luminex Software xPONENT 3.1 (Austin, TX) for data acquisition. The Median Fluorescent Intensity data using a 5-parameter logistic or spline curve-fitting method (APPENDIX B) was used for calculating cytokine concentrations in samples.

Table 1 List of chemokines, cytokines and growth factors analysed in this study

Chemokine	Abbreviation
Chemokines	
Monocyte Chemoattractant Protein-1	MCP-1/CCL2
Regulated upon Activation, Normal T cell Expressed, and Secreted	RANTES/CCL5
Macrophage Inflammatory Protein-1α	MIP-1α/CCL3
Macrophage Inflammatory Protein-1β	MIP-1β/CCL4
Macrophage Inflammatory Protein-2	MIP-2/CXCL2
Keratinocyte chemoattractant	KC/CXCL1
Interferon gamma induced protein	IP-10/CXCL10
Pro-inflammatory cytokines	
Interleukin-1α	IL-1α
Interleukin-1β	IL-1β
Interleukin-2	IL-2
Interleukin-6	IL-6
Interleukin-7	IL-7
Interleukin-9	IL-9
Interleukin-12P40	IL-12P40
Interleukin-12P70	IL-12P70
Interleukin-15	IL-15
Interleukin-17	IL-17
Tumour necrosis factor-α	TNF-α
Inteferon-γ	IFN-γ
Anti-inflammatory cytokines	
Interleukin-4	IL-4
Interleukin-5	IL-5
Interleukin-10	IL-10
Interleukin-13	IL-13
Growth factors	
Granulocyte-colony stimulating factor	G-CSF
Granulocyte macrophage-colony stimulating factor	GM-CSF

5.2.7 Statistical analysis

Statistical analysis was carried out using the Student's t-test and mean differences at the $p<0.05$ were considered to be statistically significant. The data were expressed as means±standard error of the mean (SEM) obtained from three biological replicates for the nitric oxide production assay and two replicates for the cytokine and chemokine analysis. Statistical analysis was carried out using the GraphPad Prism 7.0d Software.

5.2.8 Data analysis

Permutation analysis was used to compare cytokine levels in macrophages that were a) untreated, b) LPS-treated, c) treated with heat/chemical attenuated *S.* Typhimurium*,* d) non-solar irradiated, and e) solar-irradiated *S.* Typhimurium. Where the cytokine and chemokine samples were undetectable the lowest detectable level was assigned; and where sample concentrations were higher than the range available for analysis they were assigned the upper limit value of the range. Spearman's rank correlation was used to assess any relationship between the chemokine and cytokine using GraphPad Prism 7.0d Software. Hierarchal clustering and heat map construction was performed with CIMminer (Bethesda, MD) using correlation (Clark *et al.*, 2015).

5.2.9 A network of potential protein interactions

Using the STRING 10.5 database (Swiss Institute of Bioinformatics, Lausanne, Switzerland), networks of possible protein interactions were created using significantly expressed proteins assessed in the hierarchical clustering from our analysis and were expanded to include downstream targets and, Gene Ontology (GO) analysis was carried out.

5.3 Results

5.3.1 Nitric oxide production

Supernatants from macrophages co-cultured with heat-chemical, solar radiated for 4 (SI4) and 8 h (SI8), did not contain significant NO_2- after 3 h p.i. (Fig. 35). However, detectable levels of NO_2- was observed at 24 h p.i. (Fig. 35). The heat/chemical treated *S.* Typhimurium induced a significantly ($p<0.0001$) higher level of nitrite concentration (32.4 ± 2.4 μM) at 24 h p.i. in the macrophages when compared to other treatments. The non-solar irradiated samples produced slightly higher levels of NO_2- while SI4 produced the least. However, there were no significant differences ($p>0.05$) among these three groups (NS, SI4, and SI8).

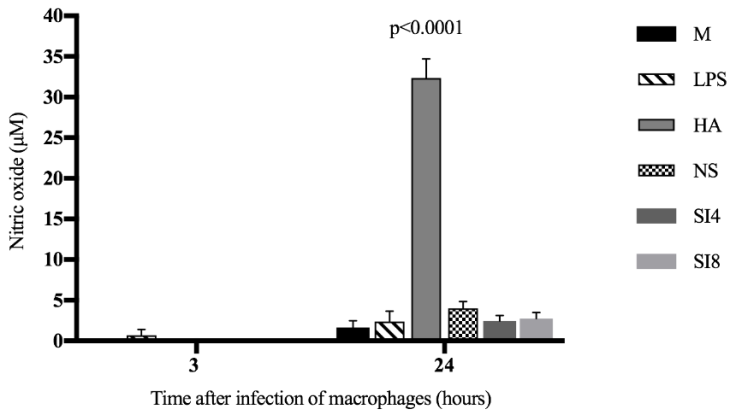


Figure 35 Nitric oxide production of macrophages that were a) untreated (M), b) LPS-treated, c) treated with heat/chemical attenuated *S.* Typhimurium (HA), d) non-solar irradiated (NS), e) 4h-solar-irradiated (SI4), and f) 8h-solar irradiated *S.* Typhimurium (SI8) after 3 and 24 h post-infection. Data shown are mean ± SEM of three independent experiments. Significant differences between the treated and non-treated macrophages are indicated on the graph where a $p<0.05$ was considered significant.

5.3.2 Chemokine, cytokine and growth factor production

Multiplex chemokine and cytokine assays were carried out to assess the expression of chemokines, cytokines and growth factors produced by RAW264.7 murine macrophages that were untreated, stimulated with LPS and heat/chemical attenuated, non-solar irradiated and solar irradiated *S.* Typhimurium. There was an increase in expression of most chemokines and cytokines during late infection (24 h) than in early infection (3 h) periods (Fig. 36-38).

5.3.3 Chemokine expression

Figure 36 shows the levels of the chemokines MCP-1. RANTES, MIP-1α, MIP-1β, MIP-2, KC and IP-10 produced by macrophages during early and late infection (i.e., 3 and 24 h p.i.). The levels of the chemokines were highly variable and lower expression levels were noted during early infection (3 h) for chemokines MCP-1. RANTES, MIP-1β, MIP-2, KC, and IP-10 than in the late infection periods. However, MIP-1α showed high expression levels during both early and late infection.

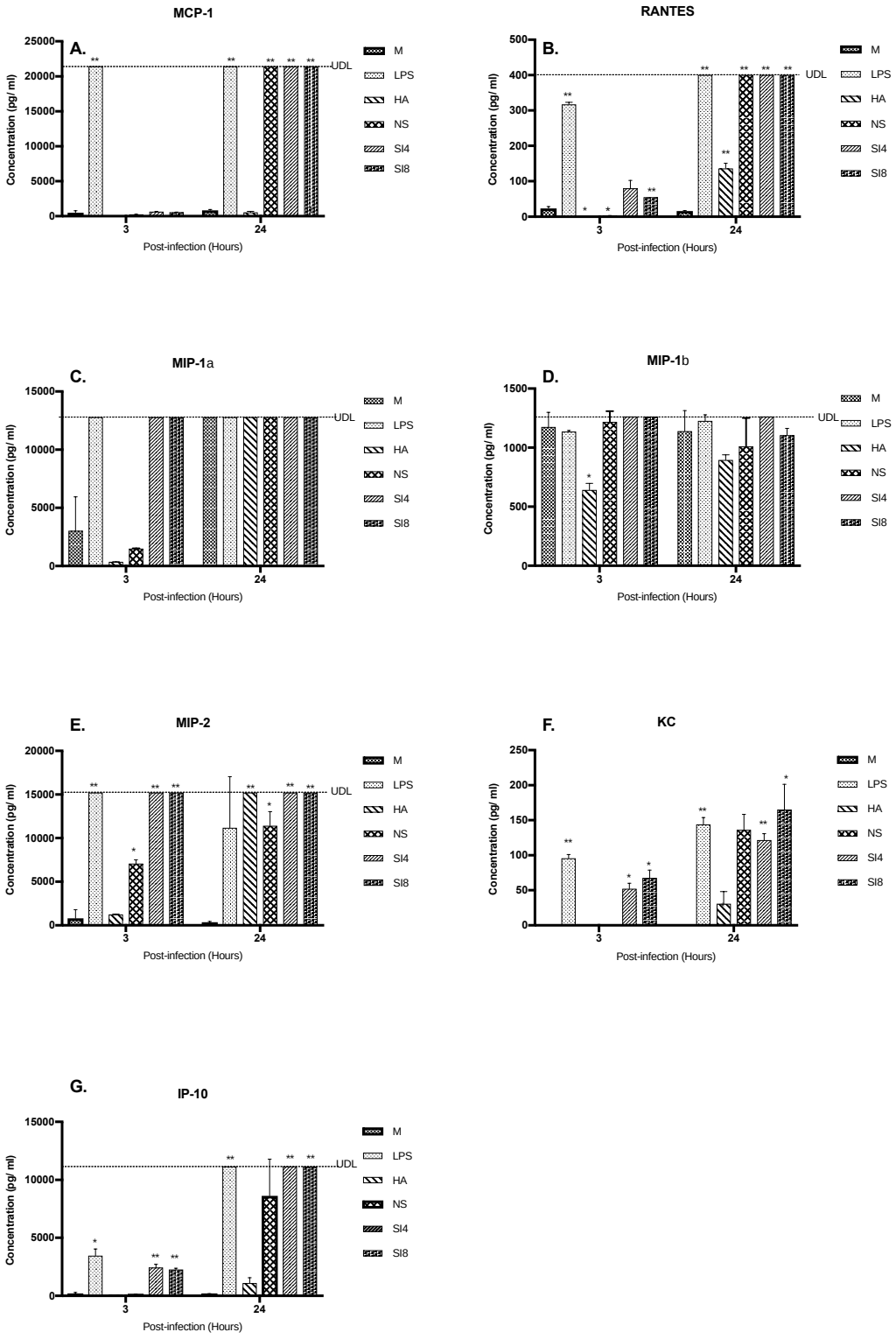


Figure 36 Chemokine levels assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA), non-solar irradiated *S.* Typhimurium (NS) and solar irradiated (SI4 and SI8); A) MCP-1), B) RANTES, C) MIP-1α, D) MIP-1β, E) MIP-2, F) KC, and G) IP-10. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper detection limits (UDL) are represented on the graphs.

During early infection (3 h p.i.), the highest MCP-1 expression was observed in macrophages treated with LPS (>21445 pg/ml) whereas the least MCP-1 expression was noted in heat-chemically treated macrophages (24.27 ± 4.82 pg/ml). After 24 h p.i., a significant ($p<0.01$) increase in MCP-1 production was observed in the LPS, NS, SI4 and, SI8-treated macrophages compared to the untreated macrophages. The values were above the detectable limit of 21444 pg/ml. The lowest MCP-1 expression was noted in heat –chemically treated macrophages (560.37 ± 133.92 pg/ml) (Fig. 36A).

There was a significant ($p<0.01$) increase in RANTES production in LPS-treated macrophages (316.99 ± 5.58 pg/ml) compared to the untreated macrophages during both early and late infection (Fig. 36B). Minimal expression of RANTES was noted for HA (>1.59 pg/ml) and NS (2.48 ± 0.13 pg/ml). There was a slight increase in RANTES production for SI4, and SI8 to 49.95 ± 21.98 pg/ ml and 54.65 ± 0.00 pg/ml, respectively. After 24 h p.i., significant increases ($p<0.01$) in RANTES were noted in all samples. The macrophages treated with LPS, SI4, and SI8 produced high amounts of RANTES; the values were above the detectable limit of 400 pg/ml (Fig. 36B) whereas, the macrophages treated with HA produced moderate amounts of RANTES in comparison to LPS-treated macrophages (136.39 ±14.55 pg/ml) (Fig. 36B).

High amounts of MIP-1α were noted in all the solar irradiated samples (SI4 and SI8) during early infection with the values being above the detectable limit of 12790 pg/ml (Fig. 36C). However, HA-treated macrophages showed the lowest of MIP-1α expression (107.00 ± 3.93 pg/ml). An increase in MIP-1α was noted in all samples after 24 h p.i. (Fig. 36C). The chemokine, MIP-1β was above the upper detectable limit of 12790 pg/ml in all the samples including the negative controls after 24 h of infection (Fig. 36C). At 3 h p.i. the highest expression of MIP-1β was observed for SI4 and SI8 where the values were above the detectable limit of >1263 pg/ml. The least MIP-1β expression was found in HA-treated macrophages (642.56 ± 56.12 pg/ml). During late infection, the highest MIP-1β expression (>1263 pg/ml) was noted in SI4-treated macrophages and the least in HA-treated macrophages (896.16 ± 43.39 pg/ml). However, it is important to note that the untreated macrophages (negative controls) also exhibited high expression levels of MIP-1β during early and late infection (Fig. 36D)

During early infection, significant increases ($p<0.01$) in MIP-2 production was noted in LPS and solar irradiated bacteria (SI4 and SI8) (>15230 pg/ml). However, the least MIP-2 production was observed in macrophages treated with heat attenuated samples (1274.00 ± 46.67 pg/ml). After 24 h post-infection a notable decrease in MIP-2 was observed in macrophages treated with NS (11196 ± 5837.88 pg/ml) and the expression levels were above the detectable limit of 15230 pg/ml in HA, SI4, and SI8-treated (Fig. 36E).

At 3 h p.i. KC production was highest in LPS treated macrophages (97.70 ± 5.46 pg/ml). However, KC expression in macrophages treated with NS and HA *S.* Typhimurium was below the detectable limit of 2.65 pg/ml. After 24 h p.i. there were significant increases in the amount of KC for the LPS, NS, SI4, and SI8 treated samples (Fig. 36F). The least KC expression (30.65 ± 17.49 pg/ml) was observed in HA-treated macrophages.

During early infection, IP-10 expression was highest in LPS-treated macrophages (3466 ± 577.00 pg/ml), and the least was observed in HA-treated macrophages (138.52 ± 2.10 pg/ml). During late infection, increased expression levels of IP-10 was found in LPS, SI4, and SI8 (>11152 pg/ml), treated samples whereas the lowest value was noted in HA-treated macrophages (1105.84 ± 465.50 pg/ml).

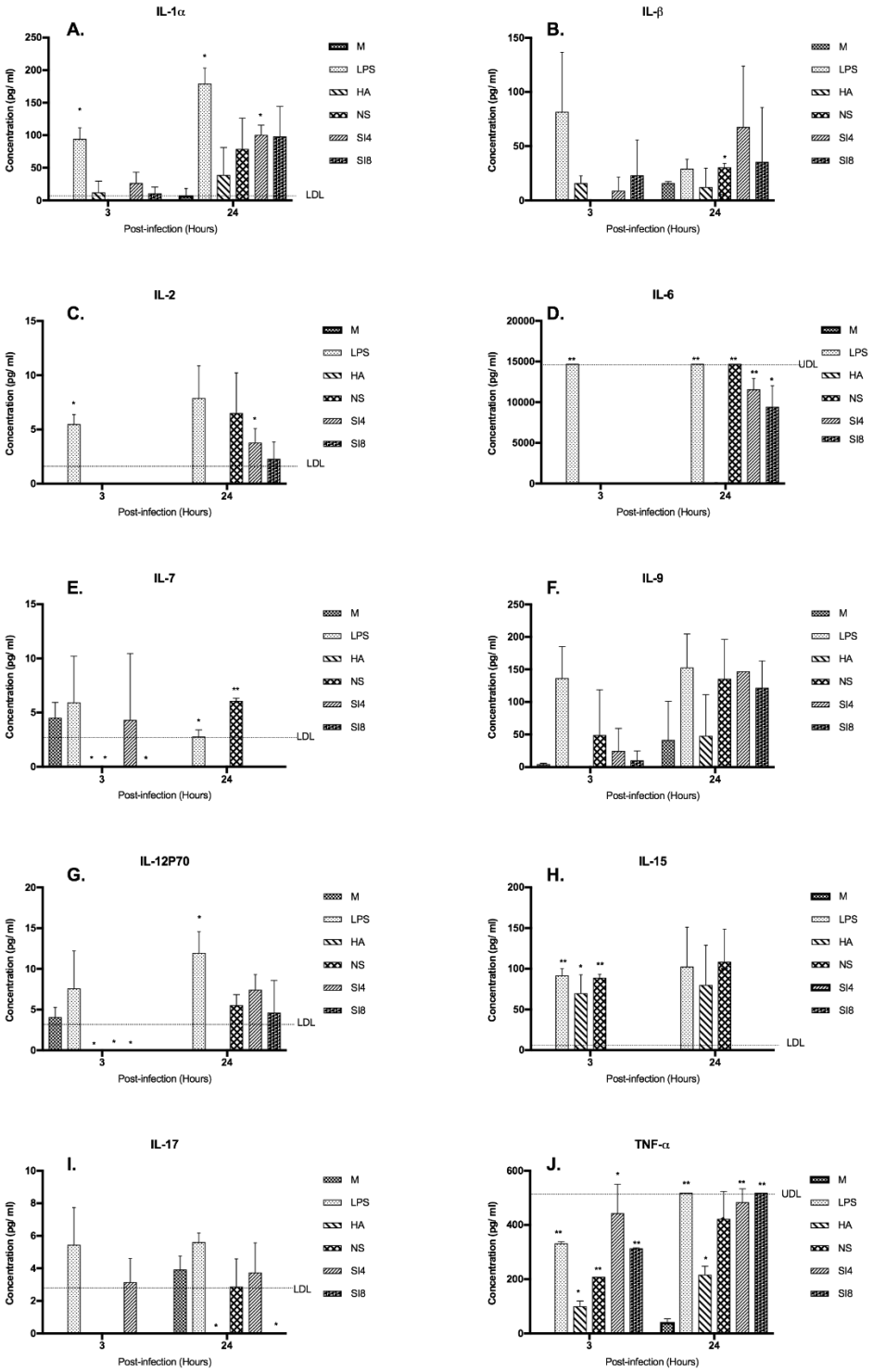


Figure 37 Cytokines involved in a pro-inflammatory immune response as assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA), non-solar irradiated *S.* Typhimurium (NS) and solar irradiated *S.* Typhimurium (SI4 and SI8). A) IL-1α, B) IL-1β, C) IL-2, D) IL-6, E) IL-7, F) IL-9, G) IL-12P70, H) IL-15 I), IL-17, and J) TNF-α. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper and lower detection limits (UDL and LDL) are represented on the graphs.

5.3.4 Classical activation (Pro-inflammatory response)

The cytokines responsible for a pro-inflammatory immune response, namely, IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-9, IL-12P40, IL-12P70, IL-15, IL-17, TNF-α, and IFN-γ were quantified. The expression levels of the cytokines were highly variable amongst the different treatments. The LPS-treated macrophages showed the highest expression of pro-inflammatory chemokines at 3 h and 24 h p.i. The macrophages treated with heat-chemical attenuated *S.* Typhimurium expressed the lowest levels of pro-inflammatory chemokines. Higher expressions of pro-inflammatory chemokines were also noted during late infection (24 h p.i.) (Fig. 37).

After the 3 h infection point, the macrophages infected with heat-chemical attenuated (HA) and solar irradiated *S.* Typhimurium (SI 4 and SI 8) produced minimal amounts of IL-1 (Fig. 37A). Macrophages stimulated with LPS expressed the highest value of IL-α (94.53 ± 16.95 pg/ml). IL-1α production for NS treated macrophages was below the detectable limit of 4.38 pg/ml. After 24 h post-infection, the LPS-treated macrophages produced a statistically significant increase ($p = 0.01$) in IL-1α compared to the untreated macrophages, and the HA-treated macrophages showed the least IL-1α expression (Fig. 37A).

At 3 h p.i., the highest IL-1β expression was observed in LPS-treated macrophages (81.67 ± 54.94 pg/ml) and the NS-treated macrophages expression level IL-1β was below the detectable limit of 8.83 pg/ml. During late infection, the production of IL-1β was highest in SI4-treated macrophages (67.74 ± 56.04 pg/ml), and the lowest levels were recorded in HA-treated macrophages (Fig. 37B).

During early infection, the LPS-treated macrophages induced the highest expression of IL-2 (5.50 ± 0.88 pg/ml). The IL-2 expressed by macrophages treated with HA, NS, SI4, and SI8 were below the detectable limit of 1.68 pg/ml. However, after 24 hours of infection, the highest increase in IL-2 expression was observed in LPS treated macrophages (7.91 ± 2.96 pg/ml) while the least was recorded for HA-treated macrophages (<1.68 pg/ml) (Fig. 37C).

For IL-6, early infection of the LPS treated macrophages produced the highest level at >14725 pg/ml. Undetectable levels of IL-6 (<3.22 p/ml) were recorded for the HA, NS, SI4, and SI8-treated macrophages. There was a significant increase ($p<0.01$) in IL-6 expression after 24 h for the LPS and NS-treated macrophages with a maximal production of >14725 pg/ml. The least expression of IL-6 was noted in HA-treated macrophages (97.04 ± 28.19 pg/ml). The *S.* Typhimurium samples solar irradiated for 8 h (SI8) induced lower amounts of IL-6 (9450.50 ± 2570.33 pg/ml) in macrophages when compared to 4h-solar irradiated *S.*Typhimurium (SI4) (11599.50 ± 1330.07 pg/ml) (Fig. 37D).

The highest amount of IL-7 was expressed in the LPS treated samples (5.96 ± 4.26 pg/ml) during early infection, and the least was observed in macrophages treated with HA, NS, and SI8. Except for the LPS (2.80 ± 0.61 pg/ml) and NS-treated macrophages (6.07 ± 0.26 pg/ml),

IL-7 was not expressed in all samples after 24 h p.i. The macrophages treated with HA, NS, SI4, and SI8 showed minimal levels of IL-7 expression (<2.76 pg/ml) (Fig. 37E).

The expression of IL-9 was highest in LPS treated samples, and very low amounts were detected in HA and SI4 samples. During late infection, the highest amounts of IL-9 was detected in SI4 and the least in macrophages treated with HA (Fig. 37F).

IL-12P40 was not produced in all the samples during the early and late infection periods (data not shown). However, small amounts of IL-12P70 were produced ranging from 0 to 15 ng/ml. in all the samples. There was an insignificant increase in the LPS-treated macrophages (7.62 ± 4.62 pg/ml) in comparison to the non-treated macrophages (Fig. 3G). No IL-12P70 was produced in the macrophages treated with non-solar and solar-irradiated *S.* Typhimurium at 3 h p.i. After 24 hours post-infection, no IL-12P70 was detected in HA and the SI8 samples. The highest IL-12P70 was noted in LPS-treated macrophages (11.99 ± 2.59 pg/ml) (Fig. 3G) after 24 h.

At 3 h p.i., LPS-treated macrophages (91.88 ± 8.17 pg/ml) showed the highest significant increase ($p<0.01$) IL-15 in comparison to non-treated macrophages. Macrophages treated with solar irradiated *S.* Typhimurium showed very little expression of IL-15 expression (<62.97 pg/ml) during early and late infection (Fig. 3E). A non-significant increase in IL-15 production was noted in LPS, HA and NS-treated macrophages during late infection (Fig. 37H).

Very low amounts of IL-17 were produced in all the samples (<10 pg/ml). The highest value of IL-17 was recorded in LPS treated macrophages at 3 h p.i. and 24 p.i (5.45± 2.28 pg/ml and 5.62 ± 0.56 pg/ml), respectively. Interleukin-17 was below the detectable limits of 2.87 pg/ml in HA and SI8-treated macrophages during early infection. At 24 h p.i., IL-17 remained below the lower detectable threshold in HA and S8-treated macrophages. However, there was an increased expression in NS and SI4-treated macrophages (2.88 ± 1.711 pg/ml and 3.75 ± 1.82 pg/ml), respectively (Fig. 37I).

IFN-γ was not detected in all samples except in the LPS-treated macrophages (2.6 ±0.09 pg/ml) at 3 h p.i. During late infection, very small amounts of IFN-γ was produced in macrophages treated with SI4 treated bacteria (2.84±0.53) (not shown in the figure).

Tumour necrosis factor (TNF-α) was released by the macrophages during early and late infection for LPS, HA, NS, SI4, and SI8 treated samples (Fig. 37J). During early infection, there were significant increases in TNF-α in the LPS (91.88 ± 8.17 pg/ml; $p<0.01$) and HA (69.96 ± 22.83 pg/ml; $p=0.02$) treated samples compared to the non-treated macrophages. However, the production of TNF-α was below the detectable limit of 2.49 pg/ml in HA, SI4 and, SI8-treated macrophages. During late infection, the LPS, SI4 and SI8 treated macrophages exceeded the upper detectable limit of 518.77 pg/ml. The least TNF-α was noted in macrophages treated with HA.

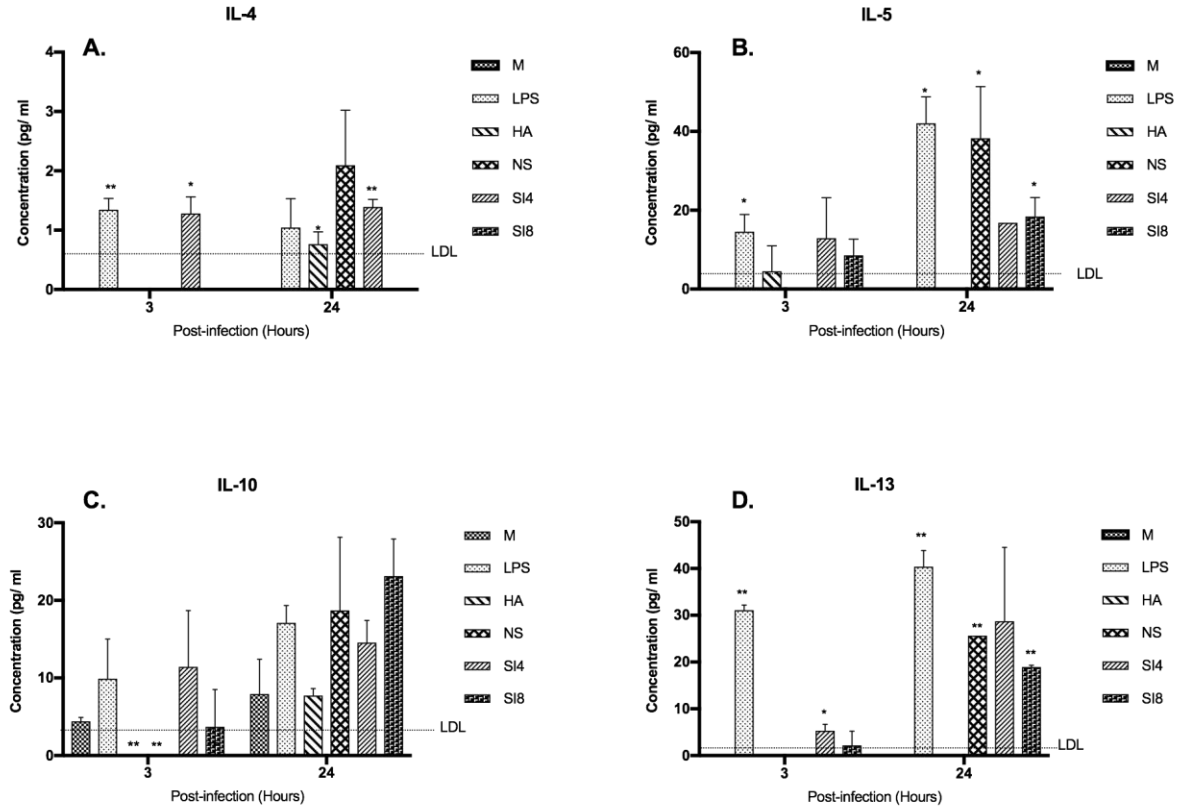


Figure 38 Levels of the cytokines involved in the anti-inflammatory immune response assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA), non-solar irradiated *S.* Typhimurium (NS) and solar irradiated *S.* Typhimurium (SI4 and SI8). A) IL-2, B) IL-4, C) IL-5, D) IL-7, E) IL-9, F) IL-10, G) IL-13, and h) IL-17. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The lower detection limits (LDL) are represented on the graphs.

5.3.5 Alternative activation (anti-inflammatory response)

The following cytokines IL-4, IL-5, IL-10 and, IL-13 responsible for alternative activation (anti-inflammatory response) were tested for at 3 and 24 h p.i. (Fig 38). Lower expression levels of anti-inflammatory than pro-inflammatory cytokines were observed (<60 pg/ml). Higher expression levels of the anti-inflammatory cytokines were observed during late infection. The macrophages treated with LPS and non-solar irradiated *S.* Typhimurium showed very high expression levels of anti-inflammatory chemokines whereas the heat-chemical attenuated *S.* Typhimurium induced minimal expression.

Very low amounts (<2 pg/ml) of IL-4 were observed in all the samples during early infection. The LPS (1.35 ± 0.19 pg/ml) and SI4 (1.28 ± 0.28 pg/ml) samples were the only samples that induced detectable amounts of IL-4 at 3 h p.i. Interleukin-4 production was below the

detectable limit of 0.58 pg/ml in HA, NS, and SI8-treated macrophages. However, after 24 hours post-infection, a significant increase in IL-4 production in comparison to the untreated macrophages was observed in HA-treated macrophages (0.78 ± 0.21 pg/ml; p=0.02) (Fig. 38A).

The expression of IL-5 production was highest in LPS (14.57 ± 4.37 pg/ml) treated macrophages and very small amounts were noted in HA, NS, SI4, and SI8 samples after 3 h. However, after 24 h p.i., there was an increase in IL-5 production in macrophages treated with LPS (42.09 ± 6.69 pg/ml) and NS (38.27 ± 13.10 pg/ml) samples. The production of IL-5 in HA-treated macrophages was below the detectable limit of 4.14 pg/ml during late infection (Fig. 38B).

During early infection, the highest amount of IL-10 was expressed in the macrophages treated with LPS (9.90 ± 5.11 pg/ml) whereas the HA and SI8 were below the detectable limits of 3.53 pg/ml. However, after 24 h p.i., there was a marked increase in IL-10 expression in all the samples and the highest amount was noted in LPS treated macrophages (17.11 ± 2.23 pg/ml). The least was observed in macrophages treated with HA (7.74 ± 0.91 pg/ml) (Fig. 38C).

The expression of IL-13 was highest in macrophages treated with LPS-treated macrophages (31.11 ± 1.10 pg/ml) both during early and late infection (40.38 ± 3.47 pg/ml). IL-13 expression was below the detectable limit of 1.66 pg/ml for HA-treated macrophages during early and late infection (Fig. 38D). During late infection, a moderate amount (in comparison to LPS) was produced in the NS (25.63 ± 0.00 pg/ml), SI4 (28.74 ± 15.80 pg/ml) and SI8-treated macrophages (18.92 ±0.38 pg/ml).

5.3.6 Growth Stimulating Factors

During early infection, the highest expression (>11097 pg/ml) of GCSF was observed in LPS-treated macrophages while the lowest (6.85 ± 3.26 pg/ml) was found in HA-treated macrophages. After 24 h p.i., GCSF was produced in very high amounts in macrophages treated with LPS, NS, SI4, and SI8 during late infection (>11097 pg/ml). The lowest GCSF expression was noted in HA-treated macrophages (2483 ± 335.02 pg/ml) (Fig. 39A).

At 3 h p.i., LPS-treated macrophages showed a significant increase ($p<0.0$) in GM-CSF expression in comparison to the non-treated macrophages. The lowest expression levels were observed in macrophages treated with HA, NS, SI4, and SI8 (<26.64 pg/ml). At 24 h p.i., the highest increase was observed in LPS-treated macrophages, and the lowest seen in HA and SI8 (<26.64 pg/ml) (Fig. 39B).

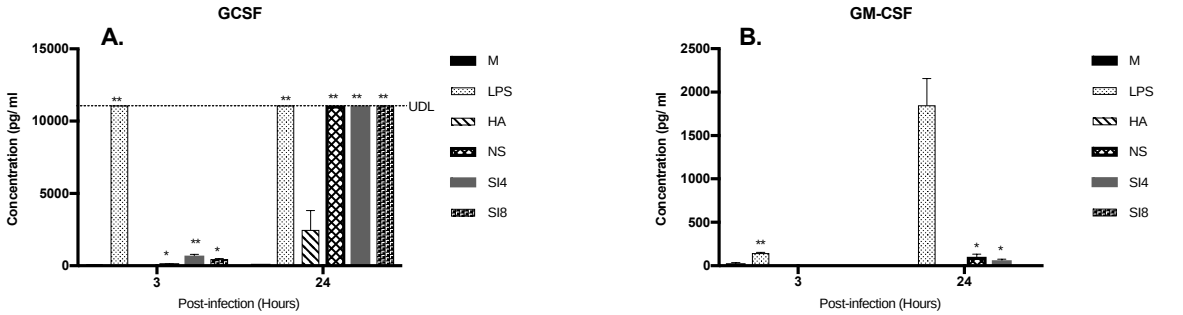


Figure 39 Protein levels of the growth factors as assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA) and, non-solar irradiated *S.* Typhimurium (SI4 and SI8); A) granulocyte-colony-stimulating factor (GCSF) and, B) granulocyte-macrophage-colony-stimulating factor (GM-CSF). Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper detection limits (UDL) are represented on the graphs.

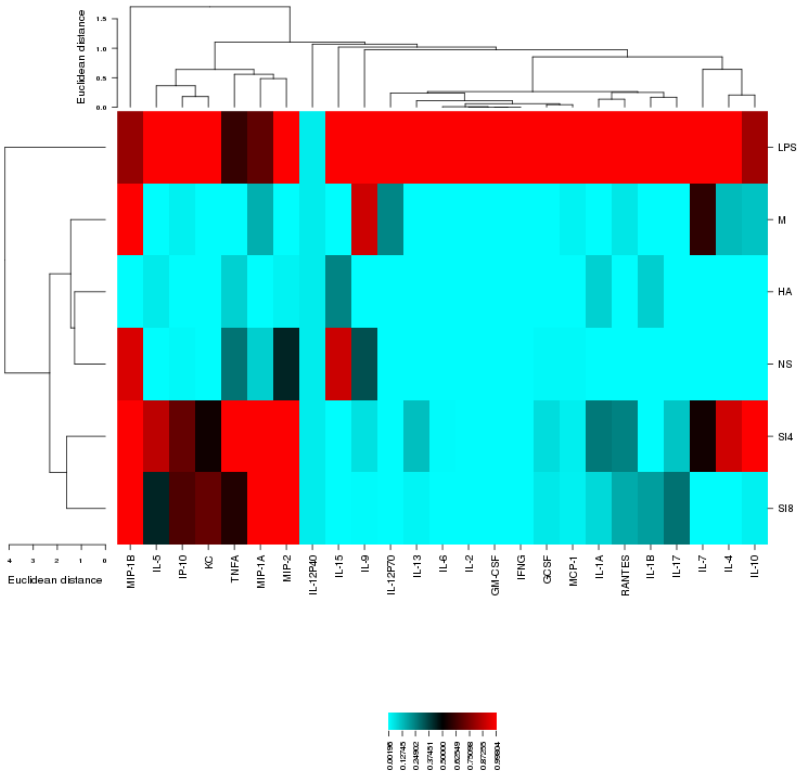
5.3.7 Hierarchal clustering

Chemokine, cytokine and growth factor expression were normalised in GraphPad Prism. Heat maps were then constructed from the Luminex results using CIMminer NIH Software. Unbiased clustering was carried out using the Euclidean Distance.

The length of the dendrogram is inversely proportional to correlation. During early infection, three clusters were formed: The M, HA, and NS-treated macrophages formed the largest cluster, SI4 and SI8-treated macrophages clustered into one group and the LPS-treated macrophages did not fall into any cluster. Moreover, the following proteins that were highly expressed were also clustered into one group; MIP-1α, MIP-1β, MIP-2, KC, IP-10, IL-5 and TNF-α (Fig 40A).

At late infection two significant clusters for the samples were observed; the first one consisting of samples with low expression levels, namely, the untreated macrophages and those treated with HA samples. The second cluster included samples showing high levels of protein expressions that included macrophages treated with LPS, NS, SI4, and SI8 bacteria. A close relationship was noted in macrophages treated with SI4 and SI8 bacteria (Fig 40B). There was an increase in highly expressed proteins during late infection and this resulted in the formation of several clusters; the most significant cluster consisted of IL-10, IL-6, IL-9, KC, TNF-α, GCSF, RANTES, IP-10, and MCP-1; one cluster was composed of MIP-1α and MIP-1β and another cluster consisted of MIP-2. However, IL-12P40 formed the quiescent group (no expression in all samples) (Fig. 40B).

A.



B.

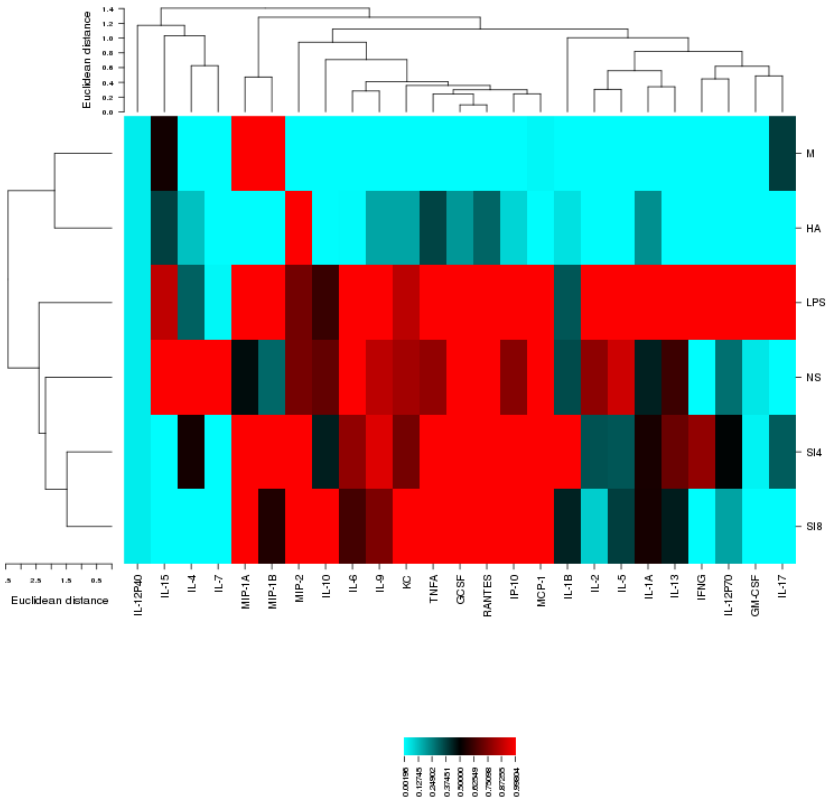


Figure 40 Hierarchal clustering of RAW264.7 macrophages treated with (LPS), heated attenuated (HA) and, non-solar irradiated *S.* Typhimurium (SI4 and SI8 during A) Early infection (3 h p.i.) and B) Late infection (24 h p.i.)

5.3.8 Network analysis and functional enrichment analysis

A network of potential protein interactions was constructed using the STRING 10.5 database. (Figs. 41). The cluster of highly expressed proteins observed during late infection (Fig 40) including NOS2 (the protein coding for enzyme Nitric Oxide Synthase 2) was used to develop the networks (Figs. 41A-E). A highly extensive network was formed for the LPS-treated macrophages and were composed of both anti- and pro-inflammatory proteins. After 24 h p.i. a much more diverse and intricate network was developed, and IL-6 appears to be occupying the central node with multiple downstream protein interactions.

Tables 2-5 show some functional enrichments obtained from STRING 10.5 which can be expected from the network of potential protein interactions expressed during late infection periods in macrophages treated with the endotoxin, LPS, heat attenuated (HA), non-solar irradiated and solar-irradiated *S.* Typhimurium (Tables 2-5). The typical primary biological processes associated with the networks of potential protein interactions highly expressed during late infection included the cytokine-mediated pathway, inflammatory responses, positive regulation of the immune response, response to molecules of bacterial origin and positive regulation of leukocyte chemotaxis (Tables 2-5).

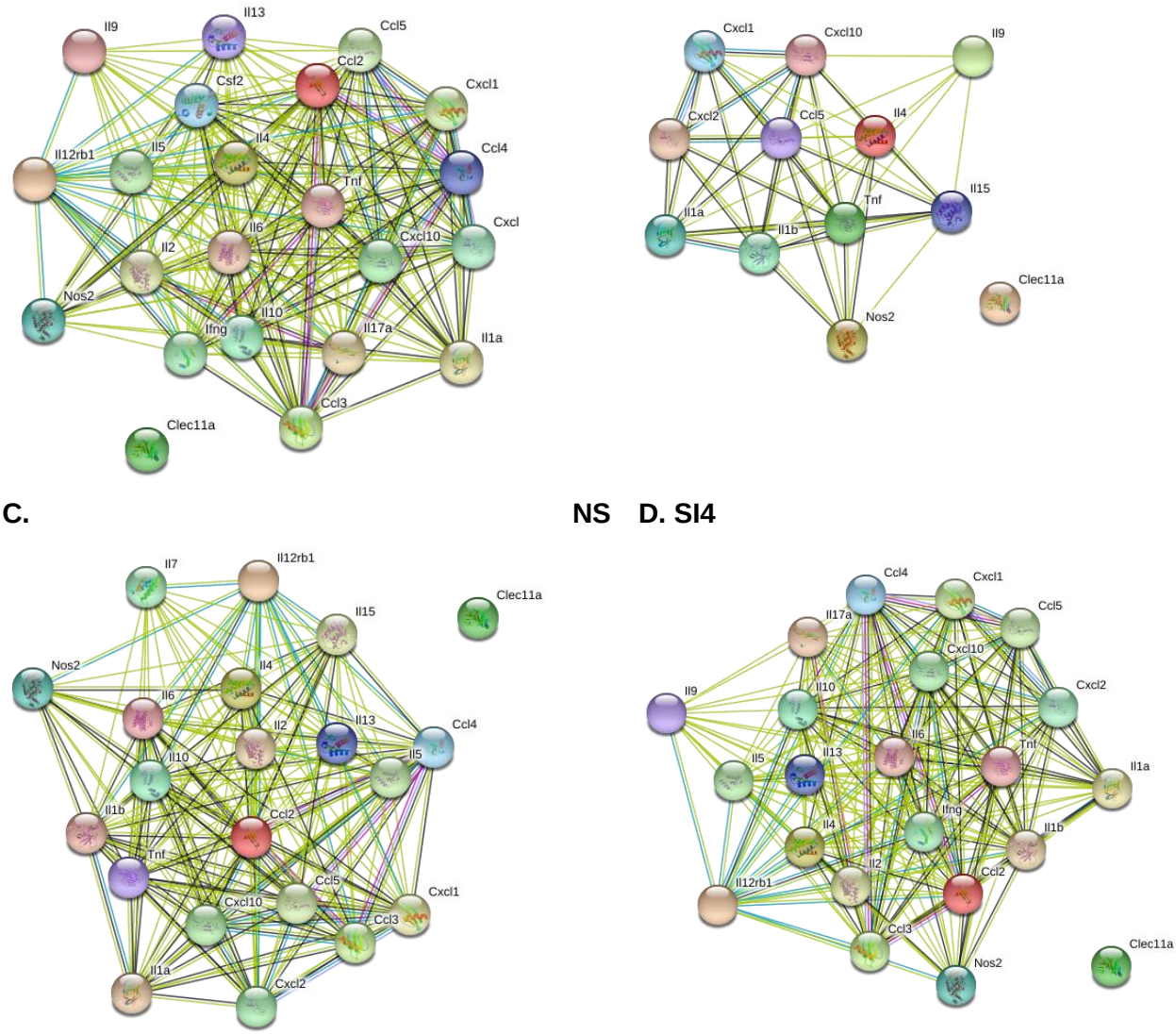


Figure 41 Network String Analysis derived from hierarchal clustering. Using the STRING 10. 5 databases, a network of potential protein interactions expressed in macrophages treated with (A) LPS, (B) Heat/chemical treated (HA), (C) non-solar irradiated (NS), (D) 4h-solar irradiated, and (E) 8h-solar irradiated *S.* Typhimurium at 24 h p.i. was clustered. Key for lines: Light green, association by text mining; pink association by experiment; black, association by co-expression, dark green, association neighbourhood, light blue association from curated databases.

To determine what biological processes and pathways that are induced by each of the treatments, a gene ontology analysis on genes upregulated by LPS, HA, NS, SI4 and SI8 was performed (Tables 2-6). Gene ontology analysis of the genes induced by LPS, HA, NS, SI4, and SI8 all reached high statistical significance as shown by the very low P-values. This is likely due to a large number of genes expressed. The highest statistical significances were observed for an immune response in NS-treated samples, with the lowest p-value of 3.55E-26 (Table 3). The heat attenuated (HA)-treated macrophages showed the highest p-value for immune response (p=1.75E-12) (Table 3). Amongst the solar irradiated samples, the *S.* Typhimurium irradiated for 4 hours showed higher statistical significance for an immune response (p=3.80E-25) (Table 5) when compared to *S.* Typhimurium that was solar irradiated for 8 hours (p=1.36E-21) (Table 6).

The LPS, HA, NS, SI4, and SI8 treatment, IL-10 treatment produced common gene lists that were enriched in the biological processes of immunity and defence, cellular response to LPS, inflammatory response, cytokine and chemokine mediated signalling pathways (Tables 2-6).

Table 2 Biological Process (GO) induced by LPS during late infection (24 h p.i.)

Biological Process	Gene count	P-value
immune response	20	3.80E-25
positive regulation of cytokine production	16	1.44E-22
defence response	17	1.72E-17
inflammatory response	14	3.03E-17
response to molecule of bacterial origin	13	8.94E-17
cellular response to lipopolysaccharide	11	1.27E-16
response to lipopolysaccharide	12	2.19E-15
positive regulation of JAK-STAT cascade	9	2.19E-15
positive regulation of tyrosine phosphorylation of STAT protein	7	3.87E-12
positive regulation of intracellular signal transduction	13	3.87E-12
regulation of immunoglobulin production	7	8.86E-12
positive regulation of protein secretion	9	1.08E-11
positive regulation of peptidyl-tyrosine phosphorylation	8	1.03E-10
positive regulation of transport	12	1.55E-10
leukocyte chemotaxis	7	1.76E-10
positive regulation of leukocyte mediated immunity	7	2.70E-10
positive regulation of secretion by cell	9	4.23E-10
positive regulation of phosphate metabolic process	12	4.70E-10
regulation of B cell activation	7	7.67E-10
regulation of lymphocyte activation	9	8.55E-10
regulation of cell killing	6	1.61E-09
positive regulation of myeloid leukocyte differentiation	6	1.76E-09
positive regulation of biosynthetic process	14	1.76E-09
positive regulation of metabolic process	17	1.76E-09
positive regulation of a cellular metabolic process	16	1.80E-09
regulation of inflammatory response	8	2.13E-09
regulation of response to stress	11	4.91E-08
regulation of nitric oxide biosynthetic process	5	6.09E-08
regulation of reactive oxygen species metabolic process	6	2.03E-07
positive regulation of adaptive immune response	5	5.47E-07
response to lipid	9	5.68E-07
positive regulation of MHC class II biosynthetic process	3	1.88E-06

Table 3 Biological Process (GO) induced by heat/chemical attenuated (HA) *S,* Typhimurium **during late infection (24 h p.i.)**

Biological Process	Gene Count	P-Value
immune response	11	1.75E-12
cytokine-mediated signaling pathway	7	1.10E-08
response to molecule of bacterial origin	7	3.22E-08
cellular response to lipopolysaccharide	6	3.22E-08
positive regulation of response to an external stimulus	7	7.23E-08
positive regulation of cytokine biosynthetic process	5	7.27E-08
inflammatory response	7	1.06E-07
response to bacterium	7	3.86E-07
response to lipopolysaccharide	6	7.92E-07
leukocyte migration	5	1.90E-06
positive regulation of fever generation	3	2.10E-06
regulation of acute inflammatory response	4	2.24E-06
regulation of cell proliferation	8	7.35E-06
regulation of response to stress	7	3.20E-05
regulation of intracellular signal transduction	7	6.98E-05
regulation of reactive oxygen species metabolic process	4	7.54E-05
positive regulation of a homeostatic process	4	8.68E-05
chronic inflammatory response to antigenic stimulus	2	0.000104
protein kinase B signalling	3	0.000104
regulation of granulocyte chemotaxis	3	0.000104
extrinsic apoptotic signaling pathway in the absence of ligand	3	0.000104
positive regulation of tyrosine phosphorylation of STAT protein	3	0.000168
positive regulation of defence response	4	0.00017
regulation of nitric oxide biosynthetic process	3	0.000175
sequestering of triglyceride	2	0.00018
positive regulation of metabolic process	9	0.000183

Table 4 Biological Process (GO) induced by non-solar irradiated (NS) *S.* Typhimurium during late infection (24 h p.i.)

Biological process	Gene count	P-value
immune response	20	3.55E-26
positive regulation of cytokine production	14	1.52E-18
inflammatory response	14	1.49E-17
immune system process	18	2.23E-16
defence response	16	2.30E-16
regulation of response to an external stimulus	15	1.03E-15
cellular response to lipopolysaccharide	10	7.56E-15
positive regulation of leukocyte differentiation	10	1.20E-14
response to lipopolysaccharide	11	7.65E-14
regulation of lymphocyte proliferation	10	8.35E-14
positive regulation of immune system process	13	1.23E-13
positive regulation of lymphocyte proliferation	9	1.31E-13
positive regulation of JAK-STAT cascade	8	1.84E-13
regulation of immune effector process	11	3.70E-13
regulation of protein transport	11	2.94E-10
positive regulation of tyrosine phosphorylation of STAT protein	6	3.49E-10
regulation of B cell activation	7	5.33E-10
positive regulation of T cell activation	7	3.35E-09
positive regulation of peptidyl-tyrosine phosphorylation	7	3.80E-09
positive regulation of phosphate metabolic process	11	4.66E-09
regulation of natural killer cell chemotaxis	4	5.12E-09
positive regulation of calcium ion transport	6	2.13E-08
regulation of nitric oxide biosynthetic process	5	4.64E-08
chemokine-mediated signaling pathway	5	4.64E-08
regulation of T cell differentiation	6	4.66E-08
regulation of acute inflammatory response	5	5.76E-08
regulation of reactive oxygen species metabolic process	6	1.50E-07
negative regulation of apoptotic process	9	4.19E-07
positive regulation of immune response	7	4.19E-07
cellular response to interferon-gamma	4	8.30E-07
regulation of cell migration	8	9.08E-07
regulation of granulocyte chemotaxis	4	1.93E-06
extrinsic apoptotic signaling pathway in absence of ligand	4	1.93E-06
response to alkaloid	5	3.30E-06
regulation of cytokine secretion	5	3.43E-06
regulation of chronic inflammatory response	3	3.60E-06
eosinophil chemotaxis	3	3.60E-06
granulocyte chemotaxis	4	3.72E-06
chronic inflammatory response	3	8.47E-06

response to gamma radiation	4	8.66E-06
cellular response to interleukin-1	4	1.05E-05
negative regulation of cell death	8	1.19E-05
MAPK cascade	5	1.25E-05
regulation of membrane protein ectodomain proteolysis	3	3.12E-05
cellular response to an oxygen-containing compound	7	3.50E-05
cellular response to high-density lipoprotein particle stimulus	2	3.73E-05
negative regulation of complement-dependent cytotoxicity	2	3.73E-05
cellular response to tumor necrosis factor	4	3.93E-05
positive regulation of immunoglobulin mediated immune response	3	9.01E-05

Table 5 Biological Process (GO) induced by 4h-solar irradiated (SI4) *S.* Typhimurium during late infection (24 h p.i.)

Biological Process	Gene Count	P-value
immune response	20	3.80E-25
positive regulation of cytokine production	16	1.44E-22
defense response	17	1.72E-17
inflammatory response	14	3.03E-17
response to molecule of bacterial origin	13	8.94E-17
cellular response to lipopolysaccharide	11	1.27E-16
positive regulation of JAK-STAT cascade	8	2.88E-13
leukocyte chemotaxis	8	1.82E-12
positive regulation of response to a stimulus	16	2.20E-12
regulation of immune system process	14	3.15E-12
regulation of production of a molecular mediator of immune response	8	3.71E-12
regulation of immunoglobulin production	7	7.25E-12
regulation of inflammatory response	9	4.28E-11
positive regulation of tyrosine phosphorylation of STAT protein	6	4.02E-10
regulation of nitric oxide biosynthetic process	6	4.59E-10
regulation of intracellular signal transduction	13	5.26E-10
regulation of B cell activation	7	6.47E-10
regulation of cell killing	6	1.50E-09
positive regulation of B cell activation	6	3.11E-09
regulation of reactive oxygen species metabolic process	7	3.60E-09
positive regulation of peptidyl-tyrosine phosphorylation	7	4.89E-09
positive regulation of chemokine biosynthetic process	4	5.54E-09
regulation of natural killer cell chemotaxis	4	5.54E-09
response to glucocorticoid	7	7.05E-09
lipopolysaccharide-mediated signaling pathway	5	7.25E-09
positive regulation of inflammatory response	6	7.53E-09
positive regulation of calcidiol 1-monooxygenase activity	3	7.24E-08
response to toxic substance	6	3.30E-07
positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	5	4.85E-07
positive regulation of acute inflammatory response	4	5.05E-07
positive regulation of reactive oxygen species metabolic process	5	5.32E-07
regulation of interferon-gamma production	5	7.04E-07
positive regulation of cell death	8	7.62E-07
positive regulation of immunoglobulin mediated immune response	4	8.57E-07
regulation of vascular endothelial growth factor production	4	9.79E-07
positive regulation of natural killer cell chemotaxis	3	9.93E-07
positive regulation of nitric oxide biosynthetic process	4	1.42E-06
regulation of interleukin-6 production	5	1.48E-06

positive regulation of MHC class II biosynthetic process	3	1.64E-06
positive regulation of isotype switching to IgG isotypes	3	1.64E-06
positive regulation of killing of cells of other organisms	3	1.64E-06
regulation of granulocyte chemotaxis	4	1.99E-06
extrinsic apoptotic signaling pathway in the absence of ligand	4	1.99E-06
positive regulation of B cell proliferation	4	2.19E-06
response to abiotic stimulus	9	2.28E-06
positive regulation of fever generation	3	2.48E-06
cell activation	7	8.70E-06
positive regulation of interleukin-6 production	4	1.20E-05
MAPK cascade	5	1.32E-05
regulation of oxidoreductase activity	4	1.94E-05

Table 6 Biological Process (GO) induced by 8h-solar irradiated (SI8*)* *S.* Typhimurium during late infection (24 h p.i.)

Biological Process	Gene Count	P-value
immune response	17	1.36E-21
inflammatory response	13	1.14E-16
response to molecule of bacterial origin	12	5.04E-16
positive regulation of cytokine production	12	8.30E-16
cytokine-mediated signaling pathway	10	1.90E-13
regulation of protein transport	10	2.34E-09
regulation of natural killer cell chemotaxis	4	6.30E-09
lipopolysaccharide-mediated signaling pathway	5	6.38E-09
response to heat	6	6.46E-09
protein kinase B signalling	5	1.44E-08
regulation of localization	13	1.56E-08
positive regulation of calcium ion transport	6	1.56E-08
positive regulation of ion transport	7	1.99E-08
lymphocyte chemotaxis	4	1.07E-07
cell surface receptor signaling pathway	11	1.13E-07
regulation of defense response	8	1.13E-07
positive regulation of leukocyte differentiation	6	1.24E-07
positive regulation of cell proliferation	9	1.50E-07
positive regulation of immune effector process	6	1.51E-07
positive regulation of intracellular signal transduction	9	1.85E-07
positive regulation of JAK-STAT cascade	5	1.88E-07
positive regulation of inflammatory response	5	4.03E-07
regulation of intracellular signal transduction	10	4.03E-07
positive regulation of defense response	6	4.23E-07
positive regulation of acute inflammatory response	4	4.43E-07
positive regulation of lymphocyte migration	4	4.43E-07
positive regulation of lymphocyte activation	6	7.21E-07
cellular response to interferon-gamma	4	7.52E-07
regulation of production of molecular mediator of immune response	5	7.87E-07
regulation of signalling	12	7.87E-07
positive regulation of fever generation	3	2.46E-06
granulocyte chemotaxis	4	3.06E-06
eosinophil chemotaxis	3	3.45E-06
regulation of nitric oxide biosynthetic process	4	3.60E-06
regulation of reactive oxygen species metabolic process	5	4.33E-06
positive regulation of smooth muscle cell proliferation	4	4.84E-06
positive regulation of a homeostatic process	5	5.04E-06
negative regulation of a multi-organism process	5	5.04E-06
regulation of immunoglobulin production	4	5.16E-06

cellular response to interleukin-1	4	8.16E-06
MAPK cascade	5	8.21E-06
cellular response to drug	4	8.62E-06
cellular response to organic substance	9	8.89E-06
T cell migration	3	9.41E-06
positive regulation of cytokine biosynthetic process	4	1.03E-05
positive regulation of cell-cell adhesion	5	1.15E-05
regulation of homeostatic process	6	1.18E-05
positive regulation of monocyte chemotaxis	3	1.18E-05
response to organonitrogen compound	7	1.44E-05
regulation of phosphate metabolic process	9	1.63E-05

5.4 Discussion

In this study, nitric oxide, chemokine, cytokines and growth stimulating factors secreted by RAW 264.7 macrophage cells *in vitro* following their stimulation by LPS, heat attenuated (HA), non-solar (NS) and solar irradiated *S.* Typhimurium for 4 (SI4) and 8 (SI8) hours were assessed. The 4h-solar irradiation was selected because this it represents the time required to eliminate *S.* Typhimurium from infected water. The 8h-solar irradiation time point was selected because this is the normal time SODIS users expose their water samples to solar irradiation. Therefore, these time points were used to assess the effect of SODIS water spiked with S. Typhimuriums to induce iNOS expression and to ascertain the extent of macrophage activation.

5.4.1 Nitric oxide production

The production of NO_2^- in the infected RAW264.7 cells was suppressed in the presence of living *S.* Typhimurium (Fig 35). However, there was increased production of NO_2^- with the heat-chemically inactivated *S.* Typhimurium (Fig. 35). These findings are in agreement with similar studies which showed that chicken macrophage HD11 cells infected with normal *Salmonella enteritidis* suppressed the production of NO_2^- whereas infection by heat-killed *S. enteritidis* (HKSE) stimulated a significant amount of NO_2^- production (He *et al.*, 2011; He *et al.*, 2012). It was suggested that a lack of metabolic activity of the heat killed *S. enteriditis*, perhaps, stimulated NO_2^- production (He *et al.*, 2012). In another study, *S.* Typhimurium and other typhoid causing organisms such as *S.* Enteriditis, *S.* Dublin, and *S.* Choleraesuis down-regulated nitrite ion production in murine macrophages whereas non-typhoidal strains were unable to down-regulate nitrite ion production (Hulme *et al.*, 2012). Live *S.* Typhimurium is known to induce iNOS expression in macrophages via flagellin or Toll-like receptor 5 (TLR 5) and LPS / TLR4 pathways that lead to NF-κβ activation (Andersen-Nissen *et al.*, 2005).

However, the mechanism by which solar irradiated pathogens inhibit NO_2- production is still unclear because they are not viable upon infection and therefore needs further investigation. Nitric oxide synthatase (NOS2 or iNOS) plays an important role in both the innate and adaptive response systems in hosts. The expression of iNOS in macrophages is controlled by microbial products and cytokines, mainly by transcriptional induction. iNOS expression has previously been documented in humans, cows, horses, rats, mice, sheep, goats and chickens. However, in humans iNOS has been observed in patients with infectious and inflammatory diseases. Prolonged production of nitric oxide by iNOS endows macrophages with cytotoxic and cytostatic activity against bacteria, viruses, fungi, helminths, protozoa and tumour cells (MacMicking *et al.*, 1997).

5.4.2 Chemokine expression

The solar irradiated *S.* Typhimurium did not alter the ability of the macrophages to express chemokines, such as MCP-1, RANTES, MIP-1α, MIP-1β, MIP-2, KC and IP-10 (Fig. 36A-G). Chemokines have a crucial role in the innate immune response (Sokol & Luster, 2015). Chemokines are essential for the positioning of innate immune sentinels at mucosal barriers and the recruitment of the first line of innate immune effector cells to sites of infection and inflammation (Sokol & Luster, 2015). The expression of chemokines can also be an advantage to SODIS users because it has been found that chemokine function is also necessary to translate an innate immune response into an adaptive immune response. Innate immune stimuli, through activation of pattern recognition receptors (PRRs), can activate a genetic program that elicits the expression of chemokines from macrophages, and can also modulate the expression of chemokine receptors on dendritic cells (DCs). The induction of chemokine and chemokine receptor expression brings about the movement of antigen-loaded DCs from the tissue into the lymphoid tissue to activate T and B cells to initiate the adaptive immune response (Sokol & Luster, 2015).

Testing of the 7 chemokines used in this study showed that 6 of them including MCP-1, RANTES, MIP-1α, MIP-1β, MIP-2, and IP-10 were highly expressed by macrophages treated with solar and non-solar irradiated *S.* Typhimurium. Ssemakalu *et al.* (2015a) also showed that there were high levels of chemokine expression in dendritic cells (JAWS II) stimulated with solar and non-solar irradiated *V. cholera.* However, the chemokine expression levels in dendritic cells treated with solar irradiated *V. cholerae* were lower than their non-solar irradiated counterparts (Ssemakalu *et al.*, 2015a). Ssemakalu *et al*. (2015a) postulated that the mode of microbial inactivation dramatically influences the nature and magnitude of chemokines and cytokine secretion.

Monocyte chemotactic chemokine (MCP-1) was produced at the highest detectable expression levels in macrophages treated with LPS, non-solar and solar irradiated *S. Typhimurirum*. This observation suggests that solar irradiation did not alter the ability of *S.* Typhimurium to elicit the expression of MCP-1. Chemokines such as MCP-1 are usually secreted in response to signals such as pro-inflammatory cytokines where they play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes (Callewaere *et al.*, 2007). In this study, the macrophages treated with LPS, non-solar irradiated and solar irradiated *S.* Typhimurium showed higher expression levels of pro-inflammatory cytokines (Fig 3) and thus this could be a contributing factor in the high expression of MCP-1 in these samples. The heat attenuated samples showed low expression levels of pro-inflammatory chemokines (Fig. 3) and corresponding low expression levels of MCP-1.

MCP-1 targets the chemotaxis of memory T-cells (Carr *et al.*, 1994). The production of MCP-1 in host cells by solar irradiated pathogens can lead to the chemo attraction of memory cells (Carr *et al.*, 1994). This will stimulate rapid host protection by initiating cognate antigen-mediated activation and direct killing of infected cells (Lauvau & Soudja, 2015). MCP-1 has also been shown to cause apoptosis in macrophages enhancing the cytotoxicity of RAW264.7 macrophages through upregulation of membrane FasL (Wang *et al.*, 2014). Therefore, the high MCP-1 expression levels is perhaps a contributing factor to high cytotoxicity levels observed in RAW264.7 macrophages treated with both solar and non-solar irradiated *S.* Typhimurium *(Chihomvu et al., Unpublished-a).*

The expression pattern for RANTES was similar to that of MCP-1. The macrophages treated with LPS, non-solar and solar irradiated *S*. Typhimurium produced high levels of RANTES whereas, the heat attenuated samples expressed it in low levels. RANTES is a pro-inflammatory chemokine and is usually found at inflammatory sites. It is also closely linked with the function of cytotoxic T lymphocytes (CTL), which are essential in eliminating many intracellular pathogens (Kim *et al.*, 1998a). The ability of solar irradiated pathogens to elicit RANTES production may be beneficial to consumers of SODIS water since RANTES is known to be a chemotactic and activating factor for human eosinophils (Alam *et al.*, 1993). Eosinophils contain a full complement of mediators required to regulate both the innate and adaptive immune responses (Giembycz & Lindsay, 1999; Jacobsen *et al.*, 2007; Jacobsen *et al.*, 2012). Moreover, RANTES can regulate leukocyte activation (such as T cells and macrophages) at high concentrations. The mechanism of this activation is postulated to be through RANTES binding to GAG and subsequent self-aggregation on the cell surface which then leads to non-specific cross-linking of signalling molecules and ultimately to cell activation (Appay *et al.*, 2000).

The high levels of the CC-MIP family of chemokines (MIP-1α, MIP-1β and, MIP-2) induced by both non-solar and solar irradiated *S.* Typhimurium is of particular interest. The expression of MIP-chemokines in macrophages treated with heat attenuated, solar and non-solar irradiated *S.* Typhimurium could have been a result of pro-inflammatory exosomes as shown by Hui *et al.* (2018). Pro-inflammatory exosomes produced by macrophages infected with *S.* Typhimurium contain LPS which lead to TLR4 signalling pathways in macrophages upon release. Moreover, pro-inflammatory exosomes could have been responsible for releasing chemokines such as RANTES, MIP-2, CXCL1, and MCP-1 (Hui *et al.*, 2018) in macrophages treated with LPS, non-solar irradiated and solar irradiated *S.* Typhimurium. This is in contrast to the findings of Ssemakalu et al. (2015a) which showed that the level of chemokines produced in dendritic cells treated with solar irradiated *V. cholerae* was lower than that of non-solar irradiated bacteria. One of the reasons cited for finding higher expression levels of CC-MIP family of chemokines in non-solar irradiated *V. cholerae* is perhaps due to the ability of *V. cholerae* to secrete cholera toxins.

The ability of solar irradiated pathogens to elicit pro-inflammatory chemokines such as keratinocyte chemoattractant (KC or CXCL1) and IP-10, can be of benefit to the SODIS user because the KC chemokine is a powerful chemoattractant that plays a significant role in both the generation of effector T cells (e.g. CD4+, CD8+, Treg cells) and their delivery to sites of tissue inflammation (Dufour *et al.*, 2002). Effector cells play an important role in the immune response to infection because CXCL10 is a chemokine generally associated with a pro-inflammatory response and it induces chemotaxis, apoptosis, cell growth inhibition and angiostasis (Dyer *et al.*, 2009).

5.4.3 Classical activation (Pro-inflammatory response)

In this study, we investigated the profiles of pro-inflammatory cytokines secreted by macrophage cells *in vitro* following their treatment with heat/chemical attenuated, solar and non-solar irradiated bacterial cultures of *S.* Typhimurium. Cytokines are small secreted proteins released by cells that have a specific effect on the interactions and communication between cells. Cytokines can act on cells that secrete them (autocrine action) or on nearby cells (paracrine action) and in some instances on distant cells (endocrine action) (Zhang & An, 2007). In this study, the expression of the following pro-inflammatory cytokines were analysed; IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-9, IL-12P40, IL-12P70, IL-15, IL-17, TNF-α, IFN-γ. Higher expression levels of the pro-inflammatory cytokines were observed after the 24 h post-infection period.

Interleukin-I (IL-I) is a prototypic pro-inflammatory cytokine. There are two forms of IL-1, IL-1α, and IL-1β. IL-I affects nearly every cell type, often together with other pro-inflammatory

cytokines, such as TNF (Dinarello, 1997). Although IL-1 can upregulate host defences and function as an immune adjuvant, IL-1 is a potent pro-inflammatory cytokine. The margin between clinical benefit and unacceptable toxicity in humans is exceedingly narrow (Dinarello, 1997). In this study, the expression of IL-α expressed by macrophages treated with solar and non-solar irradiated *S.* Typhimurium was moderate in comparison to the LPS treated macrophages, and the lowest IL-α was observed in heat attenuated *S.* Typhimurium (Fig.37A). The induction of IL-1α may be beneficial to the immune system of the SODIS consumer. However, the expression profile of IL-1β was not similar to that of IL-1α (Fig. 37B).

In this study IL-2 was expressed in macrophages treated with LPS, non-solar and solar irradiated *S.* Typhimurium (Fig. 37C). The results suggests that solar irradiated *S.* Typhimurium can be of benefit to consumers of SODIS water because IL-2 cytokine plays a crucial role in regulating the adaptive immune response (Smith, 1988), thus long term protection against *S.* Typhimurium is possible.

Previous studies of this cytokine mainly focused on T-cells because T cells are the main cells that express IL-2. However, recent findings have shown that IL-2 can be produced by other cell types (e.g. macrophages) (Malek, 2008).

The non-solar irradiated *S.* Typhimurium produced the highest levels of IL-6 amongst the infected samples. The 8h-solar-irradiated samples induced lower IL-6 when compared to 4h-irradiated samples and this is an indication that the duration of solar irradiation on *S.* Typhimurium does influence the pathogen's ability to elicit the expression of IL-6 in macrophages. However, the heat/chemical attenuated *S.* Typhimurium was not able to induce IL-6 expression. Interleukin-6 is a pleiotropic cytokine produced mainly by monocytes/macrophages (Horii *et al.*, 1988). Interleukin-6 affects adaptive immune responses when it stimulates the differentiation of T-cells (Tormo *et al.*, 2012) and B cells and promotes immunoglobulin production (Hilbert *et al.*, 1989). The results in this study suggest that solar-irradiated pathogens can elicit IL-6 expression during late infection, thus bridging the gap between the innate and adaptive immunity. These results are similar to those produced by Ssemakalu et al. (2015a) who showed that solar irradiated *V. cholerae* expressed lower IL-6 levels than non-solar irradiated *V. cholerae* in dendritic cells.

Interleukin, was produced in small amounts (>10 pg/ml) in all the samples. However, during late infection solar irradiated *S.* Typhimurium did not induce IL-7 production (Fig. 37E). Interleukin-7, like IL-2, is another critical homeostatic cytokine involved in the adaptive immune response that provides signals for T cell survival and proliferation (Bazdar *et al.*, 2015). The production of IL-9 was high during late infection in macrophages treated with LPS, non-solar and solar-irradiated *S.* Typhimurium. However, the heat attenuated *S.* Typhimurium induced

very low levels of IL-9 (Fig. 37F). Interleukin-9 might be of benefit to the SODIS user by down-regulating the inflammatory response (Goswami & Kaplan, 2011).

The IL-12 family of cytokines has been recognised as essential regulators of host immunity (Trinchieri *et al.*, 2003). No IL-12P40 was expressed in all the samples. However, IL-12P70 was expressed in the LPS, NS, SI4, and SI8-treated macrophages. None of the heat attenuated (HA) samples were able to elicit the expression of IL-12P70 (Fig. 37G). This suggests that heat attenuation of *S.* Typhimurium may suppress the expression of IL-12P70, but SODIS treatment does not. Interleukin-12 stimulates the antimicrobial activity of macrophages (Hamza *et al.*, 2010). A critical attribute of IL-12 cytokines is that they mediate their biological actions by binding Janus kinases (JAKs) associated heterodimeric receptors and activating JAK-STAT signalling pathways (Trinchieri *et al.*, 2003). IL-12 also induces naïve CD4+ T cells to differentiate into Th1 cells, a T-helper subset that is implicated in the etiology of some human autoimmune diseases. High levels of IL-12 and Th1 cells are detected in the aqueous and vitreous humour of patients with autoimmune uveitis, suggesting a role for IL-12-induced expansion of Th1 cells in this group of sight-threatening intraocular inflammatory diseases (el-Shabrawi *et al.*, 1998)

Interleukin-15 was produced in macrophages treated with LPS, HA, and NS during early and late infection. However, the macrophages treated with heat attenuated *S.* Typhimurium did not express IL-15 (Fig. 37H). Therefore, solar irradiation does have the ability to prevent IL-15 expression in macrophages. Thus the SODIS user might not experience the benefits associated with IL-15 production such as stimulation of T cell and NK cell proliferation as well as enhancement of B cell expansion and antibody production (Doherty et al., 1996).

IL-17 was produced in low levels in the macrophages treated with LPS, NS and SI4. However, the heat attenuated and 8 h solar irradiated *S.* Typhimurium did not induce IL-17 expression in macrophages (> 6pg/ml) (Fig. 37I). This data suggests that heat treatment and prolonged solar irradiation did affect the organisms' ability to induce IL-17 production in macrophages. Thus, consumers of water treated with SODIS may not obtain the immunological benefits associated with IL-17 production. Interleukin-17 derived from innate and adaptive sources can fight against pathogen invasion at different phases and locations of infection, which may enhance the defensive immune response. It has been previously shown that IL17 signalling in macrophages elicits a new cytokine profile characterized by the production of GM-CSF, IL-3, IL-9, MIP-1β, RANTES and notably IL12p70 (Barin *et al.*, 2012). Another study suggested that IL-17A is involved in the activation of macrophages that are in the process of adopting the different profiles of both the M1 and M2 states (Nakai *et al.*, 2017).

TNF-α is an effective pro-inflammatory cytokine secreted by classically activated macrophages. TNF-α plays a crucial role in cellular differentiation, proliferation and multiple

pro-inflammatory effects (Fujiwara & Kobayashi, 2005). Tumour necrosis factor was highly expressed during late infection in macrophages treated with LPS, NS, SI4, and SI8. The lowest expression of TNF was noted in macrophages that were co-cultured with heat/chemical attenuated *S.* Typhimurium (Fig. 37J). The low production of TNF-α with heat/chemical attenuated *S.* Typhimurium shows that the pathogen was unable to elicit an immunological response. Conversely, solar irradiated cultures of *S.* Typhimurium induced higher levels of TNF-α and this is important in the development of the M1 phenotype of macrophages (Gordon & Martinez, 2010).

5.4.4 Alternative activation (anti-inflammatory response)

The immune system's response to pathogens involves the rapid induction of pro-inflammatory cytokines that initiate an innate response against microbial invasion. However, high levels of pro-inflammatory responses give rise to systemic metabolic and hemodynamic disturbances which are harmful to the host. Therefore, the immune system has developed anti-inflammatory mechanisms which serve to counteract the production of pro-inflammatory cytokines to limit tissue damage and to maintain or restore tissue homeostasis (Moore *et al.*, 2001; Mosser & Zhang, 2008). In general, lower amounts of anti-inflammatory cytokines were produced during early infection than in late infection of the macrophages.

Interleukin-4 is required in macrophages adapting to the M2 phenotype (Murray *et al.*, 2014). It is a multifunctional pleiotropic cytokine mainly produced by activated T cells but also by mast cells, basophils, and eosinophils (Nelms *et al.*, 1999). This could be the reason why very minute amounts of IL-4 was noted in all the samples (<3 pg/ml). It was interesting to find that *S.* Typhimurium that was solar irradiated for 8 h was not able to elicit IL-4 expression during early and late infection (Fig. 38A). Therefore, prolonged solar treatment might alter the ability of *S.* Typhimurium to induce IL-4 expression in macrophages.

IL-5 controls the expression of genes involved in the proliferation, cell survival and maturation and effector functions of B cells and eosinophils. Thus, IL-5 plays an essential role in innate and adaptive immune responses (Kouro & Takatsu, 2009). In this study, IL-5 was secreted in cells infected with LPS, solar-irradiated and non-irradiated pathogens during late infection while the heat attenuated *S.* Typhimurium did not induce the expression of IL-15 in late infection (Fig. 4B). The results show that heat attenuation and solar irradiation negatively impacts the ability of *S.* Typhimurium to induce the IL-5 cytokine in macrophages.

Interleukin 10 (IL-10) is a highly effective anti-inflammatory cytokine that plays a role in preventing inflammatory and autoimmune diseases (Moore *et al.*, 2001; Sabat *et al.*, 2010). IL-10 expression in innate immune cells is mainly induced through pattern recognition receptors (PRRs) that are specific for pathogen-derived products. The PRRs trigger the

expression of target genes that initiate APCs (such as macrophages), phagocytic, anti-microbial and scavenger function as well as stimulate a cascade of signalling events leading to cellular infiltration of the inflammatory milieu to the site of infection or tissue damage (Kawai & Akira, 2010). The increased production of IL-10 by solar irradiated pathogens (especially the 8-h solar irradiated *S.* Typhimurium) during the late infection period (Fig. 38C), may contribute to regulating the pro-inflammatory responses induced by the pathogen (Fig. 640B). Heat attenuation, on the other hand, reduces the capacity of *S.* Typhimurium to produce the expression of this cytokine in macrophages.

Interleukin-13 is a cytokine that has an approximately 25% homology shares many structural characteristics with IL-4 (Zurawski & de Vries, 1994). The significant increase in IL-13 in macrophages treated with solar irradiated pathogens during late infection (Fig. 38D) may play an anti-inflammatory role during late infection because IL-13 inhibits the production of pro-inflammatory cytokines and chemokines by monocytes/macrophages both *in vitro* and *in vivo* (Juha *et al.*, 1998).

Some cytokines responsible for the adaptive immune response such as IL-2, IL-4, IL-5 IL-9, IL-10, IL-13, and IL-17 were expressed by macrophages treated with SI4 and SI8. Therefore, there is a high probability that solar irradiated *S.* Typhimurium can elicit an adaptive immune response in SODIS consumers. The adaptive response usually starts if the innate immune system fails to clear the pathogen (Cologne, 2016). Therefore, avirulent non-replicating solar-irradiated *S.* Typhimurium (Chihomvu *et al.*, Unpublished-a) can prime the adaptive immune so that a more efficient and faster response can occur in the case of an invasive from an *S.* Typhimurium infection of the host.

5.4.5 Growth stimulating factors

Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) can enhance the production of granulocytes or antigen presenting cells (APC) (Mehta *et al.*, 2015). In this study, there was a high amount of GC-SF produced by macrophages treated with LPS, non-solar, and solar irradiated pathogens (Fig. 39). However, a lower amount was produced in HA-treated macrophages compared to the control (LPS). These results probably show that solar irradiating *S.* Typhimurium enhances its ability to produce an immune reaction. However, this was not the case with GM-CSF. Macrophages treated with *S.* Typhimurium (irrespective of treatments) showed low expression levels of GM-CSF. Therefore, the probability exists that *S.* Typhimurium (whether dead or alive) might have mechanisms preventing the expression of GM-CSF in this case.

5.4.6 Hierarchal clustering

To compare the chemokine and cytokine profiles associated with cytokine signalling of macrophages treated with HA, NS, and SI4 and SI8, cluster analysis was performed on all cytokine and pathogen profiles. Early infection (3 h p.i.) showed that innate activation by LPS was not related to other treatments. The solar irradiated samples SI4 and SI8 showed classical activation by high expression of MIP-1α, IP-10, KC, TNF-α, MIP-1β, and MIP-2 during early infection (Figure 40A). On the other hand, the heat attenuated *S.* Typhimurium showed the lowest levels of expression and clustered with the macrophages (Figure 40A). Macrophages treated with non-solar irradiated and solar irradiated *S.* Typhimurium showed high expression levels of chemokines and cytokines clustered with the positive control (LPS-treated macrophages). Moreover, the hierarchal clustering also showed that most cytokines were late response types since the inflammatory response was more significant after 24 h p.i. (Figure 40B).

The variability we found between the two treatments, heat/chemical inactivation and solar irradiation of *S.* Typhimurium*,* most likely reflects the differences in pattern recognition receptors caused by each treatment. Heat/chemical attenuated *S.* Typhimurium induced fewer cytokines and chemokine responses than LPS and solar irradiated pathogens, consistent with previous studies comparing infection of solar irradiated *V. cholera* in dendritic cells (Ssemakalu *et al.*, 2015a).

IFN-γ was not expressed in macrophages treated with *S.* Typhimurium*,* whether the cultures were heat treated (HA), solar irradiated (SI4 and SI8) or non-solar irradiated (NS). However, IFN-γ was produced in LPS treated-macrophages. This evidence suggests that *S.* Typhimurium may be suppressing the expression of IFN-γ. Mutations in IFN-γ induces a greater susceptibility to systemic non-typhoidal *Salmonella* infections (Jouanguy *et al.*, 1999), and the lack of IFN-γ expression may prevent the M1 phenotype in these individuals. The alternative pathway in the absence of IFN-γ usually results in the development of a replication-permissive M2 phenotype (Martinez *et al.*, 2006).

5.4.7 Functional enrichment analysis

The five most highly significant GO biological process terms amongst all 5 samples (i.e., macrophages treated with LPS, heat attenuated, non-solar irradiated and solar irradiated *S.* Typhimurium-treated macrophages) were immune response, positive regulation of cytokine production, cellular response to lipopolysaccharide, inflammatory response and response to molecule of bacterial origin (Tables 2-6).

Another common GO biological process term common in all samples except HA-treated macrophages were ‘positive regulation of the Janus Kinas (JAK)-signal transducer” and activators of transcription (STAT) pathway. These pathways have critical roles in the

regulation of the immune system, especially the fate of T helper cells. T helper cells play a central role in immune regulation. JAKs are associated with cytokine receptors, which are activated upon stimulation, and they phosphorylate STAT proteins, enabling them to be transported to the nucleus. Several regulators, such as protein tyrosine phosphatase (PTPs), suppressors of cytokine signalling (SOCS) and protein inhibitors of activated STATs (PIAS) families have been described to modulate the function of the JAK-STAT pathway (Seif *et al.*, 2017).

It is interesting to note that 8h- solar irradiated *S.* Typhimurium is most likely (high P-values) to positively regulate the following processes: leukocyte differentiation, intracellular signal transduction, lymphocyte migration, fever generation, and immunoglobulin production. These biological processes are all associated with the immune system which suggests that consumption of SODIS treated water may stimulate the innate and adaptive immune response in

in SODIS users. On the other hand, significance levels (P-values) were lower for heat treated *S.* Typhimurium because of the fewer cytokine and chemokines that were differentially expressed.

5.5 Conclusion

This study showed that both non-solar and solar irradiated *S.* Typhimurium suppresses the expression of iNOS in macrophages whereas heat/chemical attenuated *S.* Typhimurium elicited high levels of iNOS. The chemokine and cytokine profiles showed that solar irradiated *S.* Typhimurium may elicit a stronger immune response in macrophages than heat attenuated samples. The immune response elicited by solar inactivated *S.* Typhimurium was almost as strong as the live *S.* Typhimurium. These findings are significant because it has recently been postulated that live *Salmonella* bacterial strains form better vaccines because they elicit a stronger immune response. Thus immunity lasts longer (Lee *et al.*, 2012). Therefore, SODIS consumers can benefit tremendously by drinking SODIS treated-water due to the strong immune response and the fact that immunity against *S.* Typhimurium may last longer. Finally, the profile of cytokines and chemokines secreted by macrophages in response to solar irradiated *S.* Typhimurium is an (M1 phenotype) which results in a pro-inflammatory immune response. Further host cell interactions using a proteomic approach need to be carried out to further substantiate the effect of solar irradiated *S.* Typhimurium on macrophages.

Chapter 6: Solar irradiated *Campylobacter jejuni* induces nitric oxide production, chemokine and cytokine release in RAW264.7 murine macrophages

Abstract

Campylobacter jejuni is the leading cause of gastroenteritis worldwide. It is a waterborne pathogen which is highly susceptible to solar disinfection (SODIS). SODIS of water contaminated with bacteria results in the breakdown of the bacteria and the release of antigenic materials. Therefore, the immunological effects of consuming SODIS-treated water need to be investigated. This study examined the effect of solar irradiated *C. jejuni* on early and late production of nitric oxide (NO), pro-inflammatory cytokines and chemokines in RAW264.7 cells *in vitro*. *Campylobacter jejuni* was suspended in mineral water and exposed to SUVR for 0, 4 and 8 h. Heat/chemical attenuated samples were prepared by using a combination of heat and chemical conditions (0.5% formalin at 60°C for 1 h). Thereafter, the macrophage RAW264.7 cell line was treated with LPS, heat/chemical attenuated, non-solar and solar irradiated *C. jejuni*. After 3 and 24 h, post-infection (p.i.) the nitric oxide was measured using Greiss reagent, and pro-inflammatory cytokines in the tissue culture media from each treatment were quantified using the Luminex 200. The following proteins were analysed: G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES and TNF-α. The results showed that solar irradiation of *C. jejuni* does not affect the iNOS activity of RAW 264.7 macrophages. However, the macrophages treated with both non-solar irradiated and solar irradiated *C. jejuni* showed an immunosuppressive response.

Keywords: *Campylobacter jejuni,* chemokines, cytokines, macrophages, SODIS

6.1 Introduction

The absence of potable water in many developing countries has led people to drink water contaminated with zoonotic pathogens such as *Campylobacter jejuni*, the leading cause of campylobacteriosis (Bartram *et al.*, 2005; Bartholomew *et al.*, 2014). Several home water treatment methods such as filtration, boiling and solar disinfection (SODIS) have been implemented to prevent this untenable situation. Solar disinfection is one of the cheapest and most accessible means of treating water (Borde *et al.*, 2016). Solar disinfection involves filling water into a clear glass or plastic bottle, shaking the bottle for a few seconds to increase the number of reactive oxygen species, and then exposing the bottle to the sun for a period of 6 to 8 hours. Thereafter the water is deemed safe to drink. The spread of Campylobacteriosis can easily be prevented by solar irradiating contaminated water before consumption because *C. jejuni* is highly susceptible to the sun's irradiation. The organism's high sensitivity is due to photo-oxidative damage to the DNA, proteins and membranes (Sinton *et al.*, 2007).

The consumption of solar- disinfected water has been associated with several health benefits such as the reduction of diarrhoeal cases (Conroy *et al.*, 1996; du Preez, 2010). These health benefits have primarily been attributed to the detrimental effects of solar ultraviolet radiation (SUVR) on bacteria. People drinking SODIS water are thought to develop immunity to water-borne pathogens (Ssemakalu *et al.*, 2014). However, the immunological benefits of SODIS water on individuals have not been thoroughly investigated (Ssemakalu *et al.*, 2014). It has been postulated that SODIS treatment of water leads to bacterial cell death and release of antigenic materials such as lipopolysaccharides, glycopeptides and deoxyribonucleic acids (DNA) (Bessler *et al.*, 1997). The presence of such antigenic determinants in the water may initiate an immune response in SODIS users (Ssemakalu *et al.*, 2014).

A previous study that assessed the effect of solar-irradiated cultures of *V. cholerae* on dendritic cells (JAWSII) revealed that the cells produced cytokines and chemokines when infected with solar irradiated *V. cholerae* (Ssemakalu *et al.*, 2015a). It was concluded that the profile pattern of cytokines and chemokines secreted by the dendritic cells in response to solar irradiated *V. cholerae* could probably elicit a Th2 immune response. Another study showed that *Campylobacte*r-infected dendritic cells induced the activation of NF-κB that led to the production of interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, interferon-gamma, and tumour necrosis factor alpha (TNF-α) (Hu *et al.*, 2006). Active bacterial invasion of dendritic cells was not necessary for the induction of these cytokines as heat-killed *C. jejuni* stimulated similar levels of cytokine production as live bacteria (Hu *et al.*, 2006). Considering these findings, the current study focused on assessing the effect of solar irradiated *C. jejuni* on the activation of RAW264.7 murine macrophages.

Macrophages play an essential role in the innate immune system. They are phagocytic cells that can eliminate pathogens and apoptotic cells. Macrophages are also capable of secreting

cytokines. Cytokines are central to the role of macrophages as sentinels of the innate immune system that control the transition from innate to adaptive immunity. Macrophages can be "classically activated," (M1 phenotype) or "alternatively activated (M2 phenotype) (Arango Duque & Descoteaux, 2014). Once activated, the macrophages can produce nitric oxide which can inhibit or kill a variety of microorganisms (Fang, 2004). Inflammation is closely associated with the activation of macrophages: M1 macrophages exert pro-inflammatory activities while M2 macrophages are involved in inflammation resolution. M1 macrophages are pivotal in antigen presentation and secretion of pro-inflammatory cytokines (Benoit *et al.*, 2008).

Although the infection of macrophages by *C. jejuni* has been studied (Siegesmund *et al.*, 2004a; Bouwman *et al.*, 2014) there is no information on the interaction of solar-irradiated *C jejuni* with macrophages. Understanding the interaction between macrophages and solar-irradiated *C. jejuni* may provide some insight into the role of macrophages in regulating the immune system of SODIS users. This study assessed the expression of iNOS by quantifying nitric oxide, and the induction of cytokines, chemokine and growth factors that are involved in both the pro- and anti-inflammatory immune responses in macrophages treated with solar and non-solar irradiated *C. jejuni.*

6.2 Methodology

6.2.1 Bacterial culture

Campylobacter jejuni (ATCC® 33560™) was grown in Mueller-Hinton (M-H) broth (Sigma-Aldrich, Modderfontein, South Africa) and on *Campylobacter* blood-free selective agar supplemented with 1 vial of CCDA selective supplement (Thermo Fischer Scientific, Waltham, MA) at 42°C under microaerophilic atmosphere generated by an Oxoid™ Campygen gas generation kit (Thermo Fischer Scientific).

6.2.2 Preparation of heat-chemically inactivated *C. jejuni* samples.

A single colony of *C. jejuni* was inoculated in Mueller-Hinton (M-H) broth (Sigma-Aldrich). The cells were harvested and re-suspended in sterile mineral water and the optical density of the bacterial suspension was adjusted to 0.1 (approximately 10^7 cells/ml). The bacterial suspension was dispensed into aliquots of 20 ml in empty sterile conical centrifuge tubes. The bacteria were heat/chemically treated in 0.5% formalin and heated at 60°C in a rotatory incubator at 150 rpm for one hour (Baqar *et al.*, 1995).

6.2.3 Exposure of *C. jejuni* to SUVR and enumeration

Cultured *C. jejuni* was harvested by centrifugation at 4 000 xg for 15 minutes and washed thrice with autoclaved still mineral water (Oasis, Vanderbijlpark, South Africa). The pellet was suspended in sterile mineral water up to an OD_{546} of 0.1 (approximately 10^7 cells/ml). Aliquots

of 20 ml of the cell suspension were shaken and exposed to solar-irradiation in 25 cm^3 tissue culture flasks under atmospheric conditions. Similar flasks were exposed to the same atmospheric conditions except for SUVR by enclosing the samples in an opaque ventilated box (Ssemakalu, 2010a). The SODIS experiments were conducted on the roof of the laboratory at the Vaal University of Technology in South Africa (26°42'39.1"S 27°51'46.2"E - 26.710858, 27.862820) from 8.00 am – 4 pm.

6.2.4 Preparation of the macrophage cell line (RAW 264.7)

RAW 264.7 obtained from Separations (Randburg, South Africa) was grown in Dulbecco's Modified Eagles Medium (DMEM), with the addition of 10% foetal bovine serum (FBS) and 1% Penicillin /Streptomycin at 37ºC in a humidified 5% CO_2 incubator. The macrophages were seeded in 48 well plates at a density of $1x10^5$ cells/ml and incubated for 24 h at 37°C in a humidified CO_2 incubator. Thereafter, the macrophages were treated with a) LPS, b) heat/chemical attenuated *C. jejuni*, c) non-solar irradiated and, d) 4h- and 8h-solar irradiated *C. jejuni* in infection media (DMEM media and 10% foetal bovine serum without antibiotics) for 3 hours. Thereafter, the cells were washed thrice with sterile PBS and DMEM media containing 50 µg/ml of Gentamicin to eliminate external bacteria. Macrophages treated with *E. coli* LPS (Sigma-Aldrich) were used as the positive controls, and untreated macrophage cultures were considered as the negative controls.

6.2.5 Spectrophotometric analysis of nitric oxide production

Nitrite production was evaluated as an indicator of *iNOS* activity in the supernatant of RAW 264.7 macrophages that were treated as explained herafter. Briefly, the macrophages were cultured in 48-well plates with 200 µl of culture medium until the cells reached confluence. The media of each well was aspirated and the wells were washed twice with PBS. The cells were separately treated with solar-irradiated *C. jejuni* that were exposed for 4 and 8 h, non-irradiated controls and LPS suspended in phenol-free DMEM media (Thermo Fisher Scientific, Germiston, South Africa). After 3 and 24 h post-infection, the supernatants from each well were collected and centrifuged at 1000 rpm for 5 min. The presence of NO_2- in the supernatant was determined with a commercial NO_2- detection kit (Thermo Fisher Scientific). Nitric oxide was measured by adding 150 µl of cell culture medium to 20 µl of Griess reagent and 130 µl of double deionised water in a 96 well plate and incubated at room temperature for 30 minutes. Thereafter, the optical density was measured at 540 nm in a microplate reader (Biotek, Winooski, VT). The concentrations of NO_2- were calculated by comparison of the absorbance of standard solutions of sodium nitrite prepared in culture medium and assayed under similar conditions (APPENDIX A).

6.2.6 Chemokine, cytokine and growth factor measurement of macrophage supernatants by Luminex assay

Cytokine assays for G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES and TNF-α were performed on the supernatants of the treated macrophages using the Milliplex MAP Mouse Cytokine/ Chemokine Kit (Millipore, Burlington, MA) and a Luminex 200 analyser (Luminex Corporation, Austin, TX). The results were analysed using the Luminex Software xPONENT 3.1 for data acquisition. The Median Fluorescent Intensity data using a 5-parameter logistic or spline curve-fitting method was used for calculating cytokine concentrations in the samples (APPENDIX B).

6.2.7 Statistical analysis

All the experiments for the metabolic assays (Alamar Blue Assay) and NO_2^- production assays were replicated biologically three times. The Luminex assays were carried out in duplicate. Where comparison of the means was made, a student's t-test was performed using Graph Pad Prism 7.0d Software. A two-tailed p value of <0.05 was taken to indicate statistical significance.

6.2.8 Data analysis of chemokine, cytokine and growth factor levels.

Permutation analysis was carried out to compare chemokine, cytokine and growth factor levels in macrophages treated with a) LPS, b) heat/chemical attenuated, c) non-solar, and d) solar irradiated *C. jejuni*. Where readings were below the threshold, the lowest detectable level was assigned and where sample concentrations were above the range available for analysis, they were assigned the upper limit value of the range. Pearson's correlation was used to assess any relationship between protein expression levels and duration of solar irradiation on samples during early (3 h post infection) and late infection (24 h post infection) using GraphPad Prism 7.0d Software. Hierarchical clustering and heat map construction was performed with CIMminer (Bethesda, MD) using Euclidean distance to cluster both samples and protein factors (Clark *et al.*, 2015)**.**

6.2.9 A network of potential protein interactions

Using the STRING 10.5 database (Swiss Institute of Bioinformatics, Lausanne, Switzerland), networks of possible protein interactions were created using the inflammatory proteins that were found to be significantly raised in the hierarchical clustering from the analysis and were expanded to include downstream targets (Clark *et al.*, 2015).

6.3 Results

6.3.1 Nitric oxide production

Bacterial cultures that were solar irradiated for 4 and 8 hours or inactivated through a combination of heat and chemical means were not culturable on Campycount and Chocolate agar and were considered metabolically inactive (P. Chihomvu *et al., Unpublished*). Figure 42 shows the nitrite generation by RAW 264.7 cells that were treated as follows: a) media only **(M)**, b) lipopolysaccharide (LPS) of *Escherichia coli*, c) heat/chemical attenuated (i.e., 60°C for 1 hour, 5% formalin)**(HA)** d) non-solar irradiated *C. jejuni* **(NS)** and, e) *C. jejuni* solar-irradiated for 4 and 8 hours and **(SI4** and **SI8)**. Nitric oxide was not produced in macrophages treated with heat attenuated (HA) and for both periods of solar irradiated *C. jejuni* (SI4 & SI8). Nitric oxide production was only detected in LPS treated controls (0.68 ± 0.73 μM) and NS (0.17 ± 0.26 μM). After 24 hours post-infection period (24 h p.i.) a slight increase in NO_2^- production was noted in the negative control (M) (1.65 ± 0.8 μM), the LPS treated positive control (2.40±1.26 μM) and the heat attenuated controls (2.09 ± 0.40 μM). A highly statistically significant increase ($p<0.001$) of NO_2^- production was noted in macrophages treated with non-solar and solar irradiated *C. jejuni* for 4 and 8 h (NS, SI4 and SI8, respectively). However, the 8-h solar irradiated ones induced lower NO_2^- production when compared to the non-solar and 4h-solar irradiated *C. jejuni*.

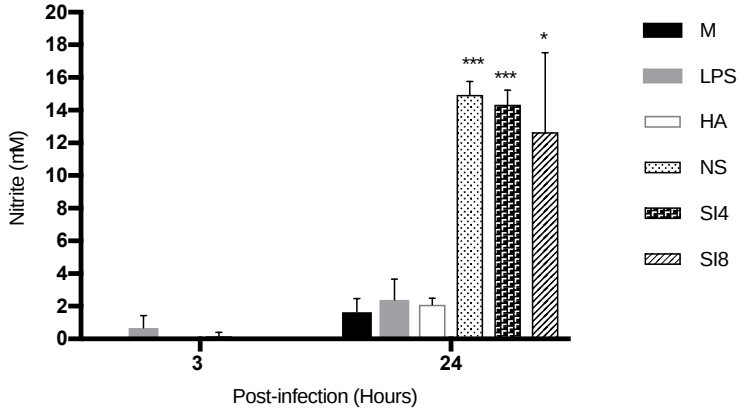


Figure 42 Nitrite generation of macrophages infected with *C. jejuni* that were treated according to the following conditions: (a) heat and chemical treatment (HA), (b) non-solar irradiated C. jejuni (NS), and (c) *C. jejuni* solar irradiated for 4 (SI4) and 8 (SI8) h. Error bars indicate standard error of the mean of triplicate biological replicates. Significant differences between the treatments are shown on the graph where a p-value < 0.05 was considered significant *$p < 0.05$ and ***$p < 0.001$.

6.3.2 Chemokine and cytokine production

Multiplex chemokine and cytokine assays were used to investigate the quantitative differences in the expression levels of cytokines and chemokines produced by RAW264.7 murine macrophages. Upon stimulation with LPS and the different treatments of *C. jejuni* the production of some of the chemokines and cytokines was up-regulated in the macrophages. The stimulatory patterns revealing quantitative differences in specific chemokine, cytokine and growth factors expression levels between the samples are shown in Figs. 43A-G, Figs. 44A-I, Figs. 45A-D and 46A-B, respectively. Basically, the macrophages produced many pro-inflammatory mediators characterized by significantly ($P \leq 0.05$) higher expression of a large number of chemokines (MIP-2, MIP-β, MIP-1α, MCP-1 and IP-10) and a fewer cytokines (IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12P40, IL-12P70, IL-13 and IL-17).

6.3.3 Chemokine expression

The expression of chemokines MCP-1, RANTES, MIP-1α, MIP-1β, MIP-2, KC, and IP-10 by macrophages during early (3 h) and late (24h) infection was assessed and is shown in Fig. 43. The macrophages treated with LPS, heat attenuated, non-solar and solar irradiated *C. jejuni* induced unusually high amounts of MIP-1α, MIP-1β, and MIP-2 and lower expression levels of MCP-1, RANTES, KC, and IP-10 (Fig 43).

The highest amount of MCP-1 was detected in LPS treated macrophages with values exceeding the upper detection limit of 12,445 ng/ml during both early and late infection (Fig. 43A). At 3 h p.i., MCP-1 was undetectable in macrophages treated with heat attenuated *C. jejuni* (HA). The non-solar (NS) and solar-irradiated samples of *C. jejuni* (SI4 and SI8) were able to elicit MCP-1 expression during early infection. After 24 hours, post-infection, the amount of MCP-1 for macrophages infected with heat attenuated *C. jejuni* (HA) increased from 10.43 ± 14.75 pg/ml to 2870 ± 980.94 pg/ml. The macrophages infected with solar-irradiated samples (SI4 and SI8) showed significant increases ($p<0.01$ and $p=0.05$, respectively) from 634.28 ± 66.751 pg/ml to 1,582.00 ± 16.97 pg/ml and from 861 ± 120.14 pg/ml to 1915.00 ± 315.37 pg/ml, respectively. The amount of MCP-1 in macrophages treated with non-solar irradiated *C. jejuni* increased from 948.47±14.75 pg/ml to 1492.50±183.14 pg/ml.

There was a significant increase ($p<0.01$) in RANTES production in macrophages treated with LPS (316.99 ± 5.58) during early infection. Minimal expression of RANTES was noted for HA (1.59 ± 0.00 pg/ml). Macrophages treated with non-solar irradiated *C. jejuni* (NS) produced 81.30 ± 3.23 pg/ml, whereas solar irradiated SI4 and SI8 samples produced 43.59 ± 7.19 pg/ml and 73.22 ± 16.21 pg/ml, respectively. During late infection, there was a slight decrease in RANTES production for NS (31.35 ± 3.23 pg/ml), SI4 (32.53 ±2.77 pg/ml), and SI8 (35.71 ± 2.50 pg/ml) during late infection when compared to early infection (Fig. 43B). Conversely, a

slight increase in RANTES production was noted in LPS-treated macrophages (400.00 ± 0.00 pg/ml).

During early infection, high levels of MIP-1α above the detectable limit of 12,790 pg/ml was observed, except for the heat/chemical treated *C. jejuni* (HA). However, after 24 hours post-infection HA induced high MIP-1α (Fig. 43C). High levels of MIP-1β was also noted in untreated macrophages (M) (1,175 ± 124.45 pg/ml), LPS (1,137.00 ± 8.49), the NS and SI4 were above the detectable limit of 1,263 pg/ml and SI8 (1,255 ± 222.03 pg/ml) (Fig. 43D). However, the heat attenuated samples induced a low production of MIP-1β (161.96 ± 10.46 pg/ml) in the macrophages. After 24 hours post-infection, the amount of MIP-1β in HA, NS and SI4 were above the detectable limit of 1263 pg/ml. The lowest amount of MIP-1β was noted in the untreated macrophages (1140 ± 173.95 pg/ml) and those treated with LPS (1227.00 ± 8.49 pg/ml).

During the early infection, significant increases in MIP-2 production in comparison to the non-treated macrophages ($p<0.01$) were found in all the samples except for the HA treatment. MIP-2 expression exceeded the upper threshold of 15,230 pg/ml in LPS, NS, SI4, and S18 samples. The least amount of MIP-2 was produced in macrophages treated with HA (78.18 ± 96.21 pg/ml). However, at 24 h p.i., a significant increase ($p<0.01$) of MIP-2 production was noted in HA, with values exceeding the upper threshold of 15230 pg/ml (Fig. 43E). A decrease in MIP-2 production was noted in treatments involving LPS (11,196.00 ± 5837 pg/ml), NS (6,960.00 ± 2182.13 pg/ml), SI4 (7,270.00 ± 630.74 pg/ml) and SI8 (10,150.00 ± 485.08 pg/ml) (Fig. 43E).

During early infection, there were notable increases ($p<0.01$) in KC production in LPS, NS, SI4, and SI8-treated macrophages. The highest expression of KC was observed in LPS-treated macrophages (95.70 ± 5.46 pg/ml) whereas KC production in HA-treated macrophages was below the detectable limit of 2.65 pg/ml. Macrophages treated with SI4 (47.08 ± 7.45 pg/ml; $p<0.01$) and SI8 (86.23 ± 2.18 pg/ml; $p<0.01$) also produced a considerable amount of KC. However, after 24 h p.i., there no detectable production of KC in NS, SI4, and SI8 was observed. However, higher amounts of KC were noted for the LPS treated samples (144.11 ± 9.81 pg/ml). A slight increase in KC production was observed in macrophages treated with HA (10.44 ± 3.17 pg/ml) (Fig. 43F).

Finally, there were significant increases ($P<0.01$) in IP-10 production in the treatments involving LPS, NS, SI4 and SI8 during early infection compared to the non-treated macrophages. A marked decrease in IP-10 expression in the samples mentioned above was noted 24 h p.i. Conversely, IP-10 production in LPS stimulated macrophages continued to increase above the detectable limit of 11,152 pg/ml (Fig. 43G).

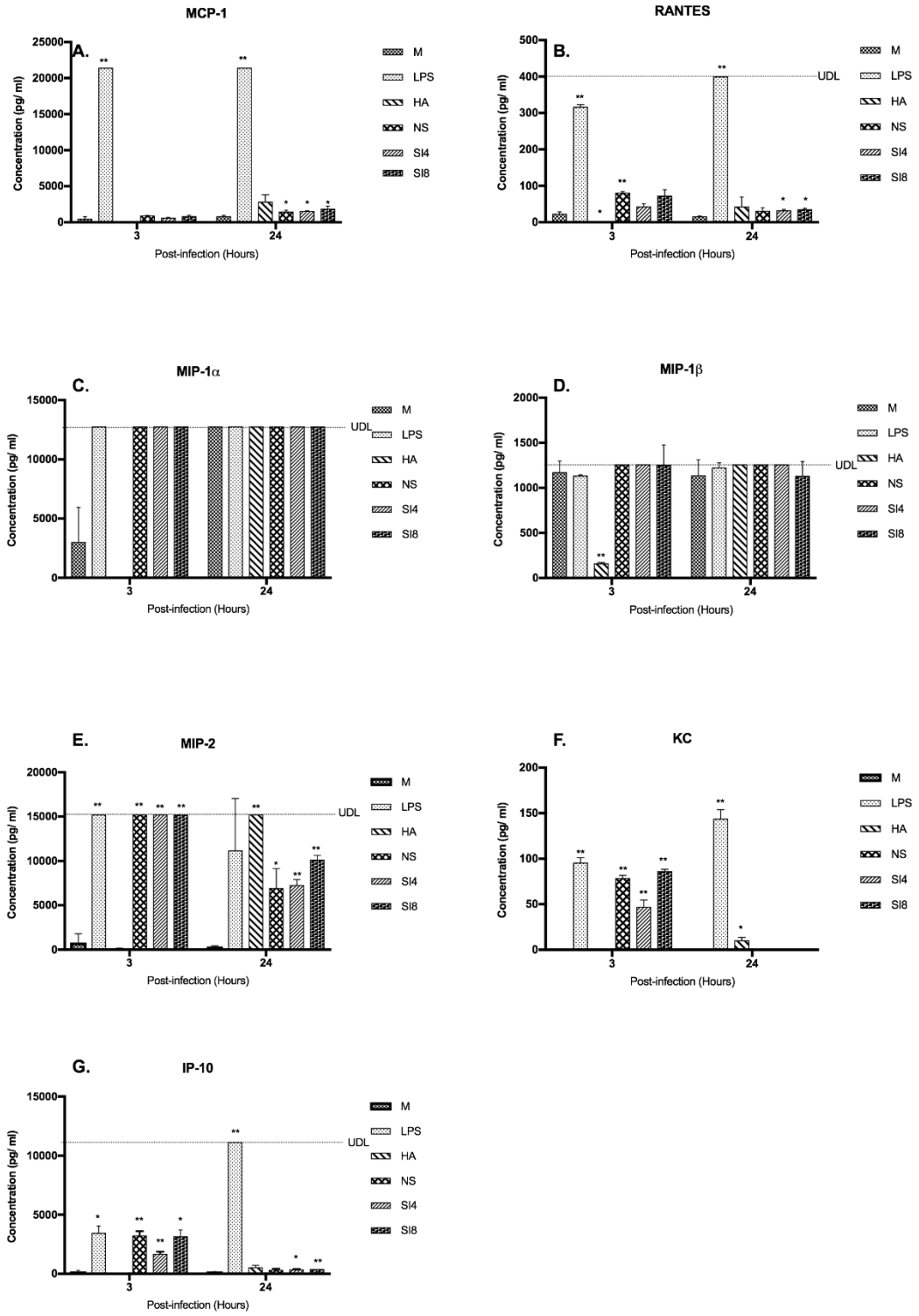


Figure 43 Chemokine levels assessed by Luminex multiplex array in RAW264.7 macrophages treated with LPS, heated attenuated (HA), non-solar irradiated (NS) and solar irradiated (SI4 and SI8) C. jejuni. A) monocyte chemoattractant protein (MCP-1), B) regulated on activation, normal T cell expressed and secreted (RANTES), C) macrophage inflammatory protein (MIP-1α), D) MIP-1β, E) MIP-2, F) keratinocyte chemoattractant (KC) and, G) interferon gamma-induced protein (IP-10) Error bars indicate standard error of the mean of 2 replicates. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper detection limits (UDL) are represented on the graphs.

6.3.4 Cytokine expression

6.3.4.1 Classical activation (Pro-inflammatory response)

Cytokines, namely, IL-1α, IL-1β, IL-6, IL-7, IL-12P40, IL-12P70, IL-15, IL-17, and TNF-α responsible for the pro-inflammatory response were assayed during early and late infection periods. The results are shown in Figure 44. Generally, very low IL- 1α expression was observed in the macrophages treated with heat/chemically treated, non-solar and solar irradiated *C. jejuni.* The macrophages treated with LPS (the positive controls) had the highest expression levels of pro-inflammatory cytokines. Moreover, higher expression levels were noted in the pro-inflammatory cytokines expression during early infection (3 h p.i.) than the late infection period (24 h p.i.).

Macrophages infected with HA, NS, SI4, and SI8 did not induce the production of IL-1α during both infection periods. However, a significant increase ($p=0.02$ and $p=0.01$, respectively) was noted in LPS treated samples during early and late infection (Fig. 44A). There was a two-fold increase in IL-1α production that increased from 94.53 ± 16.95 pg/ml to 179.48 ± 23.78 pg/ml in LPS-treated macrophages during late infection.

Macrophages stimulated with LPS expressed the highest levels of IL-β (81.67 ± 54.94 pg/ml) at 3 h p.i. whereas the least IL-1β expression (8.83 pg/ml) was below the detectable limit in SI8-treated macrophages. After 24 h p.i., a reduction in IL-1β expression was noted in the LPS, HA, NS and, SI4-treated macrophages. However, IL-1β expression increased in the SI8 sample (Fig. 44B).

There was minimal production of IL-2 in all the samples (<10 pg/ml). However, during early infection there was a significant increase ($p<0.01$) in IL-2 production in the LPS-treated macrophages compared to the untreated ones. However, the rest of the samples had values below the detectable limit of 1.68 pg/ml (Fig. 44C).

Expression of IL-6 by macrophages treated with NS, SI4, and SI8 increased during early infection (Table 7). After 3 h p.i., the highest significant increase ($p<0.01$) was noted in macrophages treated with LPS (>14,725 pg/ml), and lowest IL-6 production was observed in macrophages treated with heat attenuated *C. jejuni* (HA) (<3.22 pg/ml). There was a statistically significant difference ($p=0.02$) in IL-6 production between the non-solar irradiated sample (NS) and the sample that was solar irradiated for 4 hours (SI4). There was also a statistical difference ($p=0.04$) in IL-6 production between those treated for 4 and 8 hours. After 24 h p.i., a marked decrease in IL-6 was recorded in all the samples except for those treated with LPS (Table 7). The data was presented in table form because the range of the values obtained for IL-6 was too wide.

Table 7 Concentration (mean ± SEM) of IL-6 expressed by macrophages treated with LPS, heat/ chemical attenuated and solar irradiated *C. jejuni*

Treatment	Post-infection (Hours)	
	3	24
Negative control (untreated macrophages)	2.78 ± 3.93 pg/ml	4.74 ± 6.70 pg/ml
Positive control (LPS treated control)	>14 725 pg/ml[a**]	>14 725 pg/ml[a**]
Heat/ chemical treated (HA)	<3.22 pg/ml[b]	<3.22 pg/ml[b]
4h-solar irradiation (SI4)	62.70 ± 4.21 pg/ml[**]	4.74 ± 6.70 pg/ml
8h-solar irradiation (SI8)	121.33 ± 15.42 pg/ml	<3.22 pg/ml[b]

Notes: Please note [a] represents the upper detectable limit and [b] is the lower detectable limit.

Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. Heat/chemical inactivation refers to *C. jejuni* culture treated with a combination of heat (60°C) and 0.5 % formalin.

During early infection, the highest amount (14.99 ± 5.58pg/ml) of IL-7 was expressed in macrophages treated with solar radiation for 4 h. There was no significant difference in the level of IL-7 produced in the treated and untreated macrophages. The least, IL-7 expression was observed in macrophages treated with HA (<2.76 pg/ml). After 24 h p.i. the expression of IL-7 decreased in all the samples except for NS treated samples (5.92 ± 1.53 pg/ml) and the macrophages treated with HA, SI4, and SI8 expressed negligible amounts of IL-7 (<2.76 pg/ml) (Fig. 44D).

After 3 h p.i., there was an increase in IL-9 production in all samples. The highest IL-9 production was observed in macrophages treated with LPS (136.76 ± 48.28 pg/ml), and the lowest amount of IL-9 was found in macrophages treated with SI4 (24.60 ± 34.80 pg/ml). The only statistically significant difference in comparison to untreated macrophages was noted in the SI8 samples (66.41 ± 7.86 pg/ml). After 24 h p.i., the highest increase in IL-9 was recorded in LPS treated samples (153.09 ± 51.44 pg/ml). However, IL-9 expression in macrophages treated with HA was below detectable limit (<2.49 pg/ml) (Fig. 44E).

No IL-12P40 was produced in all the samples during the early and late infection periods (not shown in the figure). However, minimal amounts of IL-12P70 were produced in all the samples ranging from 0 to 15 pg/ml at 3 and 24 h p.i. During early infection, the highest IL-12P70 was expressed in the LPS-treated macrophages (7.62 ± 4.62 pg/ml). The IL-12P70 produced in macrophages treated with NS, SI4, and SI8 was below the detectable limit of 3.03 pg/ml.

However, after 24 h p.i., no IL-12P70 detected in HA and the SI8 samples. However, IL-12P70 was noted in macrophages treated with LPS (11.99 ± 2.59 pg/ml) and SI4 (6.08 ± 2.85 pg/ml) (Fig. 44F).

With regards to IL-15, the highest significant increase ($p=0.04$) in expression was noted in macrophages treated with LPS (91.88 ± 8.14 pg/ml) after 3 hours. The IL-15 expression in macrophages treated with HA and SI8 was below the detectable limit of 62.97 pg/ml. However, the HA sample showed a significant increase ($p<0.01$) at 24 h p.i. when compared to untreated macrophages. A slight decrease in IL-15 expression was noted in macrophages treated with NS (80.25 ± 8.28 pg/ml) and SI4 (71.44 ± 12.49 pg/ml) whereas, IL-15 expression was below the detectable limit of 62.97 pg/ml in SI8-treated macrophages (Fig. 44G).

Very negligible amounts of IL-17 were produced in all the samples. During early infection, the highest value of 5.46 ± 2.28 pg/ml was recorded in LPS treated macrophages. The IL-17 expression for HA-treated macrophages during both early and late infection periods was below the detectable limits of 2.87 pg/ml. After 24 h p.i., a slight increase in IL-17 production (5.62 ± 0.56 pg/ml) in LPS-treated macrophage, whereas, the expression of IL-17 in SI8-treated macrophages was below the detectable limit of 2.87 pg/ml (Fig. 44H).

There was a significant increase ($p<0.01$) in TNF-α production in macrophages treated with LPS (332.25 ± 5.93 pg/ml), NS (363.93 ± 8.04), SI4 (>518.77 pg/ml), and SI8 (385.01 ± 10.18 pg/ml). The least significant increase ($p=0.02$) in TNF-α expression was observed in HA-treated macrophages (15.71 ± 3.47 pg/ml). However, during late infection, the expression of TNF-α of HA-treated macrophages increased to 403.27 ± 144.29 pg/ml. A decrease in TNF-α production was observed in macrophages treated with NS (291.99 ± 50.03 pg/ml), SI4 (242.25 ± 30.02 pg/ml) and SI8 (234.45 ± 13.20) (Fig. 44I).

No IFN-γ was found in all samples except in the LPS-treated macrophages (2.6 ±0.09 pg/ml) at 3 h p.i. During late infection no IFN-γ was produced in all the samples (not shown in the figure).

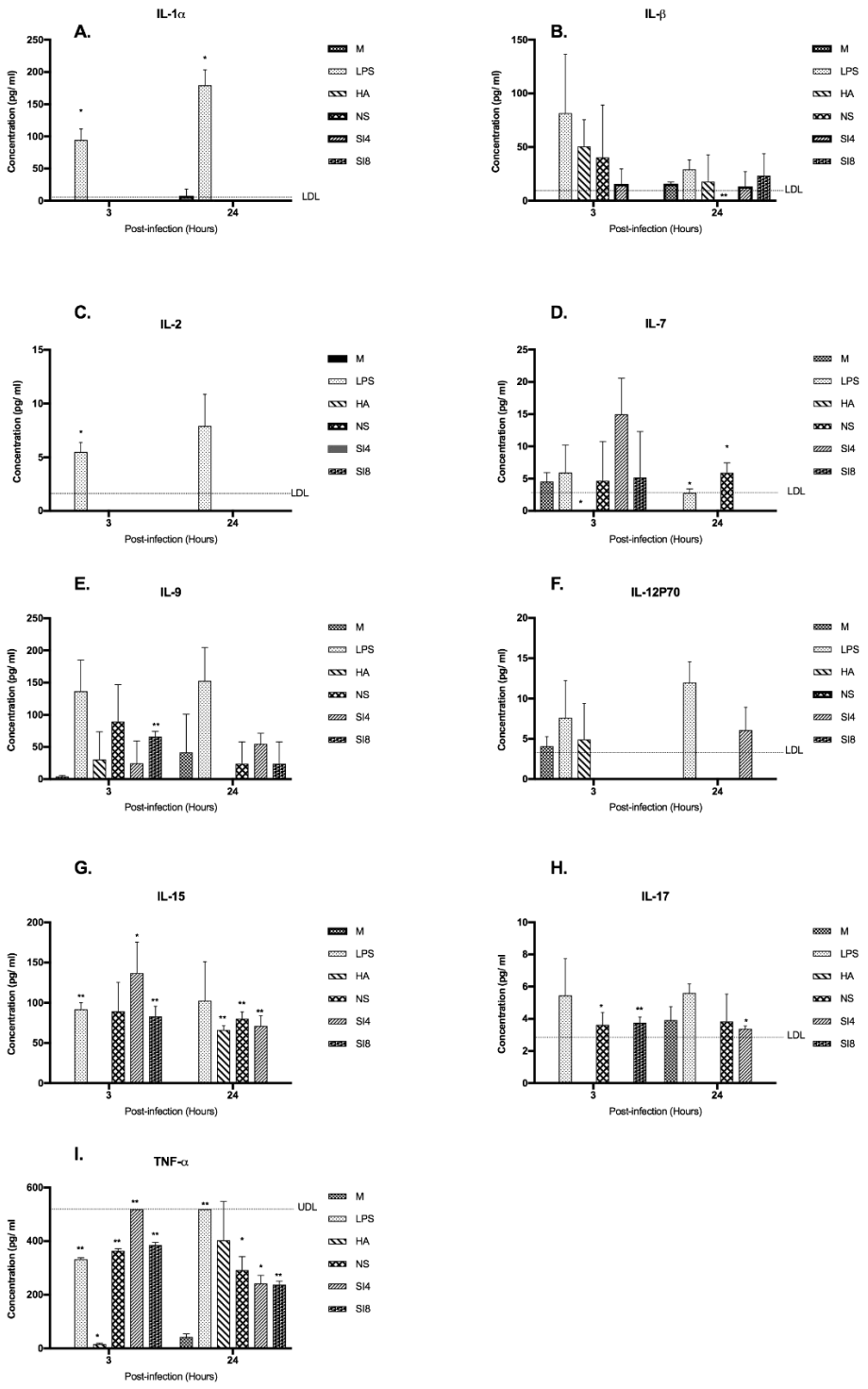


Figure 44 Pro-inflammatory cytokines assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA) and, non-solar irradiated *C. jejuni* (SI4 and SI8). A) Interleukin-1α (IL-1α), B) IL-1β, C) IL-2 D) IL-7, E) IL-9, F) IL-12P70, G) IL-15, H) IL-17 and, I) tumour necrosis factor-α (TNF-α). Error bars indicate standard error of the mean of 2 replicates. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper and lower detection limits (UDL and LDL) are represented on the graphs.

6.3.4.2 Alternative activation of macrophages

The expression of cytokines, IL-4, IL-5, IL-10, and IL-13 that are responsible for the anti-inflammatory response was assayed at 3 and 24 h p.i. In general, a lower amount of cytokines involved in the anti-inflammatory response was produced in all the treatments (Fig. 45A-D). Small quantities of IL-4 were observed in all the samples (<3 pg/ml) during early and late infection. There was a significant ($p<0.01$) difference in the means for IL-4 expression in LPS-treated macrophages (1.35 ± 0.19 pg/ml) and NS-treated macrophages (1.39 ± 0.13 pg/ml) in comparison to the control. The expression of IL-4 in the rest of the samples was below the detectable limit of 0.58 pg/ml. However, after 24 h p.i., IL-4 production was recorded in LPS (1.05 ± 0.49 pg/ml) and HA-(0.78 ± 0.61 pg/ml) treated macrophages (Fig. 45A).

At 3 h p.i. the expression of IL-5 production was highest in NS-treated macrophages (30.34 ± 12.03 pg/ml). However, the increase was non-significant relative to the non-treated macrophages whereas macrophages treated with SI4 and SI8 expressed undetectable amounts of IL-5 (<4.14 pg/ml). However, after 24 h p.i., there was an increase in IL-5 production in LPS (42.10 ± 6.69 pg/ml), while the NS-treated macrophages showed the least IL-5 expression (Fig. 45B).

During early infection, the highest amount of IL-10 was expressed in the LPS-treated macrophages (9.90 ± 5.11 pg/ml), whereas IL-10 expression in HA-treated macrophages was below the detectable limits of 3.53 pg/ml. After 24 h p.i., there was a marked increase in IL-10 production in LPS-treated macrophages (17.11 ± 2.23 pg/ml) and NS samples and the least IL-10 expression was noted in HA-treated macrophages (Fig. 45C).

A significant increase in IL-13 expression was observed in LPS-treated macrophages (30.01 ± 1.10 pg/ml) during early infection (3 h p.i.) whereas IL-13 production was below detectable limits (<1.66 pg/ml) in SI4-treated macrophages at 3 h p.i. After 24 h p.i. the highest significant increase ($p<0.01$) in IL-13 expression was noted in LPS-treated macrophages (40.38 ± 3.47 pg/ml). IL-13 expression was below detectable limits in HA-treated macrophages (Fig. 45D).

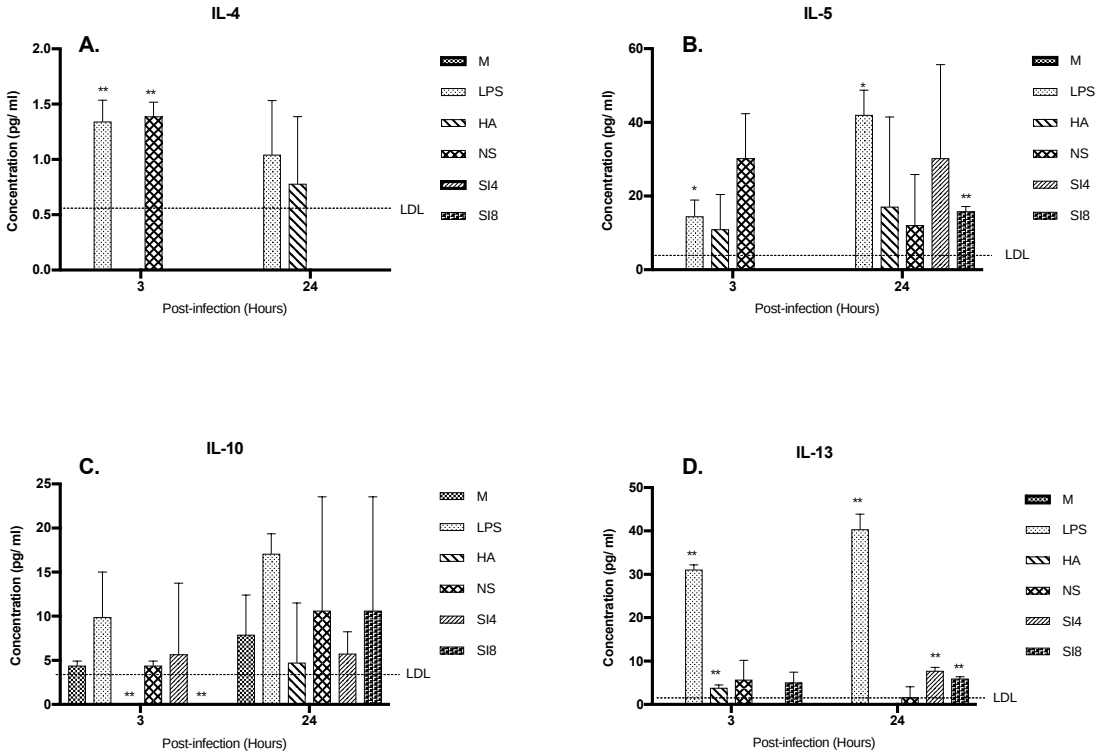


Figure 45 Anti-inflammatory cytokines assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated attenuated (HA) and, non-solar irradiated *C. jejuni* (SI4 and SI8). A) interleukin-4 (IL-4), B) IL-5, C) IL-10 and, D) IL-13. Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The lower detection limits (LDL) are represented on the graphs.

6.3.5 Growth factors

There was minimal production of GCSF and GM-CSF during early and late infection in all the samples except for the LPS treated macrophages which produced very high amounts of growth factors (Figs. 46A and 46B).

During early infection, the highest significant increase in GCSF was noted in LPS treated macrophages (>11,097 pg/ml). However, GCSF expression was below detectable limits of 0.46 pg/ml in HA-treated macrophages. After 24 h p.i., a non-significant increase in GCSF production was observed in HA-treated macrophages (6010.00 ± 4973.79 pg/ml).

At 3 h p.i., the highest increase in GM-CSF production was noted in LPS treated macrophages (147.92 ± 5.92 pg/ml) whereas, GM-CSF was below detectable limits of 26.64 pg/ml in SI4 treated macrophages. After 24 h p.i., a significant increase in GM-CSF production was noted in LPS treated macrophages (1849.50 ± 307. 59 pg/ml) and GM-CSF expression was below detectable limits in HA-treated macrophages. The non-solar irradiated (NS) and solar

irradiated *C. jejuni* (SI4 and SI4), induced low levels of GM-CSF when compared to the LPS treated samples.

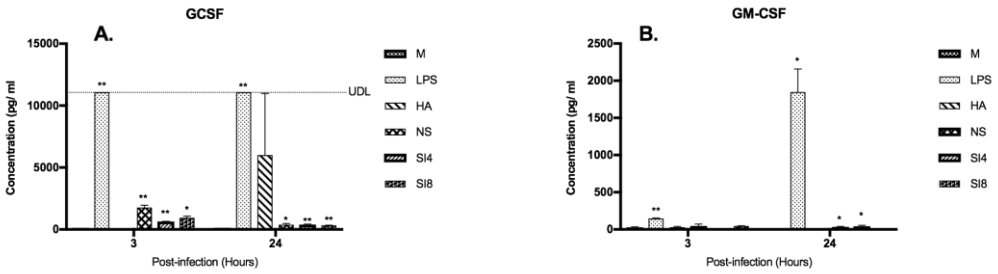


Figure 46 Growth factors assessed by Luminex multiplex array in RAW264.7 macrophages treated with (LPS), heated-attenuated (HA) and, non-solar irradiated *C. jejuni* (SI4 and SI8). A) granulocyte-colony-stimulating factor (GCSF) and, B) granulocyte-macrophage-colony-stimulating factor (GM-CSF). Significant differences are presented as *P < 0.05, **P < 0.01 obtained by student t-test in comparison to the untreated macrophage cells. The upper detection limits (UDL) are represented on the graphs.

6.3.6 Hierarchal clustering

During early infection, 3 significant clusters based on the treatment of the macrophages were observed (Fig. 47A). The first and second clusters consisted of LPS alone and HA and M, respectively. However, the third cluster had two branches; NS on its own and SI4 and SI8 (Figure 47A). During early infection, the LPS-treated macrophages cluster showed high expression levels of MIP-1β, IL-5, TNF-α, IP-10, KC, MIP-1α, MIP-2, IL-7, IL-9, IL-4, and IFN-G, IL-5. IL-10, MCP-1, IL-6, IL-1α, IL-2, GCSF, GM-CSF, IL-13, IL-17, RANTES, IL-1β and, IL-12P70 (Fig. 6A). The macrophages treated with heat attenuated *C. jejuni* (HA) showed the lowest chemokine and cytokine expression (Fig. 47A), with IL-1, IL-12P70 and IL-5 being moderately expressed in comparison to the LPS-treated macrophages. The macrophages treated with non-solar irradiated *C. jejuni* showed slightly higher expression levels of proteins when compared to the solar irradiated *C. jejuni.* The following proteins were highly expressed; MIP-1b, IL-15, TNF-A, IP-10, KC, MIP-1A, MIP-2, Il-7, IL-9, IL-4, IFN-g and, IL-5. The cluster formed by solar irradiated *C. jejuni* (SI4 and SI8) consisted of the following common cytokines and chemokines; MIP-1β, IL-15, TNF- α, IP-10, KC, MIP-1A and, MIP-2. The highly expressed proteins during early infection were used to assess the potential interactions of chemokines and cytokines (Fig 48A-D).

After 24 h p.i., there was an increase in the expression of the chemokines MIP-1α and MIP-1β in macrophages stimulated with M, HA, NS and SI4. However, the following chemokines and cytokines were down-regulated; MIP-2, KC, IP-10, IL-15, and TNF-α in NS, SI4 and SI8

samples. The heat attenuated *C. jejuni* (HA) induced an increased expression of IL-4, IL-5, IL-7, IL-15, MIP-1α, MIP-1β, MIP-2, and GCSF. Interleukin-1β, IL-6, GM-CSF and, IFN-γ were quiescent in all the samples except the LPS treated samples; There were high amounts of chemokines in cytokines in LPS treated samples except for IL-7 and IL-12P40. (Fig. 47B).

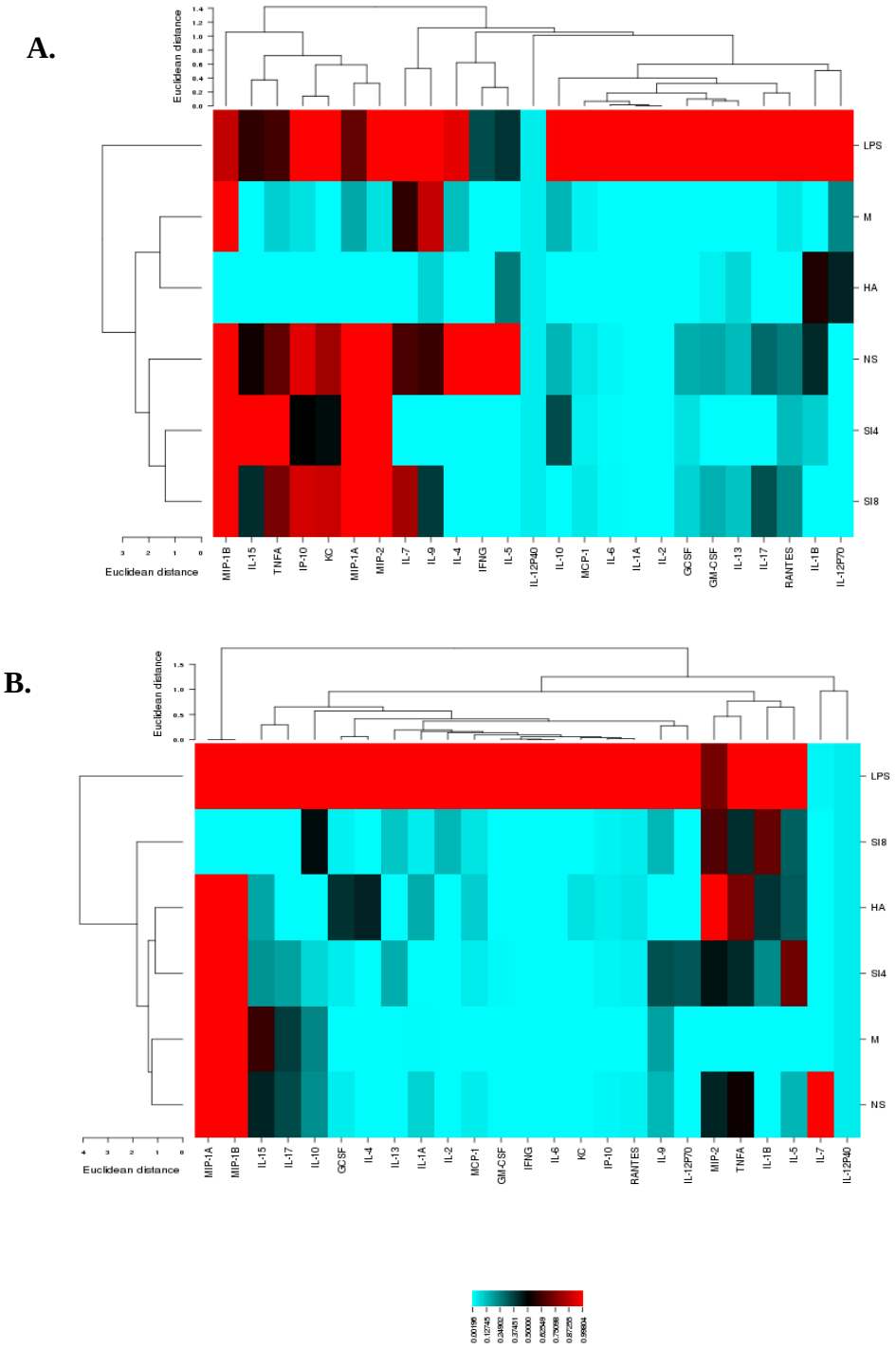


Figure 47 Hierarchal clustering of RAW264.7 macrophages treated with (LPS), heated attenuated (HA) and, non-solar irradiated *C. jejuni* (SI4 and SI8 during A) Early infection (3 h p.i.) and B) Late infection (24 h (p.i.)

6.3.7 Network analysis

A network of potential protein interactions was constructed using the STRING 10.5 database (Fig. 48). The cluster of highly expressed proteins observed during early infection (Fig. 6A) was used to develop the networks for proteins highly expressed in macrophages treated with LPS, heat/chemical attenuated, non-solar irradiated and solar irradiated *C. jejuni* (Fig 48).

A network of potential protein interactions for expressed proteins in macrophages treated with LPS-treated macrophages included NOS2 (the protein coding for enzyme Nitric Oxide Synthase 2) because the macrophages treated with LPS produced NO during early infection (Fig. 42). The proteins expressed were characterised by both pro-inflammatory and anti-inflammatory protein interactions. However, CLEC11A (GCSF) appeared to have no interactions with the other proteins (Fig. 48A). The networks from the heat attenuated treated macrophages showed triangular interaction between IL-1β, IL-5 and IL-15 (Fig. 48B).

The networks from the solar irradiated *C. jejuni* (SI4 and SI8) were mainly characterised by pro-inflammatory protein interactions, with MIP-2 (CXCL-2) occupying a central node with multiple downstream protein interactions. (Fig 48C, 48D).

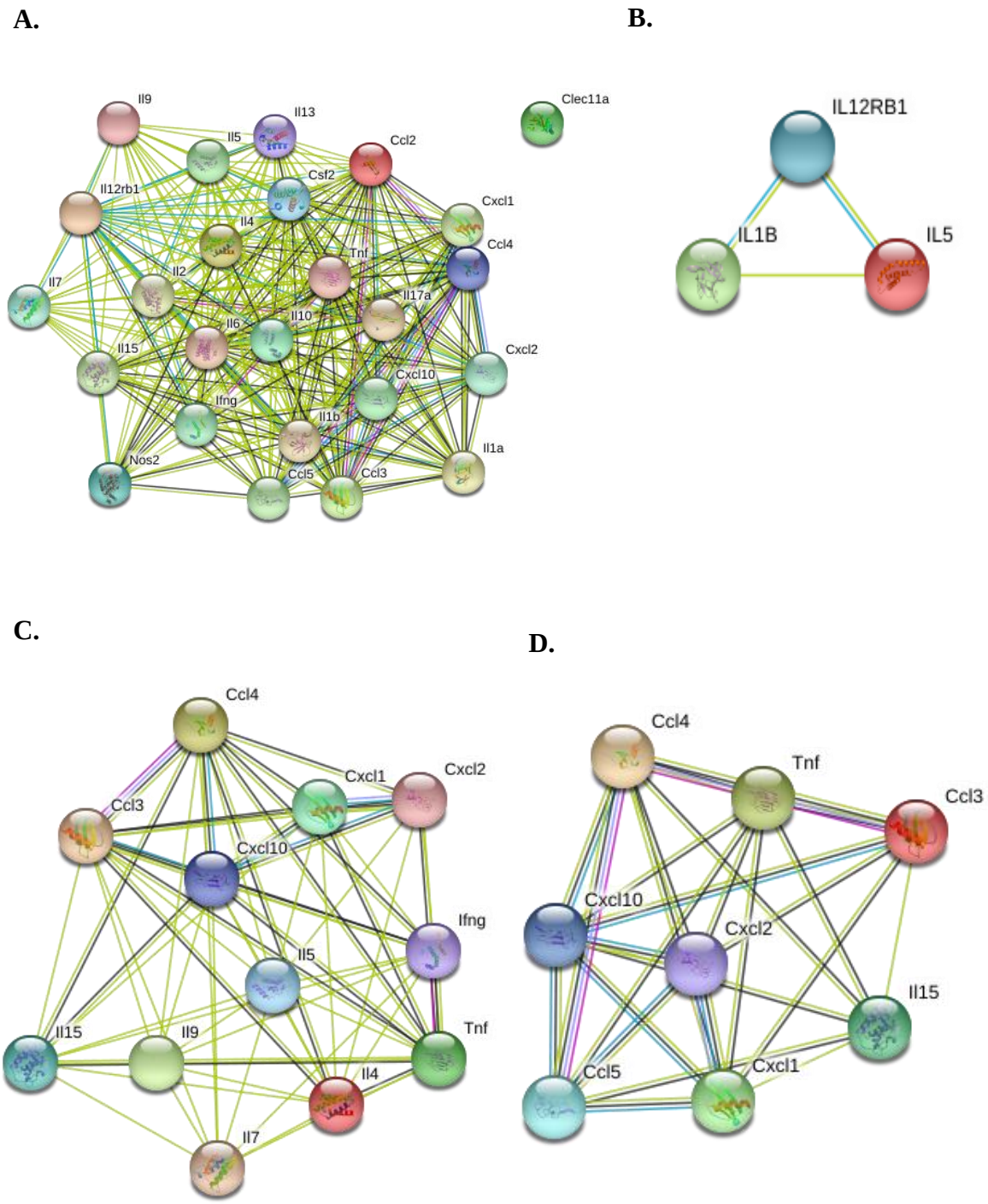


Figure 48 Network String Analysis derived from hierarchal clustering. Using the STRING 10. 5 databases, a network of potential protein interactions detected as highly expressed in RAW264.7 macrophages treated with A) LPS, B) Heat/chemical treated *C. jejuni* and, C) SI4 and D) SI8 was clustered. Key for lines: Light green, association by-text mining; pink association by experiment; black, association by co-expression, dark green, association neighbourhood, light blue association from curated databases.

The main biological processes (with high P-values) associated with the network of possible protein interactions of macrophages treated with LPS during early infection are: immune response, pro-inflammatory response, response to LPS, and molecules of bacterial origin. Additional biological processes include positive regulation of the JAK-STAT cascade, cell surface receptor signalling pathway, positive regulation of B cell activation and positive regulation of immunoglobulin production (Fig. 49).

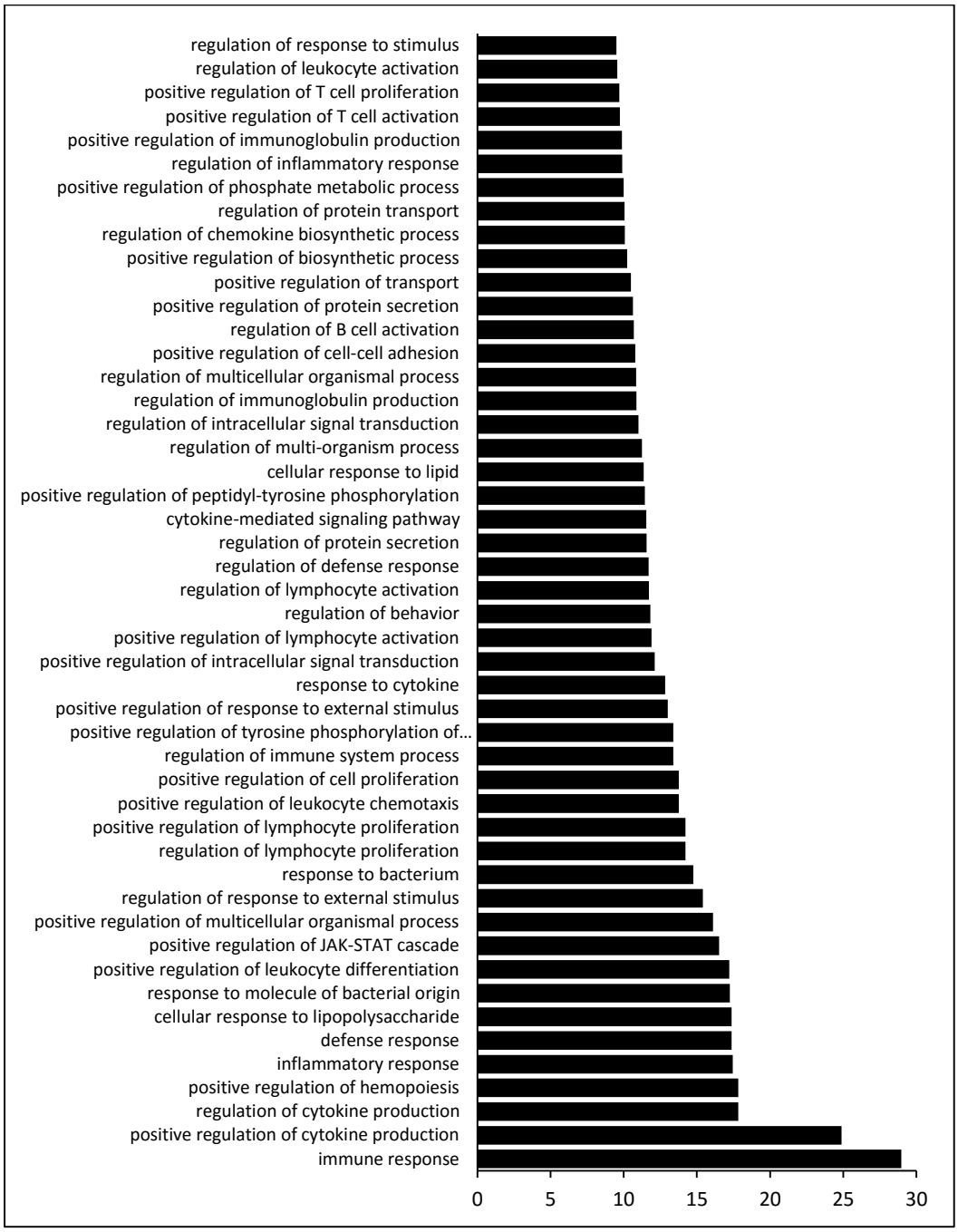


Figure 49 Biological Process (GO) induced by Lipopolysaccharide (LPS) during early infection (3 h p.i.) The x-axis indicates the amount of statistical significance [as −log(P)] in enrichment for the stated biological process.

The main biological processes associated with the network of possible protein interactions highly expressed during early infection of non-solar irradiated *C. jejuni* are immune response, positive regulation of hemopoiesis, positive regulation of leukocyte differentiation, pro-inflammatory response, response to LPS, and molecules of bacterial origin. Additional biological processes include; positive regulation of the JAK-STAT cascade, cell surface receptor signalling pathway, positive regulation of B cell proliferation and regulation of nitric oxide biosynthetic pathways (Fig. 50).

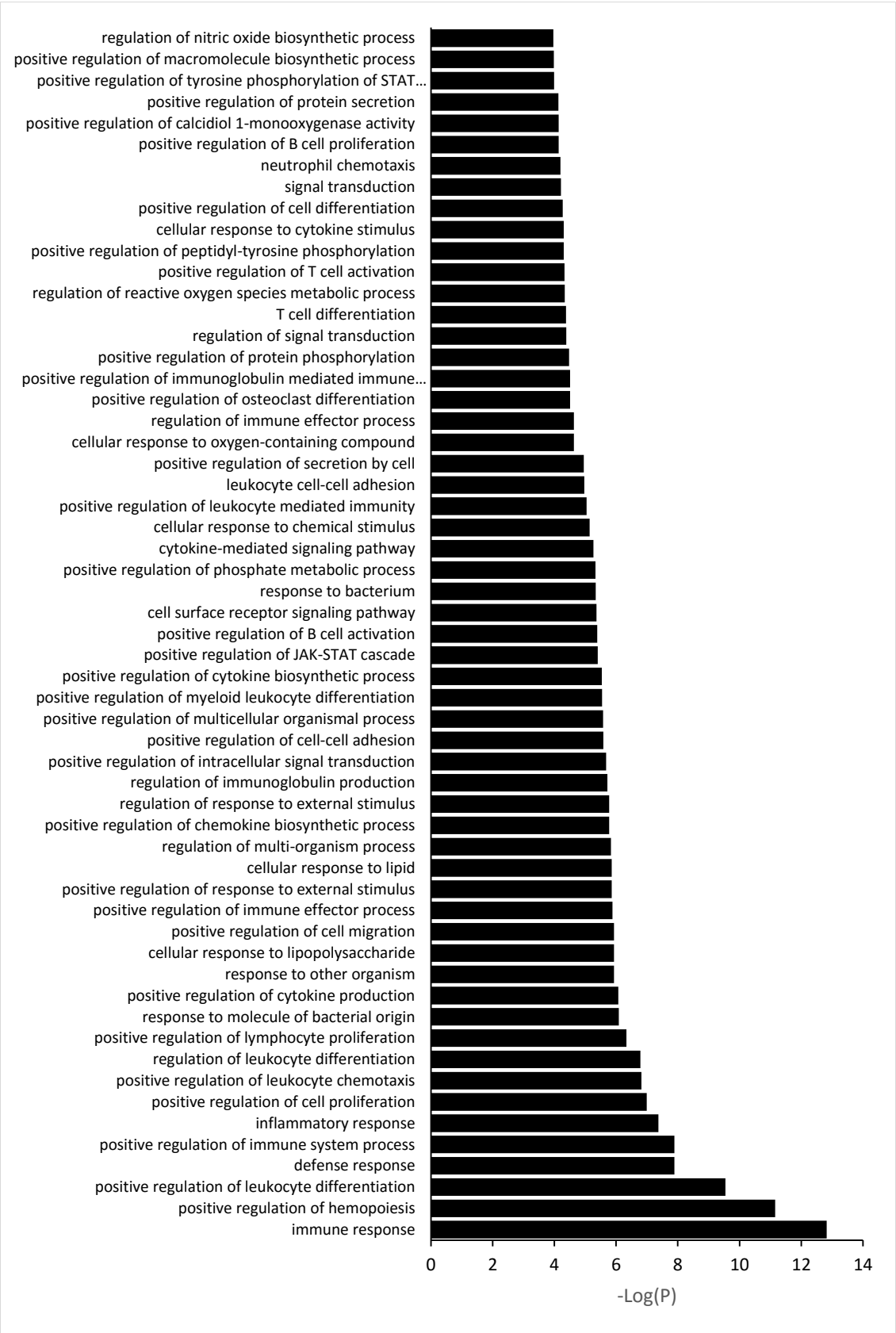


Figure 50 Biological Process (GO) induced by non-solar irradiated *C. jejuni* during early infection (3 h p.i.) The x-axis indicates the amount of statistical significance [as −log(P)] in enrichment for the stated biological process.

The primary biological processes associated with the network of possible protein interactions highly expressed during early infection of macrophages with solar irradiated *C. jejuni* are pro-inflammatory responses, positive regulation of leukocyte chemotaxis and response to molecules of bacterial origin. Additional biological processes include; response to intracellular signal transduction, positive regulation of cytokine production positive and positive regulation of hemopoiesis (Fig. 51).

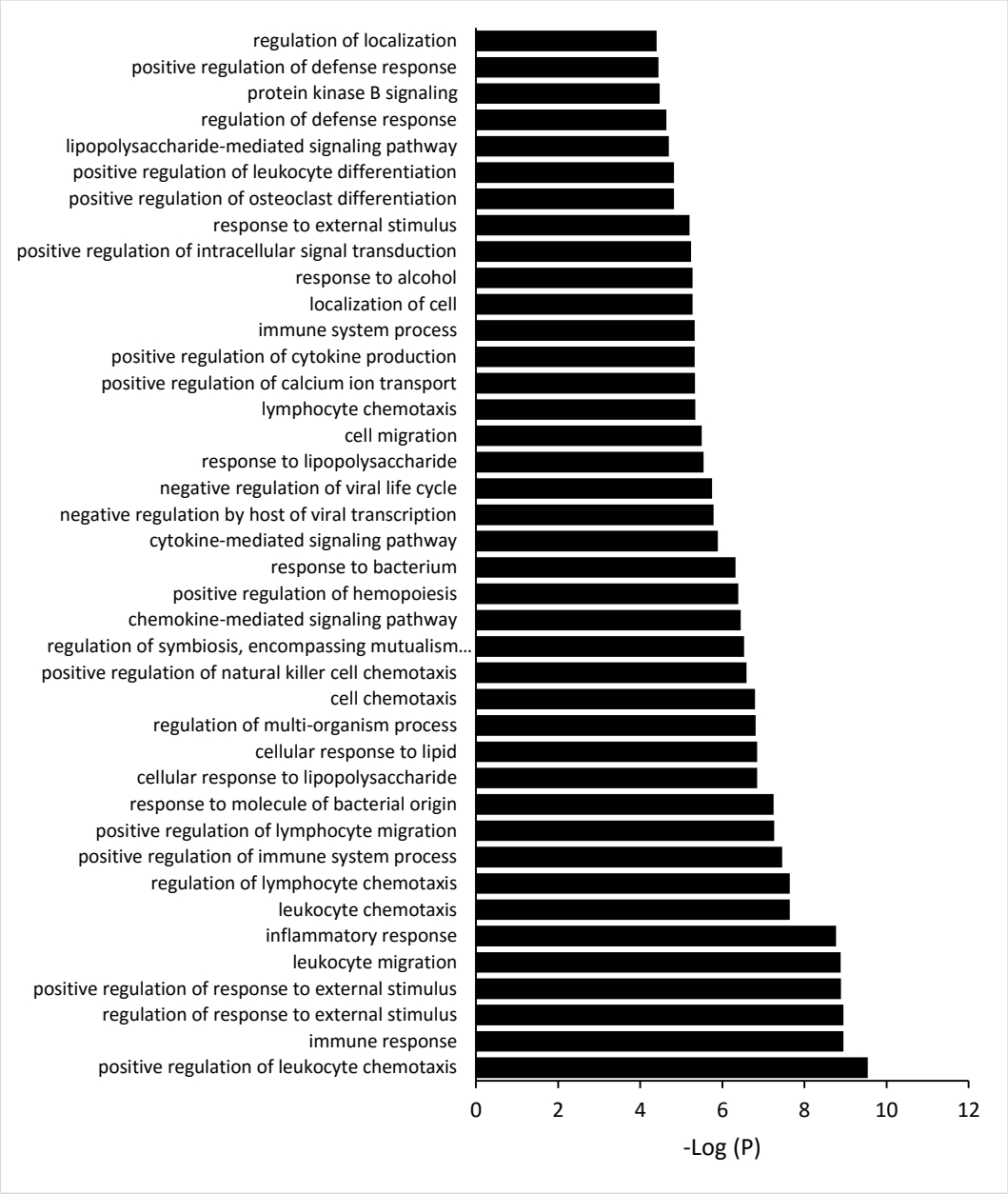


Figure 51 Biological Process (GO) induced by solar irradiated *C. jejuni* during early infection (3 h p.i.) The x-axis indicates the amount of statistical significance [as −log(P)] in enrichment for the stated biological process.

6.4 Discussion

A previous study from our laboratory assessed the immunological effects of SODIS water spiked with *V. cholerae* on dendritic cells (Ssemakalu *et al*., 2015a). This study looked at the effect of solar irradiated *C. jejuni* on RAW264.7 murine macrophages. In this study, nitric oxide production and the profiles of chemokines and cytokines secreted by macrophage cells in vitro following their stimulation with LPS, heat-chemically attenuated, non-solar and solar irradiated *C. jejuni* were assessed.

6.4.1 Nitric oxide production

Extracellular stimuli (such as fungi and bacterial endotoxins) and cytokines (IFN-γ, TNF-α and IL-1β) activate macrophages to express inducible nitric oxide synthase (iNOS) and inhibit pathogen replication by releasing a variety of effector molecules including nitric oxide (NO) (Ibiza & Serrador, 2008). In this study, no NO_2^- was produced during early infection in all the experiments. However, an increase in NO_2^-production occurred in the macrophages stimulated with non-solar- and solar-irradiated *C. jejuni* (Fig. 42) after 8 hours. The inactivated solar irradiated *C. jejuni* produced a similar amount of NO_2^- as the metabolically active non-solar irradiated *C. jejuni.* Therefore, solar irradiation does not affect *C. jejuni's* ability to elicit NO_2^- expression in macrophages and thus the immunopotency of macrophages was not impaired. Nitric oxide plays a vital role in fighting against infections (Fang, 2004). The nitric oxide and reactive nitrogen species form part of the innate immune responses that contribute to the resolution of *C. jejuni* infection (Iovine *et al.*, 2008) and the adaptive immune response (Ibiza & Serrador, 2008) and thus SODIS users can greatly benefit from iNOS expression after consumption of the solar attenuated *C. jejuni.*

Gram-negative organisms such as *S.* Typhimurium are known to induce iNOS expression in macrophages via flagellin or Toll-like receptor 5 (TLR 5) and LPS / TLR4 pathways that lead to NF-κβ activation. However, *C. jejuni* flagellin does induce iNOS expression through TLR5 (Kim *et al.*, 1997; Andersen-Nissen *et al.*, 2005), but rather, microbe-associated molecules such as LPS (TLR4 pathways) in *C. jejuni* are responsible for iNOS expression in macrophages (Albina *et al.*, 1993; Sarih *et al.*, 1993).

6.4.2 Chemokines

Chemokines are low-molecular-weight proteins that stimulate the recruitment of leukocytes. Chemokines are secondary pro-inflammatory signal molecules that are induced by primary pro-inflammatory mediators such as IL-1 or tumour necrosis factor (TNF) (Graves & Jiang, 1995). In this study, the LPS, non-solar and solar-irradiated *C. jejuni* stimulated the production of the chemokines, MCP-1, MIP1-α, MIP-1β, and MIP-2 in the RAW264.7 cells. This is

expected since these chemokines are essentially chemo-attractants of neutrophils which are the first immune cells to migrate into infected tissue sites (De Filippo *et al.*, 2008). In this study, both non-solar and solar irradiated *C. jejuni* induced an almost similar chemokine expression profile in the macrophages suggesting that macrophages infected with solar irradiated *C. jejuni* are able to induce the chemotactic abilities of macrophages. However, heat/chemical attenuation of *C. jejuni* showed some variation in the production of MIP1-α, MIP-1β, MIP-2, MCP-1, RANTES, KC, and IP-10 (Fig 43A-G) especially during early infection showing that this treatment does alter the immunomodulatory effects of *C. jejuni.*

6.4.3 Cytokine expression

Immuno-regulatory molecules such as cytokines are crucial in orchestrating the immune response network as well as in the maintenance of homeostasis in the host cells (Kim & Austin, 2006). In general, there was an M1 phenotypic expression of interleukins in macrophages treated with non-solar and solar-irradiated *C. jejuni.* The M1 phenotype is associated with high levels of pro-inflammatory cytokines (Figs. 44A-1). A higher pro-inflammatory response was noted during early infection (3 h. p.i) than in late infection. Thus, there is a possibility that both non-solar and solar irradiated *C. jejuni* may have an immunosuppressive effect since the pro-inflammatory response was reduced during the late infection period.

6.4.3.1 Pro-inflammatory response

Macrophages infected with both non-solar and solar-irradiated *C. jejuni* did not express any IL-1α during early and late infection (Fig. 44A). However, IL-1β was expressed in relatively high amounts during initial infection but decreased as time progressed (Fig. 44B). Members of the Interleukin-1 family are among the most potent cytokines produced by innate immune cells. IL-1 is a multifunctional pro-inflammatory cytokine (Dinarello *et al.*, 1983) and IL-1β, is processed by caspase-1 (IL-1β-converting enzyme) (Kostura *et al.*, 1989). Caspase-1-induces pyroptosis in macrophages and this type of cell death is an innate immune effector mechanism against intracellular bacteria (Miao *et al.*, 2010). Therefore, this suggests that the Caspase-1 pathway which leads to pyroptosis may have been initiated in macrophages stimulated with solar and non-solar irradiated *C. jejuni.* Ssemakalu *et al.*, (2015a) showed that IL-1β expression was below detectable limits in dendritic cells treated with solar irradiated *V. cholera*. A possible explanation for the difference of expression levels of IL-1β, is that *C. jejuni* is an intracellular organism whereas *V. cholerae* is an extracellular pathogen.

Figure 3C shows that, with the exception of the LPS treated samples, the other treatments did not elicit IL-2 production in the macrophages. *Campylobacter jejuni*, irrespective of how it was treated, the pathogen could not elicit IL-2 expression in the macrophages. Thus, it may be

suggested that *C. jejuni* inhibits IL-2 production in macrophages. Even with LPS treatment very little IL-2 (<10 p/ml) was produced. IL-2 is an essential factor involved in the adaptive immune response and it enables the generation of memory T cells which can undergo secondary expansion when they re-encounter an antigen (Williams *et al.*, 2006; Bachmann *et al.*, 2007).

Campylobacter jejuni solar-irradiated for 8 hours induced relatively high amounts of IL-6 at 3 h.p.i while IL-6 was absent in the 24-hour cultures (Table 7). Interleukin-6 is a pleiotropic cytokine produced mainly by monocytes/macrophages (Horii *et al.*, 1988). It regulates physiological functions of multiple immune and non-immune cell types and represents critical interphase between immune, endocrine and neural systems (Shabo *et al.*, 1988). Interleukin-6 affects adaptive immune responses when it stimulates the differentiation of T-cells (Tormo *et al.*, 2012) and B cells and promotes immunoglobulin production (Hilbert *et al.*, 1989). Therefore, this suggests that solar-irradiated pathogens can elicit IL-6 production at the onset, thus bridging the gap between the innate and adaptive immunity. This can be an advantage for long-term consumers of SODIS-treated water, because IL-6 plays an important role in the adaptive immune response thus long-term immunity against *C. jejuni* may be elicited in SODIS users. These results are similar to that of Ssemakalu *et al.* (2015).

Higher amounts of IL-7 were expressed in macrophages that were untreated, treated with LPS, NS SI4 and SI8 during early infection than in late infection. (Fig. 44D). Thus IL-7 could be an early chemokine. During late infection IL-7 was only expressed in LPS and NS-treated macrophages. Thus, heat attenuation and solar irradiation might have altered the ability of *C. jejuni's* to elicit the expression of IL-7 in macrophages during late infection periods. Interleukin-7 is another critical homeostatic cytokine involved in the adaptive immune system that provides signals for T cell survival and proliferation (Bazdar *et al.*, 2015)

There was a high production of IL-9 during late infection in macrophages treated with LPS, non-solar and solar-irradiated *C. jejuni* (Fig. 44E). Interleukin-9 plays a vital role in down-regulating the inflammatory response (Goswami & Kaplan, 2011). Therefore, the production of IL-9 could have contributed to the immunosuppressive effects of macrophages treated with solar and non-solar irradiated *C. jejuni* during the late infection period.

No IL-12P40 was expressed in all the samples. However, IL-12P70 was expressed in LPS, HA and SI4 treated samples and none of the non-solar irradiated (NS) samples were able to elicit its expression (Fig. 44F). Therefore, it is possible that the metabolically active non-solar irradiated *C. jejuni* suppress the expression of IL-12P40 and IL-12P70. Thus, evading the bactericidal effects of IL-12 chemokines because IL-12 stimulates the antimicrobial activity of macrophages (Hamza *et al.*, 2010).

Interleukin-15 was produced in macrophages treated with LPS, NS, SI4, and SI8 during early infection (Fig. 44G). Interleukin-15 has recently been shown to be similar to IL-2 in its biological

activities. The cytokine stimulates T cell and NK cell proliferation and activation as well as enhancing B cell expansion and antibody production. Unlike IL-2, IL-15 is not expressed in lymphocytes, but instead, it is synthesized primarily by monocyte/macrophages (Doherty *et al.*, 1996). Since solar irradiated pathogens do not hamper the expression of IL-15 in macrophages, it is possible that such pathogens elicited an adaptive immune response because this interleukin plays a vital role in B cell expansion and antibody production (Doherty *et al.*, 1996).

There was low production of IL-17 in all the samples (> 6pg/ml) (Fig. 44H). It has been previously shown that IL17 signalling in macrophages elicits a new cytokine profile, characterized by the production of GM-CSF, IL3, IL9, CCL4/MIP1β, CCL5/RANTES and notably, IL12p70 (Barin *et al.*, 2012). This shows that IL17A elicits macrophage activation in a manner that is different from other T cell-derived cytokines. Additionally, it demonstrates that the responses of macrophages to IL17 signalling appear to be diverse and specialized, compared to other described IL17-responding cell types (Barin *et al.*, 2012). IL-17A is also involved in the activation of macrophages that are in the process of adopting the different profiles of both the M1 and M2 states (Nakai *et al.*, 2017).

TNF-α is a potent pro-inflammatory mediator secreted by activated M1 macrophages, which plays a variety of biologic effects, such as cell differentiation, proliferation, and multiple pro-inflammatory effects (Fujiwara & Kobayashi, 2005). Tumour necrosis factor was highly expressed during early infection in macrophages treated with LPS, NS, SI4 and SI8 (Fig. 44I). However, after 24 hours the TNF-α expression decreased, showing that TNF-α is an early cytokine. However, the heat/chemically attenuated *C. jejuni* elicited low levels of TNF during early infection. These findings corroborate with a previous study which showed that heat/chemically inactivated cultures of *V. cholerae,* did not induce the dendritic cells to secrete TNF-α (Ssemakalu *et al.*, 2015a). However, increased expression of TNF was observed during late infection. This finding shows that heat/chemical inactivation dramatically alters the immunological properties of *C. jejuni.* On the other hand, solar irradiated cultures of *C. jejuni* induced high levels of TNF-α, and this may be important in eliciting an early innate immune response (Mizgerd *et al.*, 2001) in users of SODIS-treated water.

6.4.3.2 Anti-inflammatory response

The anti-inflammatory response cytokines were produced in lower amounts than the pro-inflammatory ones in the macrophages treated with solar–irradiated *C. jejuni*,

Non-solar irradiated and heat/chemically treated *C. jejuni* were the only treatments able to elicit the production of IL-4 (Fig. 45A). Therefore, solar treatment of *C. jejuni* prevents IL-4

production in macrophages. IL-4 is required in macrophages adopting the M2 phenotype (Murray *et al.*, 2014).

Interleukin-5 is another anti-inflammatory chemokine, secreted in cells infected with LPS, solar- and non-irradiated *C. jejuni* during late infection (Fig. 45B). Interleukin-5 production may have contributed to the immunosuppressive profile of macrophages during 24 h p.i. (Fig. 47B). IL-5 controls the expression of genes involved in proliferation, cell survival, and maturation and effector functions of B cells and eosinophils. Thus, IL-5 plays an essential role in innate and adaptive immune responses (Kouro & Takatsu, 2009).

Interleukin 10 (IL-10) is also a cytokine with anti-inflammatory properties that plays a central role in controlling the host's immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis (Iyer & Cheng, 2012). The increased production of IL-10 by solar irradiated pathogens during the late infection period (Fig. 45C), may have contributed to the immunosuppressive response in macrophages during late infection (Fig. 47B)

The significant increase in IL-13 in macrophages treated with solar irradiated pathogens during late infection (Fig. 45D), may have played a role in the immunosuppressive characteristics of macrophages during late infection (Fig. 47B). Interleukin-13 also inhibits the production of pro-inflammatory cytokines and chemokines by monocytes/macrophages both *in vitro* and *in vivo*, indicating that IL-13 also has essential anti-inflammatory properties (Juha P. *et al.*, 1998). Therefore, IL-5, IL-10, and IL-13 are possibly playing a regulatory role in response to solar irradiated *C. jejuni.*

Moreover, since some cytokines such as IL-2, IL-4, IL-5 IL-9, IL-10, IL-13, and IL-17 responsible for the adaptive immune response were produced by macrophages treated with SI4 and SI8, it is highly probable that solar irradiated *C. jejuni* can induce an adaptive immune response in consumers of SODIS-treated water. The adaptive response usually starts if the innate immune system fails to clear the pathogen (Cologne, 2006). The immune system can remember the solar-irradiated *C. jejuni* and acts specifically against its antigens in users of SODIS-treated water. Therefore, in the case of a more severe and invasive infection with *C. jejuni,* the defence mechanisms of the adaptive immune system are more efficient and faster than those of the innate defence if the antigen has already been encountered. This could significantly decrease the incidence of *campylobacteriosis* in users of SODIS-treated water.

6.4.4 Growth factors

Growth factors were highly expressed in LPS treated macrophages, and a slight increase was noted in heat/chemically treated samples. Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF) can promote the

production of granulocytes or antigen presenting cells (APC) (Mehta *et al.*, 2015) thus enhancing the immune response to antigenic material. However, the non-solar and solar irradiated *C. jejuni* suppressed the expression of growth factors, especially during late infection. Thus, there is a possibility that the pathogen could suppress the production of APCs in users of SODIS-treated water and this could have an immunosuppressive effect as well.

6.4.5 Hierarchal clustering and Network String Analysis

The chemokine, cytokine and growth factor profiles of the macrophage samples were compared during early and late infection. Using an unbiased approach, a highly expressed group was identified during early infection. Then protein-protein interaction STRING 10.5 software was used to analyse the pathogenic pathways in the active group. A higher pro-inflammatory response was identified during early infection and was characterised by significant elevations in MIP-1α, MIP-1β, TNF-α, IL-15, IP-10, KC and MIP-2 expression (Fig 47A) in both the non-solar and solar-irradiated samples. The solar irradiated bacteria still managed to maintain an almost similar cytokine profile to the non-solar irradiated *C. jejuni* (Fig. 47A). During late infection, several chemokines and cytokines were down-regulated. No significant alterations were found in MIP-1α or MIP-1β expression (Fig. 47B).

These cytokines and chemokines were identified to be key essential modulators of pro-inflammatory responses, positive regulation of leukocyte chemotaxis, response to molecules of bacterial origin, positive response to intracellular signal transduction and positive regulation of cytokine production as shown in (Figs. 50, 51). These findings suggest that solar irradiated *C. jejuni* are capable of eliciting an innate immune response from as early as 3 hours post-infection. However, the macrophages treated with heat attenuated *C. jejuni* showed a very different cytokine profile compared to the non-solar and solar irradiated *C. jejuni*. This suggests that heat and chemical attenuation dramatically alters the immunomodulatory properties of *C. jejuni*.

6.5 Conclusion

The results of this study showed that the duration of solar irradiation on *C. jejuni* has no significant effect on iNOS activity, as well as chemokine and cytokine production. However, heat/ chemical treatment of C. jejuni does reduce the expression of iNOS and reduces the expression of IL-4, IFN-gamma, and IL-5 during early infection. The profile of cytokines and chemokines secreted by macrophages in response to solar irradiated *C. jejuni* suggests that it is most likely be a pro-inflammatory immune response during early infection as depicted diagrammatically in Fig. 52. However, during late infection the pro-inflammteory responses in

macrophages treated with solar irradiated *C. jejuni* is reduced, this implies a potential anti-inflammatory response during late infection periods.

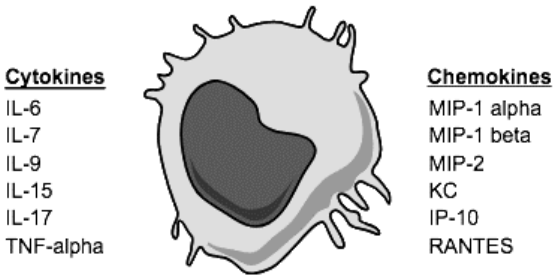


Figure 52 Schematic diagram of M1 phenotype adapted by macrophages treated with 8h-solar irradiated *C. jejuni* during early infection (Diagram constructed using Mindthegraph Software)

Moreover, the results have shown that an early innate immune response is elicited by solar irradiated *C. jejuni* as demonstrated by the high expression of IL-1ß, IL-15, TNF-alpha, and IP-10. However, mixed leukocyte assays and in vivo assays are required to elucidate further the exact immune reaction taking place in response to these solar irradiated pathogens.
In conclusion, the consumption of solar disinfected water containing solar irradiated *C. jejuni* may elicit an early immune response to consumers who practise SODIS of water.

7. Proteomic analysis reveals critical innate immune macrophage responses in the host response to solar irradiated *C. jejuni*

Abstract

Campylobacteriosis is the leading gastrointestinal disease in the world. It is a zoonotic waterborne disease that can be prevented by implementing simple home water treatment methods such as solar disinfection (SODIS). Studies have shown that *Campylobacter jejuni* is one of the most susceptible organisms to solar ultraviolet radiation (SUVR). Consumption of SODIS-water has resulted in the reduction of diarrheal incidences in areas where SODIS is being implemented. Despite previous research on the effect of SODIS on *C. jejuni*, little is known about the immunological profile of the host response when solar irradiated *C. jejuni* is ingested. Investigating the proteome expression patterns of solar irradiated *C. jejuni* may provide a better understanding of its effect on the innate and adaptive immune response of the host. In this study SWATH-mass spectrometry-based proteomics was used to investigate and compare the induced proteomic changes in macrophages treated with lipopolysaccharide (LPS), non-solar irradiated, and solar irradiated *C. jejuni*. A total of 15,077 peptides matching 2,778 proteins were identified at 1% FDR with numerous differentially expressed proteins (DEPs) detected in all the samples. Pathway analysis showed that most of the up-regulated proteins in the macrophages treated with solar irradiated *C. jejuni* were implicated in oxidation-reduction processes, endoplasmic reticulum stress, antigen processing and presentation of exogenous peptide antigens via MHC class I (TAP-dependant) and ATP-biosynthesis. The KEGG-pathways also showed that some of the up-regulated proteins such as Rab5, Rab7, Stx7, Sec61, MHCI and vATPase participated in phagosome pathways. In conclusion, our results show that solar irradiated *C. jejuni* elicits key immune responses which involve antigen processing and presentation, thus eliciting a CD8+ CTL-mediated response which may have health benefits to users of SODIS-treated water. The identification of proteins involved in the immune response to solar irradiated *C. jejuni* may facilitate the discovery of novel therapeutic targets and development of effective vaccines against Camypylobacteriosis in both animals and humans.

Keywords: *Campylobacter jejuni*, proteomics, solar disinfection (SODIS), macrophages

7.1 Introduction

Campylobacteriosis is one of the most important foodborne diseases in the world (Nic Fhogartaigh & Dance, 2013). The disease is caused by *Campylobacter jejuni*, a zoonotic waterborne pathogen endemic to Africa, Asia, and the Middle East (Kaakoush *et al.*, 2015). The symptoms of campylobacteriosis are mild diarrhoea but can lead to severe systematic infection in immuno-compromised individuals (Clark 2016).

In geographical regions with abundant solar radiation, solar disinfection (SODIS) of drinking water is one way of treating water and making it potable. SODIS involves filling transparent plastic polyethylene terephthalate (PET) bottles with water obtained from precarious water sources, shaking the bottle for 30 seconds to increase the amount of dissolved oxygen and subjecting them to the sun for at least 6 hours before consumption of the water (Solar Water Disinfection, 2002). SODIS treatment of water can be an effective method in reducing incidences of campylobacteriosis since *C. jejuni* is highly vulnerable to solar ultraviolet radiation (SUVR).

Solar disinfection of water is known to have reduced diarrhoeal cases in several regions where this water treatment method has been implemented (Conroy *et al.*, 1996; Conroy *et al.*, 1999; Mausezahl *et al.*, 2009; Meierhofer & Landolt, 2009; du Preez, 2010; Bitew *et al.*, 2018). The reduced diarrhoeal cases are due to the detrimental effects of SODIS on bacteria (McGuigan *et al.*, 1998; Boyle *et al.*, 2008; Bosshard *et al.*, 2009; Bosshard *et al.*, 2010; Heaselgrave & Kilvington, 2010; McGuigan *et al.*, 2012). For instance, Bosshard *et al.*, (2010) showed that solar disinfection significantly alters the bacterial proteins associated with vital cellular functions such as transcription and translation, cellular transport systems, amino acid synthesis and degradation, respiration, ATP synthesis, glycolysis, the TCA cycle, and chaperone functions. The protein damage pattern due to SODIS strongly resembles the destruction of proteins caused by reactive oxygen stress (Bosshard *et al.*, 2010). Sunlight probably accelerates cellular senescence and leads to the inactivation and death of solar-irradiated bacteria (Bosshard *et al.*, 2010). Despite these studies, there is still a gap in knowledge of how solar-irradiated pathogens interact with host immune cells at a molecular level.

Bacterial cell death due to SODIS may result in the release of intracellular and cellular membrane components such as lipopolysaccharides, glycopeptides, and DNA into the water. These cellular components may be potentially antigenic (Bessler *et al.*, 1997). Communities that regularly consume SODIS-treated water could be priming their immune system and lead to enhanced immunity against virulent pathogens in unsafe drinking water (Ssemakalu *et al.*, 2014).

There are several potential mechanisms by which non-solar irradiated *C. jejuni* can interact with the host. Several *C. jejuni* LOS structures are similar to human neuronal gangliosides. This molecular imitation has been postulated to lead to autoimmune disorders such as Guillain–barré syndrome (GBS). This is a paralytic neuropathic condition that occurs in approximately 1 in every 1,000 cases of Campylobacterosis (Nachamkin *et al.*, 1998; Nachamkin, 2002; Komagamine & Yuki, 2006; Yu *et al.*, 2006).

The effect of solar irradiated pathogens on macrophage activation has not been studied in detail. The alteration of the proteins in pathogens in water that is solar disinfected may be antigenic and elicit an immune response in people consuming SODIS water. This study compared the proteome of macrophages that were infected with 4h- and 8h-solar irradiated *C. jejuni*.

7.2 Materials and methods

7.2.1 Cell line and culture conditions

A murine macrophage cell line RAW264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 5% foetal bovine serum (FBS) and 1% pen/strep antibiotics (Gibco, London, UK). The cells were incubated for 48 h at 37°C in a 5% CO_2 humidified incubator before co-incubation with the (i) LPS, (ii) heat-chemically treated *C. jejuni*, (iii) solar irradiated and (iv) non-solar irradiated *C. jejuni*.

7.2.2 Bacterial Culture Preparation

Campylobacter jejuni ATCC® 33560™ was inoculated on Chocolate Blood Agar Plates and incubated at 42°C for 48 h under microaerophilic conditions in an anaerobic jar using an Oxoid™ Campygen gas generation kit (Thermo Fischer Scientific, Waltham, MA). A single colony from the incubated plate was transferred to Mueller Hinton Broth and incubated at 42°C for 48 h without shaking. The *C. jejuni* was harvested by centrifugation at 4 000xg for 15 min and washed thrice with autoclaved still mineral water. The pellet was suspended in sterile mineral water up to an OD_{546} in 0.2 ml (approximately 10^7 cells/ml).

7.2.3 Preparation of inactivated *C. jejuni*

The heat/ chemical attenuated *C. jejuni* was prepared by diluting *C. jejuni* in Mueller Hinton to an OD_{546} of 0.2 and then heating the mixture at 60°C for 1 h in 1% formalin.

7.2.4 Sample preparation and enumeration of *C. jejuni*

Aliquots of 15 ml of the cultured cell suspension were shaken for 15 seconds and exposed to solar irradiation in 25 cm^3 tissue culture flasks under atmospheric conditions. The control

flasks consisted of the same mixture were exposed to similar atmospheric conditions except for SUVR by enclosing the samples in an opaque ventilated box (Ssemakalu, 2010a). The flasks were then placed on aluminium foil and exposed to the sun for 0, 4, and 8 h. These experiments were carried out on the roof of a laboratory at the Vaal University of Technology in South Africa (26°42'39.1"S 27°51'46.2"E -26.710858, 27.862820) from 8.00am-4.00pm. The amount of solar ultraviolet irradiation (UVA +UVB radiation) was measured at 30 min intervals and captured by a Lutron 340A UV Light Meter (Lutron Electronics Company, Coopersburg, PA).

7.2.5 Macrophage treatments

Six-well tissue culture plates were seeded with 1 x 10^5 macrophages per ml and incubated for 24 h. Thereafter, the pre-seeded macrophage monolayers were washed with DPBS (Gibco) and then co-incubated with: a) Lipopolysaccharide from *E. coli* (positive control) and *C. jejuni* that was b) heat-chemically attenuated, c) non-irradiated, and d) solar irradiated for 4, and 8 h at a multiplicity of infection of 1:10 (macrophage: *C. jejuni*). The infected monolayers were co-incubated for 12 h to allow for adhesion and invasion to occur. Then the monolayers were washed with infection media, (media without antibiotics) to remove the unbound bacteria. Tissue culture media containing antibiotics (40 µg of gentamicin/ml) was then added to the cells and incubated for a further 24 h (Siegesmund *et al.*, 2004). After the 24 h post-infection period, the media was removed from infected macrophage wells, and cell scrappers were used to gently remove the macrophages. The macrophages were centrifuged at 10000 x g for 3 min and cell pellets were frozen in liquid nitrogen and kept at -80°C until used for protein extraction.

7.2.6 Protein extraction and trypsin digestion

7.2.6.1 Sample Preparation: Protein Extraction, Reduction, and Alkylation

The treated RAW 264.7 cell pellets (approximately 500,000 cells) were re-suspended in 50 µl Lysis buffer (1% SDS, 50mM Tris-HCL pH 8) in tubes. A protease inhibitor cocktail (Promega, Madison, WI) was then added to all the tubes. Cell lysis was carried out using a Barocycler 2320 EXT (Pressure Biosciences Inc., South Easton, MA) with 30 cycles: with each cycle consisting of 30 s at 45 kPsi and 15 s at atmospheric pressure (0 kPsi). The extracts were transferred to 0.5 ml tubes and placed in a bath sonicator for 5 min. Chromatin digestion was carried by the addition of 25 U benzonase (Merck, Modderfontein, South Africa), and 2 mM $MgCl_2$, and the cell lysate was incubated for 30 min at 37°C. The samples were clarified by centrifugation at 15,000 g for 10 min, and the supernatant was transferred to a 1.5 ml microcentrifuge tube. The protein concentration was assessed by using 2D-quant Assay (GE Healthcare Life Sciences, Pittsburgh, PA) as per manufacturers' instructions. The standard

curve obtained is shown in APPENDIX C. The proteins were reduced through the addition of DTT to a final concentration of 10 mM, followed by incubation at room temperature for 30 min. Subsequent alkylation was performed by addition of IAA to a final concentration of 40 mM and incubation at room temperature for 30 min in the dark. The protein sample was quenched by adding further DTT Reduction Reagent to achieve a final concentration of 20 mM DTT. The protein solution was aspirated and stored as 50 µg total protein aliquots in 0.5 ml microcentrifuge tubes and stored at -80°C (samples were processed in triplicate).

7.2.6.2 HILIC-based Automated Protein Clean-up and Digestion

Automated clean-up and digestion of the protein was performed on the KingFisherTM Duo Magnetic Handling Station (Thermo Fisher Scientific, Waltham, MA) using 96 deep-well microtiter plates, suitable for parallel processing of up to 12 samples. The reagents were laid out, as shown in Fig. 53. The device has a robotic magnetic head of 12 pins with a plastic comb that can be discarded. This avoids cross-contamination of samples between reservoirs during the binding, mixing, and transfer phases (Fig. 53). Protein digestion was carried out at 37° C in the KingFisherTM Duo Peltier block (line A) (Fig. 53) (Stoychev *et al.*, 2012).

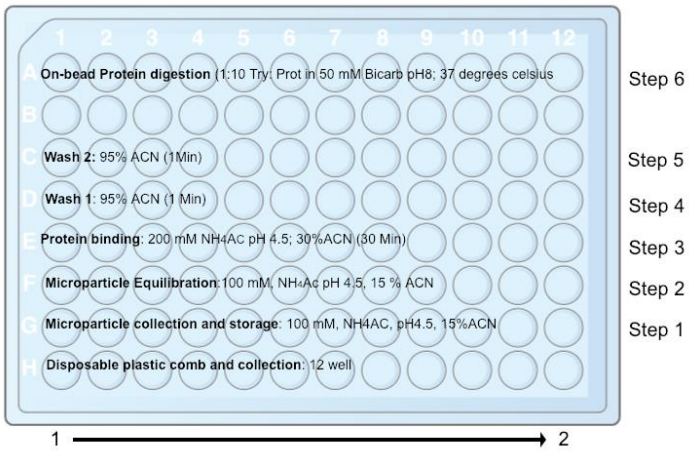


Figure 53 A KingFisher™ Duo magnetic handling station was configured for protein capture, clean-up and on-bead digestion using MagReSyn® HILIC micro-particles (Stoychev *et al.,* 2012) and placed in row G. The protein extracts were pre-mixed with Binding Buffer, in row E. The microparticles were collected from row G, transferred to row F for balancing, to row E fo r protein binding. Two consecutive washes in rows D and C removed the contaminants. The digestion continued for 4 hours in row A (peltier scheme). Micro-particles were then separated from the digested protein back to the storage place in row G. Peptide – containing supernatant from row A was recovered and dried in 0.5 ml microcentrifuge tubes.

Protein binding on MagReSyn® HILIC micro-particles was performed in 15% and 100 mM ammonium acetate, pH 4.5. Detergent and salt contaminants were removed using two 95% ACN bead washes followed by the addition of Promega sequencing grade trypsin and 4h incubation for on-bead protein digestion. The microparticles were recovered on a magnetic separator and the supernatant, containing peptides, was transferred to a 0.5 ml microcentrifuge tube. The peptides were vacuum-dried and stored at -80°C until analysis.

7.2.7 LC-MS data acquisition

Approximately 1 ug of tryptic peptides per sample were analysed using a Dionex Ultimate 3000 RSLC system (Thermo Fisher, Waltham, MA) coupled to an AB Sciex 6600 TripleTOF mass spectrometer (Sciex, Concord, Ontario, Canada). The injected peptides were de-salted online using an Acclaim PepMap C18 trap column (Thermo Fisher) (75 µm × 2 cm; 2 min at 5 $\mu l.min^{-1}$ using 2% ACN/0.2% FA). Trapped peptides were gradient eluted and separated on an Acclaim PepMap C18 RSLC column (Thermo Fisher) (75 µm × 15 cm, 2 µm particle size) at a flow-rate of 0.3 $\mu l.min^{-1}$ with a gradient of 4-40% B over 60 min (A: 0.1% FA; B: 80% ACN/0.1% FA). For Data-dependent acquisition (DDA) runs, precursor (MS) scans were acquired from m/z 400-1500 (2+-5+ charge states) using an accumulation time of 250 ms followed by 80 fragment ion (MS/MS) scans, acquired from m/z 100-1800 with 25 ms accumulation time each. For Sequential Window Acquisition of all Theoretical mass spectra (SWATH) runs, precursor scans were acquired from m/z 400-900 using 100 variable-width windows of 0.5 Da overlap between windows and fragment ions acquired from m/z 100-1800 with 25 ms accumulation time per window.

7.2.8 Protein identification and spectral library building

Raw data (.wiff files) were searched against the mouse UNIPROT sequence database (reviewed entries, downloaded on 16 June 2019) supplemented with a list of common contaminating proteins, Biognosys iRT peptide retention time standards as well as *C. jejuni* protein sequences (Uniprot). Data processing was performed using Protein Pilot (v 5.0.1). The following search settings were applied: trypsin as the proteolytic enzyme, IAA based alkylation, rapid search. False discovery rate (FDR) analysis was performed with 1% local FDR cut-off applied at PSM, peptide, and protein levels. A spectral library was constructed by importing the .group Protein Pilot output into the Skyline (v 4.2) spectral library builder. A cut-off of 0.999, corresponding to 1% peptide FDR, was applied during import. The Biognosys iRT peptides were appended to the library in order to normalize the peptide retention time. The following filters were applied for peptide and protein import into Skyline: Tryptic peptide, size 6-25 amino acids, with up-to 1 miss-cleavage and one matched cleavage site allowed. Structural modifications: Carbomedomethyl (Cys), Acetylation (N-terminal). Precursor charge

states 2-4, product charge states 1-2, product ions: b and y Ion match tolerance of 0.1 m/z. Post protein, peptide, and transition import into Skyline a decoy peptide list was generated by shuffling the sequences of all imported peptides.

7.2.9 SWATH-MS processing

SWATH data files were converted to mzML format as well as centroided using the ProteoWizard MS Convert tool. The converted SWATH mzML files were imported into Skyline (v 4.2). The following filters were applied for SWATH mzML import: Precursor charge states 2-4, product charge states 1-2, product ions: b and y, 3-6 transitions per peptide. Product m/z range 100-1800. MS1 filtering: Isotope peaks included (Count), Precursor mass analyzer (Centroid), Peaks (3), Mass accuracy (20 ppm), MS2 filtering: Acquisition method (DIA), Product mass analyser (Centroid), Mass Accuracy (20 ppm) and an isolation scheme of 100 variable windows as per the SWATH method run in Analyst. For retention time, filtering only scans within 10 min of the predicted iRT retention times were selected. Once all SWATH mzML files were imported, all repeated peptides and proteins were removed, and a peak scoring model was trained using mProphet with decoy peptides generated during spectral library building. All peaks were re-integrated using this model, and only peptides with q-values of less than or equal to 0.01 were used for further processing.

7.2.10 Protein regulation profiling

Differentially expressed proteins were detected via the Skyline external tool, MS Stats. The following filters were selected for data processing: Normalization method: equalize medians, allow missing peaks, select high-quality features, delete full protein if all features contain interferences. The MS Stats output list of differentially expressed proteins was further filtered so that only entries fitting the following criteria remained: minimum number of peptide per protein ≥ 2, minimum fold change ≥ 2, maximum adjusted p-value ≤ 0.01.

7.2.11 Functional annotation of DEPs

Functional annotation was carried out by importing the quantitative data from the proteins considered as significantly upregulated upon treatment of RAW264.7 macrophages with lipopolysaccharide (LPS), non-solar irradiated *C. jejuni* (NS), and 4h- and 8h-solar irradiated *C. jejuni* (SI4 and SI8, respectively). Functional protein association networks were evaluated using the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) v10.5 (Franceschini *et al.*, 2013) with high confidence (0.7) parameters for networks with a high number of proteins. Then, MCL clustering of the networks was analysed in Cytoscape v2.7.1 (Shannon *et al.*, 2003) using Clustermaker2 (Morris *et al.*, 2011). The clustering was

performed relative to a score at inflation factor 2.5, so that higher scoring interactions were given more weight during clustering. Differentially expressed proteins that were considered significantly upregulated or downregulated in each experimental condition were also uploaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Dennis *et al.*, 2003), Gene Ontology (GO) analysis and biological pathway analysis using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (Kanehisa & Goto, 2000).

7.3 Results

SWATH-LC-MS/MS was used to ascertain how solar irradiated *C. jejuni* affects the proteome in macrophages. It is important to note that the solar irradiated *C. jejuni* was non-viable and metabolically inactive after 4 and 8 hours of solar irradiation whereas the non-solar irradiated *C. jejuni* were metabolically active.

The macrophage proteins with at least 2-fold change (FC) ($\log_2$FC ≥ 1) and an adjusted p-value of less than 0.01 (FDR ≤ 0.01) were considered as being significant. The differentially expressed proteins (DEPs) of the treated macrophages were compared with the non-treated macrophage controls. Altogether, 19, 9, 4, 518 and 309 differentially expressed genes (DEGs) met these criteria in macrophages treated with lipopolysaccharide (LPS), non-solar irradiated C. jejuni (NS), heat-attenuated *C. jejuni* (HA) and 4h-solar irradiated (SI4) and 8h-solar irradiated (SI8) *C. jejuni*, respectively.

The common and treatment-specific host responses to infection were determined by Venn Diagrams (Figs 54A, B). Five common upregulated proteins in macrophages treated with lipopolysaccharide (LPS), non-solar irradiated (NS) and 4h- and 8h- solar irradiated *C. jejuni* (SI4 and SI8, respectively) included the ISG15 ubiquitin-like modifier (ISG15), prostaglandin-endoperoxide synthetase (Ptgs2), aconitate decarboxylase (Acod1), heme oxygenase (Hmox1) and MARKS-like 1 (Marcksl1). Treatment with SI4 or SI8 resulted in common changes in protein content of 142 host proteins. However, 108 proteins were specific to *C. jejuni* that was solar irradiated for 4 h (SI4), and 56 proteins were specific to macrophages treated with *C. jejuni* that were solar irradiated for 8 h.

In addition, 261, 105, 10, and 3 differentially expressed proteins (DEPs) were down-regulated in macrophages treated with SI4, SI8, LPS and, NS, respectively (Fig. 54B).

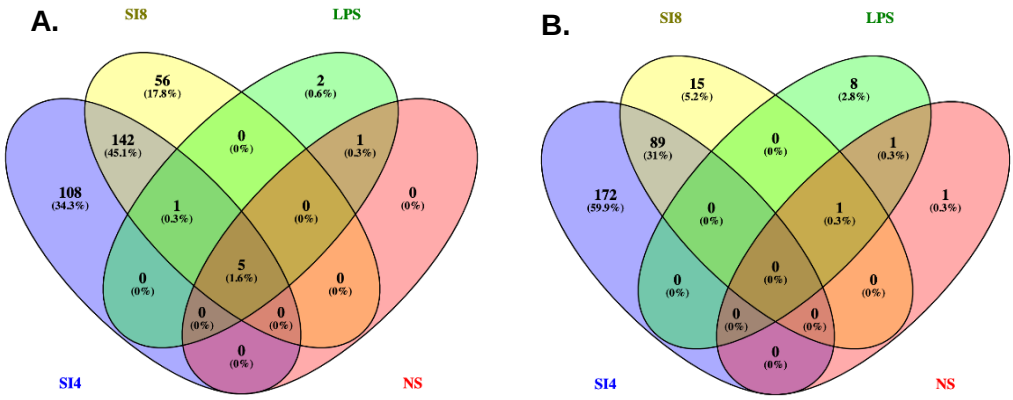


Figure 54 Venn diagram depicting the number and distribution of host proteins that were A) up-regulated and B) down-regulated [at least 2-fold increase (abs. $log_2FC \geq 1$) and maximum 1% False discovery rate (adj. p-value ≤ 0.01)] upon treatment a) Lipopolysaccharide (LPS) b) non-solar irradiated *C. jejuni* (NS) c) 4h-solar irradiated *C. jejuni* (SI4) and d) 8h- solar irradiated *C. jejuni* (SI8) Venn diagrams were obtained using VENNY 2.

7.3.1 Interaction network and functional enrichment of differentially abundant proteins

The interaction networks between the differentially expressed proteins (DEPs) in LPS-treated macrophages were significant, with p-value=0.00127 and were clustered into 2 groups, namely, regulation of cytokine and ATP synthesis coupled electron transport.

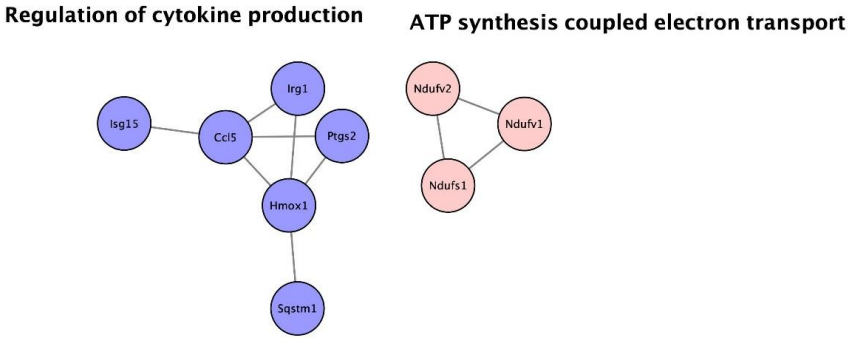


Figure 55 Protein analysis of significant (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) differentially expressed proteins (DEPs) in LPS-treated macrophages vs. untreated macrophages. STRING analysis was carried out with minimum required interaction score set to “medium confidence 0.400,” and only query proteins were included. The STRING network was retrieved in Cytospace, and MCL clustering was performed relative to the confidence score of the interaction, and GO analysis was performed on the resulting clusters. The GO-terms of the clusters are shown in Figure 55.

The network from DEPs in NS-stimulated macrophages was non-significant (p=0.0548). The DEPs in NS-treated macrophages were clustered into two groups: regulation of cytokine production and lipid transport (Fig. 56). No significant network was formed from DEPs in macrophages treated with heat attenuated *C. jejuni* (HA).

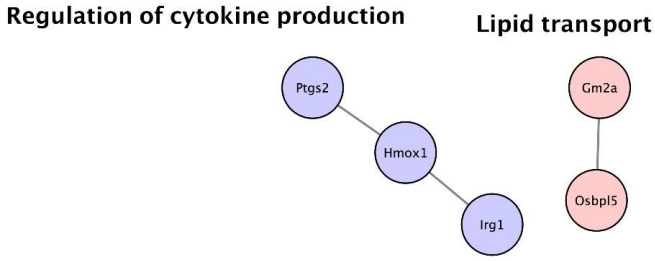


Figure 56 Protein analysis of significant (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) differentially expressed proteins (DEPs) in NS-treated macrophages vs. untreated macrophages. STRING analysis was carried out with minimum required interaction score set to "medium confidence 0.400," and only query proteins were included. The STRING network was retrieved in Cytospace, and MCL clustering was performed relative to the confidence score of the interaction, and GO analysis was performed on the resulting clusters. The GO-terms of main clusters are shown in Figure 56.

The interaction network of DEPs in SI4- and SI8-treated macrophages were highly significant with p-value < 10^{-16} for both networks (Figs. 57,58), indicating that the proteins are at least partially biologically connected. The DEPs in the networks were clustered using MCL clustering. The main clusters for SI4-treated macrophages were: protein folding, nicotinamide nucleotide metabolic process, ATP metabolic process, proteolysis in cellular catabolic process, translation, regulation of vesicle mediated transport, ribosome, regulation of biological quality and, ubiquitin mediated proteolysis (Fig. 57). The main clusters for SI8-treated macrophages were as follows: protein transport, rRNA processing, ATP metabolic processes, protein N-linked glycosylation, dicarboxylic acid metabolic pathways, mRNA processing, translation and regulation of apoptotic processes (Fig. 58).

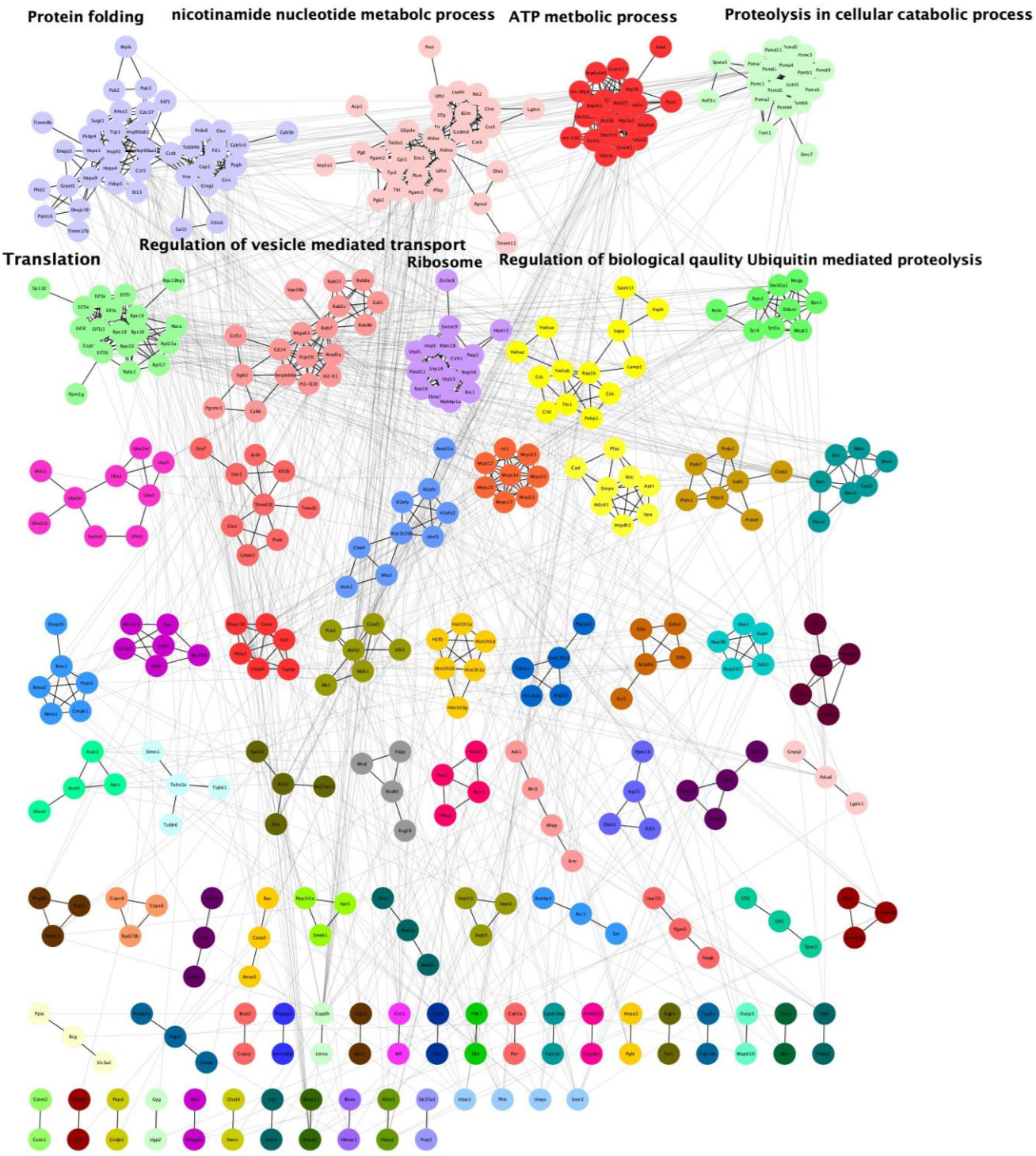


Figure 57 Protein analysis of significant (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) differentially expressed proteins (DEPs) in SI4-treated macrophages vs. untreated macrophages. STRING analysis was carried out with minimum required interaction score set to "high confidence 0.700," and only query proteins were included. The STRING network was retrieved in Cytospace, and MCL clustering was performed relative to the confidence score of the interaction. GO analysis was performed on the resulting clusters.

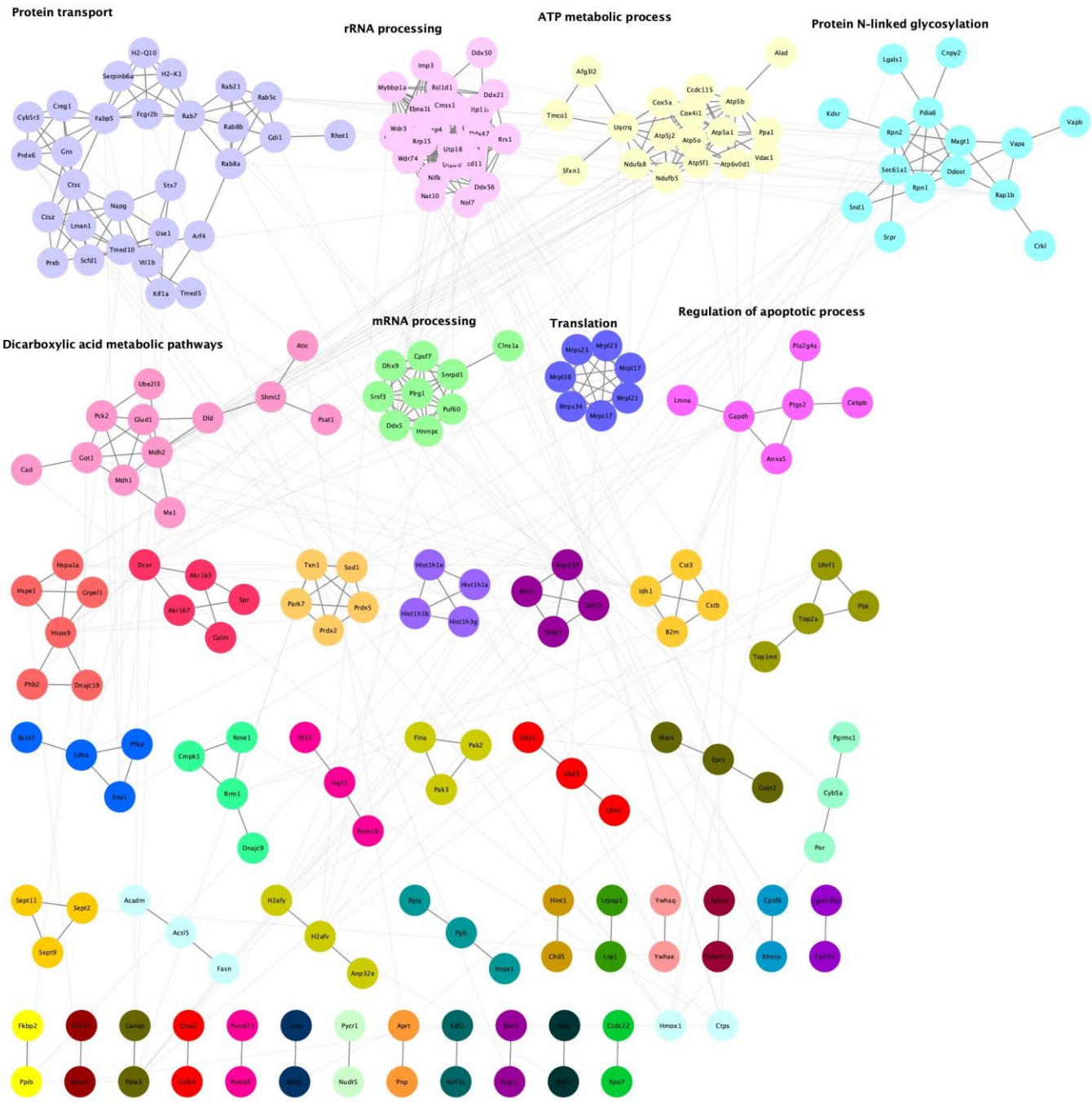


Figure 58 Protein analysis of significant (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) differentially expressed proteins (DEPs) in SI8-treated macrophages vs. untreated macrophages. STRING analysis was carried out with minimum required interaction score set to “high confidence 0.700,” and only query proteins were included. The STRING was retrieved in Cytospace, and clustering was performed relative to the confidence score of the interaction, and GO analysis was performed on the resulting clusters. The top 8 GO-terms of the main clusters are shown in Figure 58.

The DEPs in LPS-treated macrophages were enriched for GO-terms (Biological processes) identifying 1 significant term (p-adjust value < 0.05). The most significant non-redundant term was the oxidation-reduction process (GO:0055114) (Fig. 59)

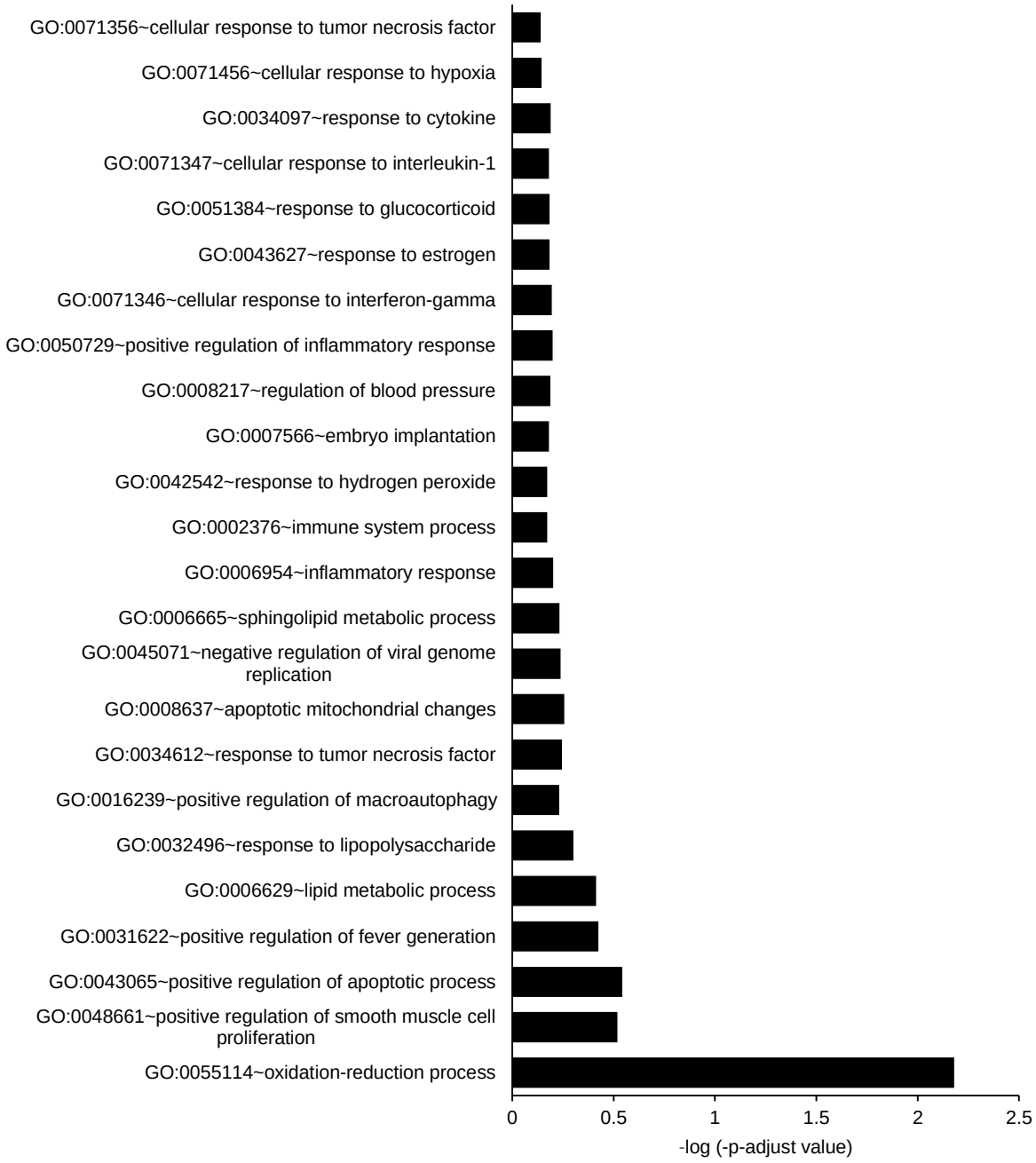


Figure 59 GO-term (Biological Processes) enrichment on DEPs in macrophages treated with lipopolysaccharide (LPS) using DAVID software.

There were 9 non-significant (p-value > 0.05) enriched terms in the NS-treated macrophages (Fig. 60). The biological processes that were enriched were a response to lipopolysaccharides (GO:0032496), response to oxidative stress (GO:0006979) and lipid metabolic process (GO0006629) (Fig. 60).

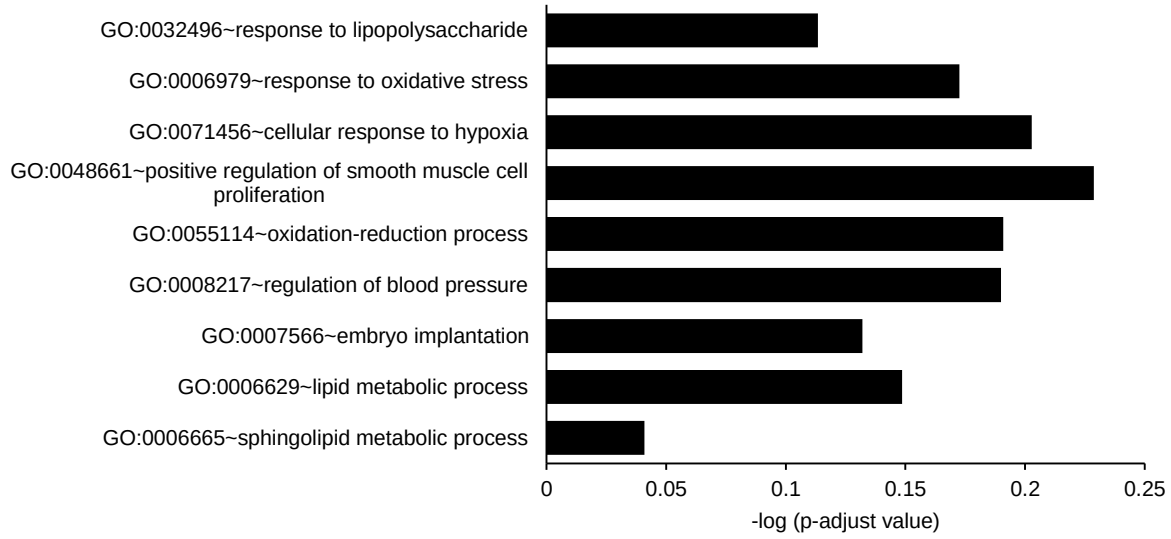


Figure 60 GO-term (Biological Processes) enrichment was on DEPs in macrophages treated with non-solar irradiated *C. jejuni* (NS) using DAVID software.

The biological process ontology presented 31 significantly enriched terms (p-adjust value < 0.05) for macrophages treated with 4h-solar irradiated *C. jejuni* (SI4). The five most significant non-redundant processes were an oxidation-reduction process (GO:0055114), protein folding (GO:0006457), antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP dependant (GO:0002479), metabolic processes (GO:0008152), and cell-cell adhesion (GO:0098609) (Fig. 61).

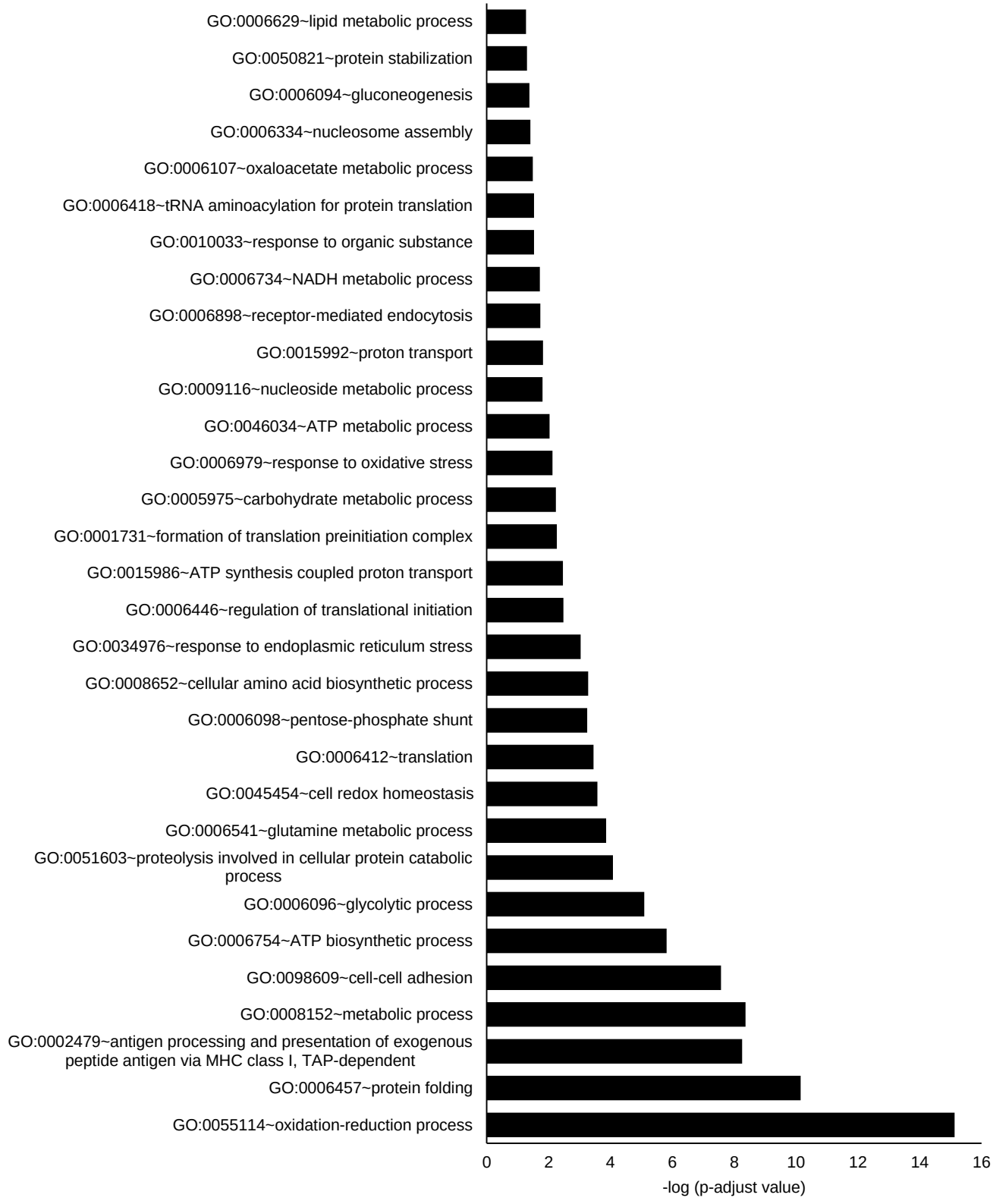


Figure 61 GO-term (Biological Processes) enrichment on DEPs in macrophages treated with 4h-solar irradiated *C. jejuni* (SI4) using DAVID software.

The DEPs in macrophages treated with 8h-solar irradiated *C. jejuni* were also enriched for GO-terms identifying 14 significant terms (p-adjust value < 0.05). The main GO-terms that were identified were oxidation-reduction process (GO:0055114), rRNA processing (GO:0006364), cell-cell adhesion (GO:00098609), protein folding (GO:0006457), and response to endoplasmic reticulum stress (GO:0034976) (Fig. 62).

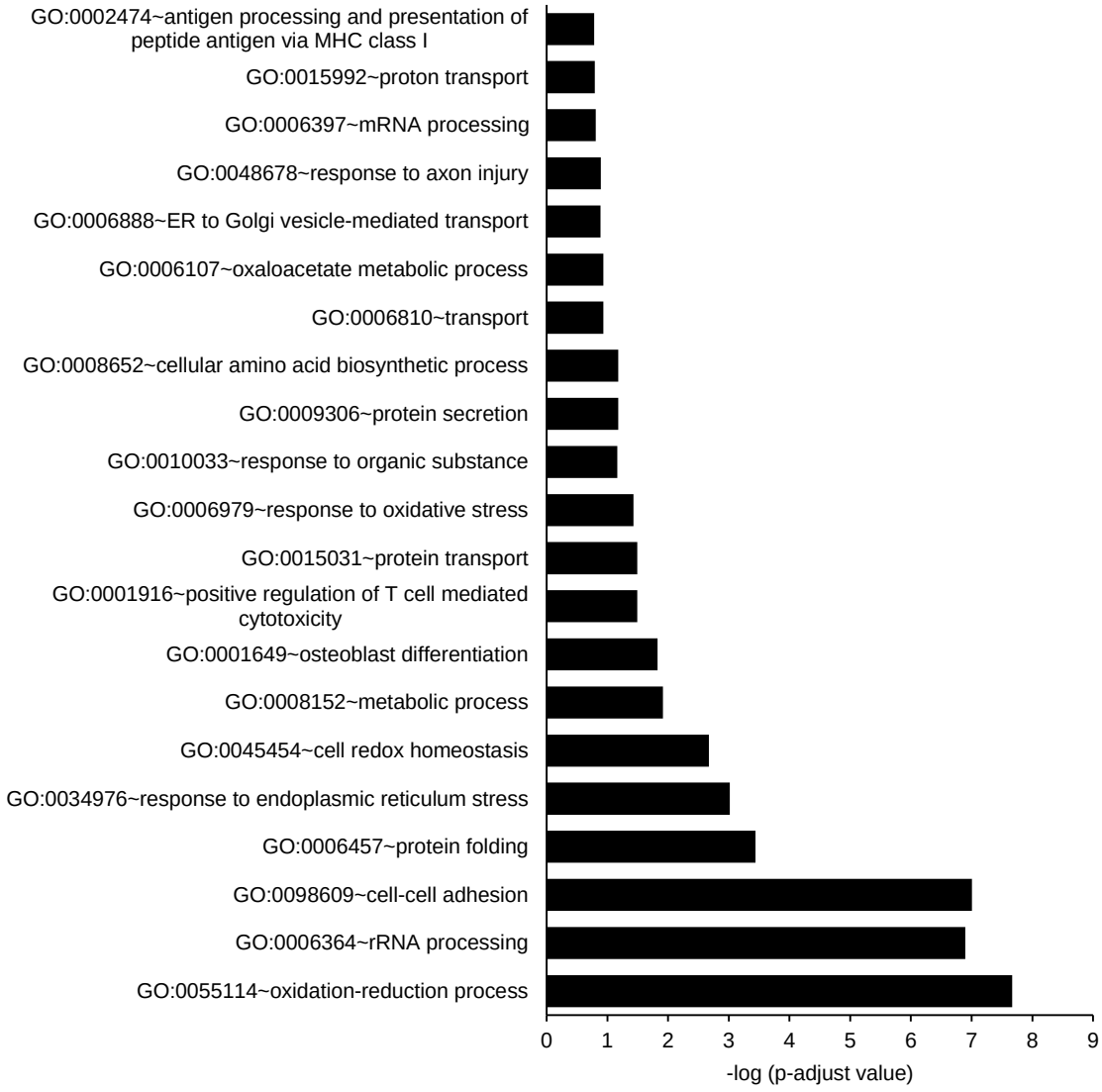


Figure 62 GO-term (Biological Processes) enrichment on DEPs in macrophages treated with 8h-solar irradiated *C. jejuni* (SI8) using DAVID software.

7.3.2 Cellular Pathway Analysis of differentially expressed proteins

To provide insights on cellular pathways associated with these differentially expressed proteins, a KEGG pathway enrichment analysis was performed using DAVID databases

(Dennis *et al.*, 2003). The main pathways enriched among differentially expressed proteins in LPS, non-solar irradiated, 4h- and 8h-solar irradiated *C. jejuni* are shown in Table 8. In LPS-infected cells, the DEPs were categorised into two **non-significant** pathways (p-adjust > 0.05), namely, Leishmaniasis (mmu0514) and TNF-signalling pathway (mmu04668). In NS-treated macrophages (Table 8) Leshmaniasis (mmu0514) was the only **non-significant** pathway that was enriched.

Sixteen significant metabolic pathways were enriched (p-adjust < 0.05) from the network formed from SI4-treated cells (Table 8), with interesting metabolic pathways consisting of oxidative phosphorylation (mmu00190), phagosome (mmu04145), protein processing in the endoplasmic reticulum (mmu04141) and antigen processing and presentation (mmu04612).

In SI8-treated cells 7 significant (p-adjust <0.05) categories were enriched, namely, ribosome biogenesis in eukaryotes (mmu03008), protein processing in endoplasmic reticulum (mmu04141), oxidative phosphorylation (mmu00190), and phagosome (mmu04145). Moreover, interesting non-significant (p-adjust >0.05) pathways were associated with antigen processing and presentation (mmu04612), spliceosome (mmu03040) and lysosome (mmu04142) (Table 8).

Table 8 KEGG pathways enriched among differentially expressed proteins (DEPs) upon treatment of RAW264.7 macrophages with lipopolysaccharide (LPS), 4h-solar irradiated C. jejuni (SI4) were analysed using DAVID software

Term	Count	p—adjust (Benjamini-Hochberg procedure)
LPS		
mmu05140:Leishmaniasis	2	0.72625387
mmu04668:TNF signaling pathway	2	0.66928857
NS		
mmu05140:Leishmaniasis	2	0.394
SI4		
mmu00190: Oxidative phosphorylation	17	7.45E-07*
mmu04145: Phagosome	17	7.38E-06*
mmu04141: Protein processing in the endoplasmic reticulum	16	2.37E-05*
mmu05012: Parkinson's disease	15	2.37E-05*
mmu05016: Huntington's disease	16	1.15E-04*
mmu05010: Alzheimer's disease	15	1.25E-04*
mmu01100: Metabolic pathways	46	1.23E-04*
mmu04612: Antigen processing and presentation	9	0.00272768*
mmu01212: Fatty acid metabolism	7	0.00628003*
mmu01130: Biosynthesis of antibiotics	13	0.00995548*
mmu03008: Ribosome biogenesis in eukaryotes	8	0.01285218*
mmu01200: Carbon metabolism	9	0.01901118*
mmu05152: Tuberculosis	11	0.01996313*
mmu00630: Glyoxylate and dicarboxylate metabolism	5	0.02285376*
mmu00510: N-Glycan biosynthesis	6	0.02290893*
mmu00020: Citrate cycle (TCA cycle)	5	0.02893002*
SI8		
mmu03008: Ribosome biogenesis in eukaryotes	9	0.00272205*
mmu04141: Protein processing in the endoplasmic reticulum	12	0.00149428*
mmu00190: Oxidative phosphorylation	11	0.00107582*
mmu05012: Parkinson's disease	11	0.00147266*
mmu04145: Phagosome	11	0.0037667*
mmu05010: Alzheimer's disease	11	0.00417238*
mmu05016: Huntington's disease	10	0.0359288*
mmu04612: Antigen processing and presentation	6	0.10022136
mmu03040: Spliceosome	7	0.16169309
mmu01100: Metabolic pathways	27	0.30548945
mmu04142: Lysosome	6	0.31770444
mmu00510: N-Glycan biosynthesis	4	0.3234544
mmu05152: Tuberculosis	7	0.34632244
mmu00630: Glyoxylate and dicarboxylate metabolism	3	0.49527068
mmu00020: Citrate cycle (TCA cycle)	3	0.53411499
mmu04130: SNARE interactions in vesicular transport	3	0.53101213
mmu01130: Biosynthesis of antibiotics	7	0.51336603
mmu01200: Carbon metabolism	5	0.49581401

*represents significant KEGG-pathways (p-adjust value < 0.05)

KEGG analysis was performed to identify genes involved in antigen processing and presentation pathways, phagosome, and lysosome pathways (Figs. 63-66). Eight genes identified for these pathways were strongly up-regulated when macrophages were treated with 4h-solar irradiated *C. jejuni* (SI4) (Fig. 63) while some genes involved in the proteasome pathway and heat-shock proteins were downregulated. In SI8-treated macrophages, 5 genes were strongly up-regulated (Fig. 64). In the antigen processing and presentation signalling pathway, most of the up-regulated genes were found to be interrelated with the MHC-I processing pathway in macrophages treated with SI4 and SI8. Two DEPs associated with MHC-II processing pathway were detected in SI4-treated macrophages (AEP and CTSB/LS) (Fig. 64).

In the phagosome pathway, 17 genes were identified as strongly up-regulated upon macrophage stimulation with SI4, while 2 genes were downregulated (Fig. 65). Eleven genes

were strongly up-regulated in SI8-treated macrophages, while one was downregulated (Fig. 66).

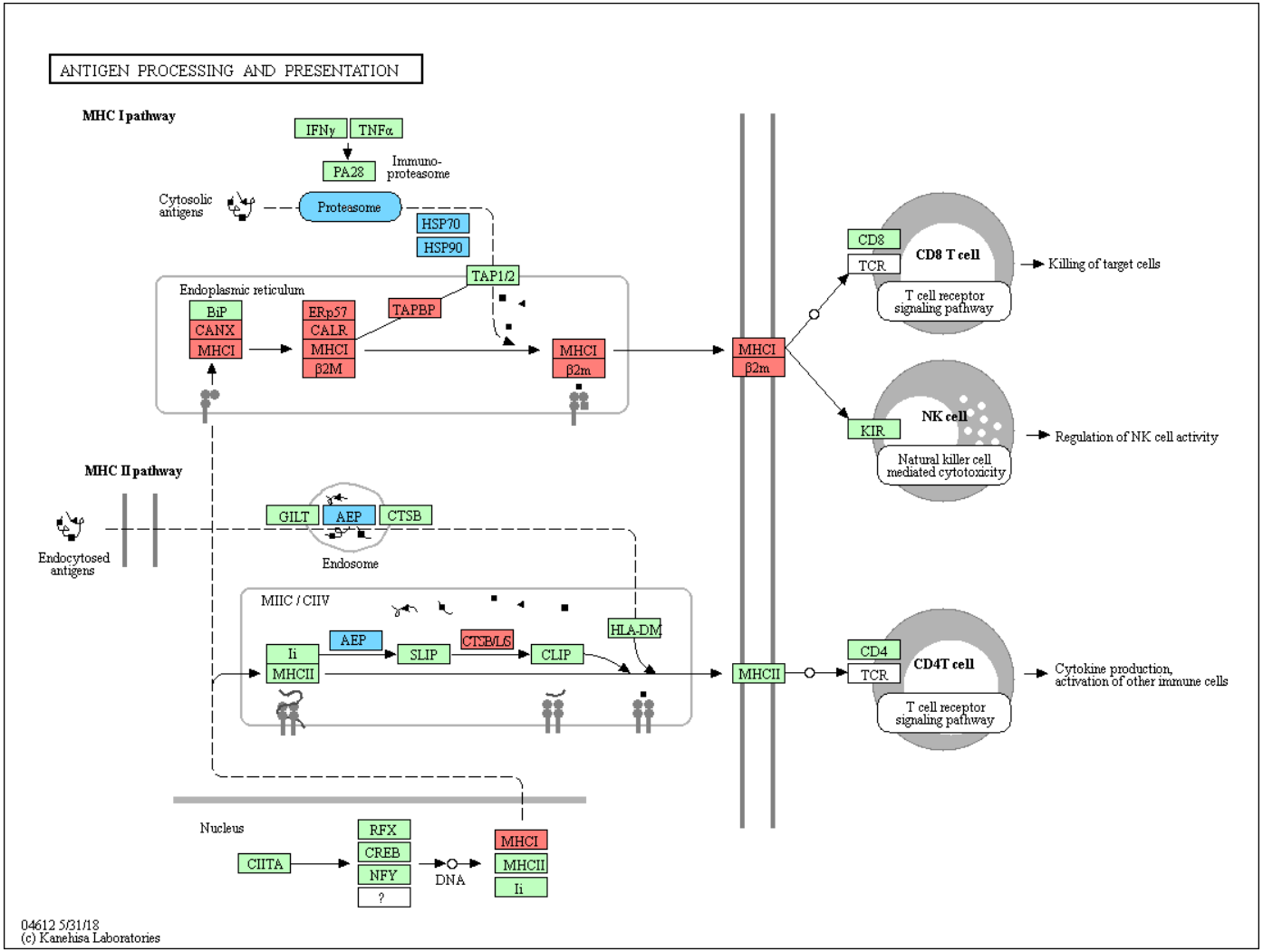


Figure 63 Significantly differentially expressed proteins (abs. $\log_2$FC ≥ 1; adj. p-value ≤ 0.01) identified by KEGG as involved in antigen processing and presentation in SI4-treated macrophages. The upregulated DEPs are highlighted in red, and the downregulated proteins are highlighted in blue.

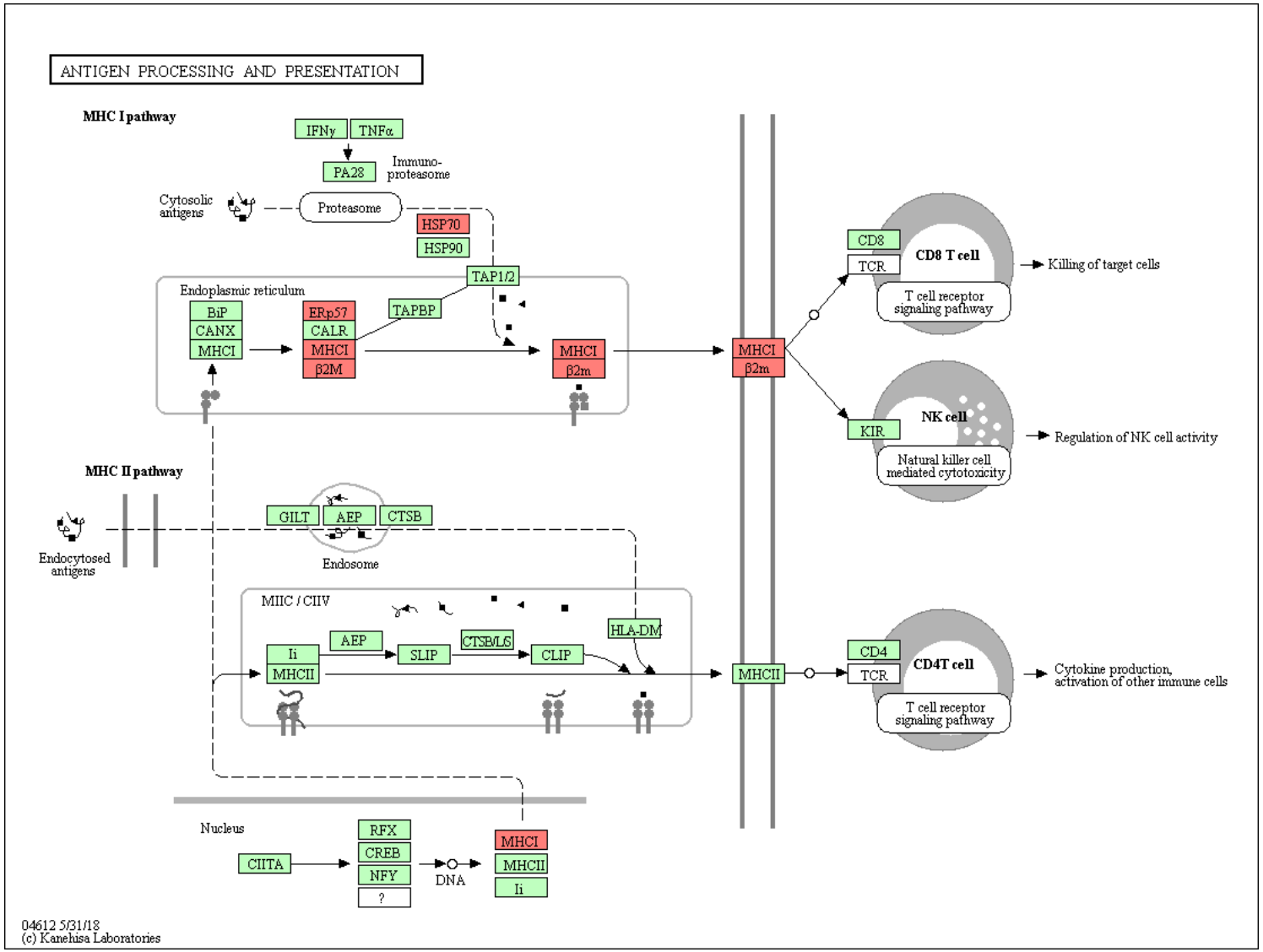


Figure 64 Significantly differentially expressed proteins (abs. $\log_2$FC ≥ 1; adj. p-value ≤ 0.01) identified by KEGG as involved in antigen processing and presentation in SI8-treated macrophages. The upregulated DEPs are highlighted in red.

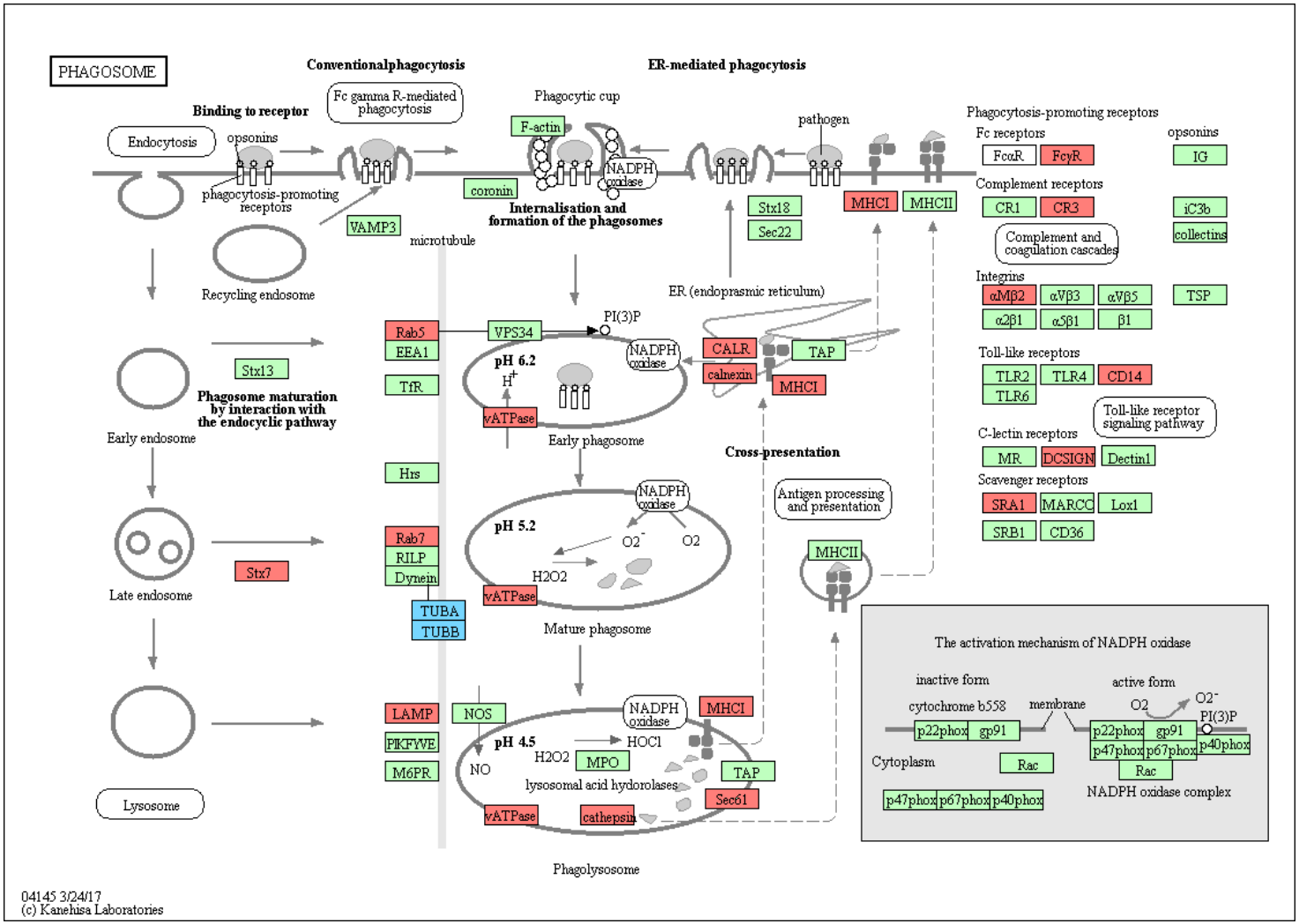


Figure 65 Significantly differentially expressed proteins (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) identified by KEGG involved in phagosome pathway in SI4-treated macrophages. The upregulated DEPs are highlighted in red, and the downregulated proteins are highlighted in blue

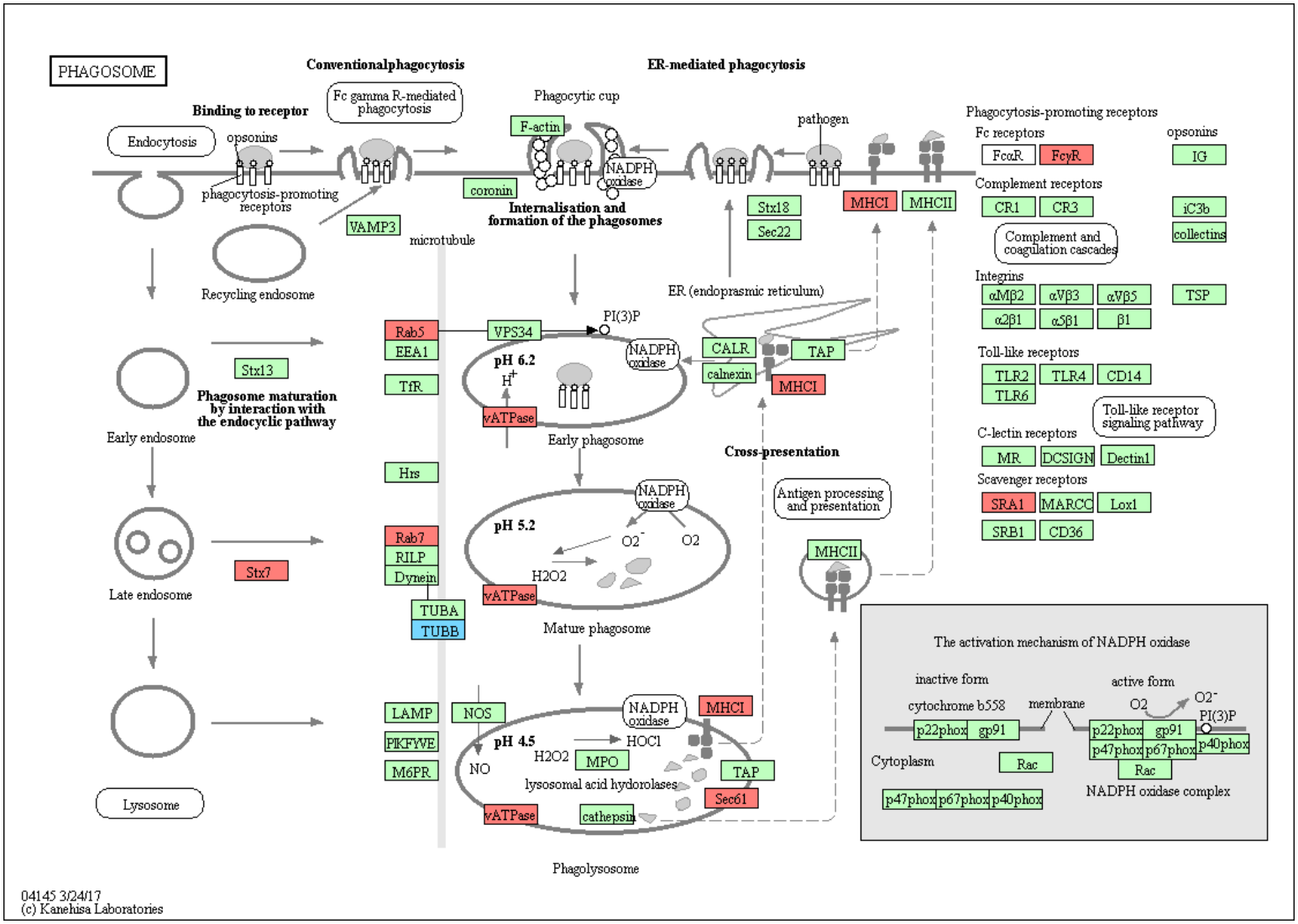


Figure 66 Significantly differentially expressed proteins (abs. $\log_2$FC ≥ 1; adj. p-value ≤ 0.01) identified by KEGG involved in phagosome pathway in SI4-treated macrophages. The upregulated DEPs are highlighted in red, and the downregulated proteins are highlighted in blue

7.4 Discussion

Although several studies have analysed the host-cell interactions between *Campylobacter jejuni* and macrophages (Bouwman *et al.*, 2014; Rodrigues *et al.*, 2016; Kim *et al.*, 2018), information on how solar irradiated *C. jejuni* interacts with host immune cells is rather limited. In this work, we provide evidence that *C. jejuni* solar irradiated for 4 h and 8h induces different pathogenicity attributes and differentially expressed proteins associated with key metabolic pathways in macrophages.

7.4.1 Interaction network and functional enrichment of differentially abundant proteins

SWATH-MS analysis identified 19, 9, 4, 518, and 309 differentially expressed proteins (DEPs) in macrophages treated with LPS, NS, HA, SI4 and SI8, respectively (Fig. 54). The number of significant DEPs in macrophages treated with LPS, HA, and NS were low. The low FDR values may have been the result of varying protein occurrence. Protein occurrence represents the frequency at which a protein is identified in multiple experiments/replicates. A higher occurrence number implies a higher probability of a true match (Zhang *et al.*, 2015). In this study, protein FDR of 1% was applied in protein confidence filtering, and most of the false protein matches in the samples with low occurrence numbers were eliminated. However, the macrophages treated with solar irradiated *C. jejuni* (SI4 and SI8) showed several significant DEPs that were statistically significant (FDR < 1%).

To further understand the interactions between the DEPs of the treated macrophage, protein interaction networks were constructed using STRING 10.0 and visualized with Cytoscape v.3.7.1. The strong association among these subnetworks may be related to the manner in which the macrophages interact with solar irradiated *C. jejuni*. For the LPS-treated macrophages, 6 macrophage proteins related to the regulation of cytokine production and 3 proteins involved in ATP synthesis were significantly up-regulated (Fig. 55). The DEPs that were identified in the first larger cluster have been shown to be involved in the pro-inflammatory response of macrophages. One of the DEPs identified was immune-responsive gene 1 protein (Irg1) which has been identified as one of the most highly up-regulated genes under proinflammatory conditions, such as bacterial infections (Tangsudjai *et al.*, 2010; Gautam *et al.*, 2011; Michelucci *et al.*, 2013; Tallam *et al.*, 2016). Another proinflammatory DEP identified in this study was RANTES (CCL5). RANTES is a chemo-attractant which draws immune cells from the peripheral blood to sites of inflammation via haptotaxis (Krensky & Ahn, 2007). The smaller cluster represents ATP synthesis coupled electron transport (Fig. 3). Most of the cellular ATP is produced by mitochondrial oxidative phosphorylation (OXPHOS) (Carroll *et al.*, 2006). The OXPHOS system consists of the identified DEPs, namely, Ndufv1, Ndufv2,

Ndufs1. These protein subunits are believed to play an essential role in proton translocation involved in the oxidation-reduction processes on the cellular membrane (Carroll *et al.*, 2006).

The network formed by DEPs in NS-treated macrophages was non-significant (p=0.0548). Nonetheless, two clusters of DEPs involved in the regulation of cytokine production and lipid transport were identified (Fig 56). However, a more significant number of DEPs were identified in macrophages treated with solar irradiated *C. jejuni* (SI4 and SI8). Proteomic analysis of the SI4-treated macrophages revealed 508 significant DEPs. A large number of these proteins were implicated in immune protein folding, ATP metabolic processes, proteolysis in cellular catabolic processes (proteasome pathway), and regulation of vesicle-mediated transport (phagosome pathway). Within the protein folding cluster, DEPs involved in antigen processing and presentation were also identified (Fig. 57).

One of the main clusters identified in SI8-treated macrophages was protein transport, rRNA processing, ATP metabolic process, protein-linked glycosylation, and apoptotic process regulation (Fig. 58). Apoptotic cell death elicits an immunosuppressive response in the host (Minton, 2015). Glycosylation is a highly regulated mechanism of secondary protein processing within cells (Arey, 2012). It plays a critical role in determining protein structure, function and stability and is also involved in immunity. Most cytokines e.g. interleukins are found in their mature state as glycosylated proteins (Chamorey *et al.*, 2002).

7.4.2 Gene ontology and pathway analysis

Gene ontology (GO) analysis revealed that upregulated DEPs in LPS-induced macrophages were involved in apoptotic processes, inflammatory response, and macro-autophagy (Fig. 59). Lipopolysaccharide (LPS) in bacteria are well-known proinflammatory stimulants in macrophages (Bonta & Ben-Efraim, 1993) and is known to induce apoptosis through the autocrine production of tumour necrosis factor-alpha (TNF-α) (Xaus *et al.*, 2000). Tumor necrosis factor (TNF) is a major proinflammatory cytokine produced by M1- macrophages, and it is the most significant upstream regulator activated during bacterial infection (Lorey *et al.*, 2017). In another study, The TNF-signalling pathway was also seen as one of the enriched KEGG pathways in LPS-treated macrophages (Table 8) (Lorey *et al.*, 2017). When the TNF pathway is inhibited, LPS-induced apoptosis occurs through the induction of nitric oxide (NO) (Xaus *et al.*, 2000). This study also showed that LPS might elicit autophagy in macrophages. This form of cell death is known to play a role in eliminating infectious agents, the modulation of the inflammatory response, antigen presentation and the regulation of T cell activation (Jang

et al., 2019). The sphingolipid metabolic process was downregulated. Sphingolipids, are a family of membrane lipids, and they participate in diverse functions such as controlling cellular processes including cell division, differentiation, and cell death (Pralhada Rao *et al.*, 2013). Additionally, sphingolipids aid in microbial pathogenesis because most microorganisms do not produce sphingolipids, but are able to utilise host sphingolipids to promote their virulence (Casadevall & Pirofski, 2003). However, sphingolipids have been shown to be key players in the host's defences against infections (Grassme *et al.*, 2003). For instance, in the case of intracellular pathogens, the host may initiate sphingolipid-mediated pathways which enable containment or dormancy of the microbe or enhance the clearance of the microorganism (Malik *et al.*, 2000; Malik *et al.*, 2003; Garg *et al.*, 2004). Thus, the downregulation of sphingolipid metabolism by solar irradiated *C. jejuni* may reduce the ability of macrophages to engulf the microbes quickly and effectively. Microbial-host sphingolipid interaction may clarify several aspects of the immune system. As signalling molecules, sphingolipids may cause actions and reactions that would either prevent host damage from the microbe (or from the host itself) without the development of a disease or promote the initiation of damage signals that could facilitate a pathological process. It has been postulated that inhibiting pathogen-used sphingolipids limits some of the microbes' pathogenicity. Therefore, a better understanding of sphingolipid-mediated microbial pathogenesis and how to change sphingolipid interactions to benefit the host can forge new therapeutic strategies and provide alternatives to conventional chemotherapy approaches to infectious disease (Heung *et al.*, 2006).

7.4.3 Metabolic pathway analysis

Metabolic pathway changes have been shown to regulate macrophage activation states and functions (Eisenreich *et al.*, 2017). The two major macrophage subtypes, M1 (proinflammatory bactericidal) and M2 (anti-inflammatory) show different metabolic signatures (Eisenreich *et al.*, 2017). Two major metabolic pathways of classically activated M1-macrophages are increased glycolysis and increased activity of the pentose phosphate pathway (PPP). The glycolysis pathway supports multiple proinflammatory functions including the synthesis of ATP to support phagocytic functions including the feeding the PPP for nucleotide and protein synthesis and the generation of reactive oxygen species by NADPH oxidase (Curto *et al.*, 2019).

Several metabolic pathway GO-terms including metabolic process (GO:0008152), glycolytic process (GO:0006096) and pentose phosphate shunt (GO:0006096) were significantly enriched in SI4-treated macrophages. (Fig. 61). Interestingly, our results show that SI4-

treated macrophages showed that DEPs involved in host glycolysis activity and PPP were downregulated at 24 h post-infection and this is characteristic of M2 macrophages. Similar results were obtained in *R. conorii* and *R. montanensis* infected THP-1 macrophages (Curto *et al.,* 2019).

The metabolic characteristics of alternatively activated macrophages (M2-macrophages) also include an intact citrate pathway (TCA cycle) and oxidative phosphorylation. However, the TCA cycle is disrupted in two places in inflammatory macrophages (M1) and thus oxidative phosphorylation is reduced (Van den Bossche *et al.*, 2017). In this study, the DEPs responsible for TCA and oxidative phosphorylation were significantly upregulated in macrophages treated with both SI4 and SI8 solar irradiated *C. jejuni* (Table 8) possibly implying that solar irradiated *C. jejuni* may elicit an anti-inflammatory response in the host. These findings correlate with previously unpublished data which show that solar irradiated *C. jejuni* elicits an apoptotic response in macrophages (Chihomvu *et al.*, unpublished-a), and apoptosis is generally associated with an anti-inflammatory response in the host. Another study also showed a decrease in the production of proinflammatory cytokines during late infection with solar irradiated *C. jejuni* (Chihomvu *et al.*, Unpublished-b).

7.4.4 Antigen processing and presentation pathways of macrophages infected with solar irradiated *C. jejuni*

The DEPs involved in the endoplasmic reticulum processing, protein processing, phagosome and antigen processing, and presentation pathways were upregulated during the late stages of infection (24 h p.i.) (Table 8) in macrophages treated with solar irradiated *C. jejuni*. The upregulation of DEPs involved in MHC class I pathway (Figs. 63-66, Table 8) in macrophages treated with solar irradiated *C. jejuni* could elicit cellular immune responses for protection in users of SODIS-treated water. MHC class I molecules may bind to endogenous-derived peptides from cytosolic antigens (Actor, 2014), (Figs 63-64), such as antigenic material produced from the SODIS-treatment of *C. jejuni* within the host cell. Protein degradation of solar irradiated *C. jejuni* may occur inherently inside the proteasome (Figs. 65-66) and the processed antigenic materials are transported to the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP) protein. The peptides are further processed and added to the MHC class I (Actor, 2014) and, once the peptide is bound to MHC class I, the complex is stabilized by a β2-macroglobulin (β2M) (Figs. 63-64) which was upregulated in SI4- and SI8-treated macrophages. The MHC class I and the β2-microglobulin

are then exported to the cell surface membrane. The entire surface molecule becomes recognisable by CD8 T cells (cytotoxic T cells) (Actor, 2014).

However, the proteins associated with the proteasome were downregulated in SI4-treated macrophages. Downregulation of proteasome activity is associated with neurodegenerative diseases as such as Huntington's disease (Zhou *et al.*, 2003) and Alzheimer's (Keck *et al.*, 2003; Oh *et al.*, 2005) and this correlates with the KEGG pathway analysis for SI4-treated macrophages (Table 8). A downregulation in proteasome activity may interfere with antigen presentation by MHC class I and recognition by the immune system. Also, this potential loss of proteasome function can cause endoplasmic stress due to the building up of misfolded proteins in the endoplasmic reticulum lumen (Curto *et al.*, 2019). In this study, the GO-term for the response to endoplasmic stress (GO:0034976) and protein folding (GO:0006457) were significantly enriched (Figs. 61, 62) suggesting that proteasome activity could have been impaired in the presence of solar irradiated *C. jejuni*. However, a previous study has shown that inhibition of proteasome activity induces the formation of alternative proteasome complexes such PA200 complexes. Alternative proteasome complexes persist until cells regain proteasome activity; thus, antigen processing and presentation through the MHC class I pathway can continue (Welk *et al.*, 2016).

Several factors such as oxidative stress may inhibit proteasome activities (Ishii *et al.*, 2005). In addition, Cuschieri *et al.* (2004) showed that pharmacological inhibition of proteasomes in macrophages could lead to inflammatory signalling dysregulation; thus, macrophages are converted into an anti-inflammatory phenotype (Cuschieri *et al.*, 2004). This correlates with our earlier research which found that solar irradiated *C. jejuni* may have some anti-inflammatory effects on macrophages (Chihomvu *et al.*, Unpublished-b). It could be assumed that solar irradiated bacteria upon internalisation by phagosomes are recognised as either endogenous or exogenous antigens which can be presented by the MHC-I pathway (Figs. 63-64), thus eliciting a CD8+ CTL-mediated response to achieve immune protection in SODIS users.

Heat shock proteins (HSP) were detected in macrophages treated with 8h-solar irradiated *C. jejuni* (SI8) (Fig. 64). Heat shock proteins are extremely preserved and ubiquitous proteins that perform an important part as molecular chaperones in the folding and storage of proteins within the cell (Craig *et al.*, 1993). HSPs also can stimulate the processing of MHC class I antigen (Fig. 66) (Murshid *et al.*, 2012). HSP / peptide interactions are generally formulated by antigen-presenting cell-specific receptors whose signalling results in the HSP-associated peptide presentation of MHC-I and may result in the response of CD8 + cytotoxic T cells (Murshid *et al.*, 2012). The antigenic peptides chaperoned by HSPs are known to be more

efficient, in terms of the magnitude of the immune response, than the free peptides for presentation by MHC-I (Murshid *et al.*, 2012). A previous study by Fagone *et al.* (2012) showed significant up-regulation of members of the HSP70 family (HSPA2 and HSPA8) as well as the HSP90 family (HSP90AA1) in M1 phenotype macrophages, indicating that heat-shock proteins may be specifically involved in the macrophage pro-inflammatory activation. Interestingly, HSPBAP1 represents the only protein that was significantly downregulated in both M1 and M2 macrophages when compared to naive macrophages. Thus, they postulated that the acquisition of a specific phenotype from the macrophage is strictly dependent on a complex pattern of expression of different HSP class members. In the current study, the heat shock protein, heat shock cognate 70 kDa protein (HSP70), was found to be upregulated following macrophage treatment with 8h-solar irradiated *C. jejuni*. The activated HSPs suggested that the internalised solar irradiated bacteria were processed and loaded onto MHC class I molecules, and this could potentially initiate the CTL-mediated response in users of SODIS-treated water. However, the heat shock proteins (HSP70 and HSP90) were downregulated in SI4-treated macrophages and may dampen the magnitude of the immune response to SI4-treated macrophages.

The folding and assembly of MHC class I molecules requires interactions in the endoplasmic reticulum with several chaperone molecules some of which are unique to MHC class I molecules (Paulsson & Wang, 2003). Some of the chaperones that were upregulated in macrophages treated with 4h-solar irradiated *C jejuni* were calreticulin (CALR) and calnexin (CANX) (Fig. 11) providing further evidence that an active MHC class I processing pathway was stimulated by solar irradiated *C. jejuni*. Calreticulin (CALR) and calnexin (CANX) are specialised lectin-binding endoplasmic reticulum chaperones that attach briefly to newly synthesised glycoproteins (Leach & Williams, 2003). It is believed that the interaction between calnexin and MHC class I molecules can stabilise the class I heavy chain and help associate it with the β2M component (Diedrich *et al.*, 2001). Additionally, a TAP binding protein (TAPBP) was upregulated in SI4-treated macrophages and this provides further evidence of MHC class I antigen loading (Ghanem *et al.*, 2010).

One of the KEGG pathways that was significantly enriched ($p < 0.05$) in SI4- and SI8-macrophage treatment was the phagosome pathway. Phagocytosis is the receptor-mediated ingestion of large particles, usually by professional phagocytes, i.e., neutrophils, dendritic cells, monocytes, or macrophages, resulting in the formation of a new cytoplasmic compartment, the phagosome. Phagosomes are created when pathogens or opsonins attach to transmembrane receptors that are spread arbitrarily on the cell surface of the phagocyte. After binding, "outside-in" signalling induces actin polymerisation and pseudopodia formation

of the phagocytes. Protein kinase C, phosphoinositide 3-kinase, and phospholipase C (PLC) are all needed for signalling and controlling particle internalization (Aderem, 2003). The prevalent upregulated GEPs were Fc receptor (FcR), Stx7, Rab5, Rab7, v-ATPase, MHCI, and SRA1 in both the SI4 and SI8-treated macrophages (Figs. 65, 66).

The Fc receptor (FcR) was also upregulated in SI4 and SI8-treated macrophages. (Figs. 65, 66). The FcR is a phagocytic receptor, which induces phagocytosis (Underhill & Ozinsky, 2002). Alternatively, activated macrophages (M1) have increased phagocytic receptor expression of FcγRI, FcγRII, and FcγRIII, resulting in increased recognition of IgG-opsonized targets (Mantovani *et al.*, 2004; Edwards *et al.*, 2006; Beyer *et al.*, 2012) and this has an anti-inflammatory effect within the phagocytic cell. The conversion from an early to a late phagosome requires Rab5 (Figs. 65, 66) (Callaghan *et al.*, 1999; Duclos *et al.*, 2000; Murray *et al.*, 2002; Vieira *et al.*, 2003; Kitano *et al.*, 2008). A previous study showed that *Mycobacterium*-containing phagosomes show incomplete luminal acidification (Sturgill-Koszycki *et al.*, 1994), increased accessibility to early endosomes, retention of the early endosome marker Rab5 and the absence of the late endosome marker Rab7 (Clemens & Horwitz, 1996), a lack of lysosomal hydrolases and cathepsins—all of which hinders phagosome maturation (Clemens & Horwitz, 1995; Via *et al.*, 1997). However, in this study, both the Rab5 and Rab7 was highly expressed in SI4- and SI8-treated macrophages inferring that phagosome maturation progressed normally. However, similar to the study carried out by Clemens *et al.*, (1996), no cathepsins were detected.

Lysosomes are vacuolar organelles in which several proteins degrade and they recycle extracellular and intracellular products in an acidic environment. Cells acquire extracellular contents via phagocytic and endocytic processes whereas intracellular materials, such as damaged organelles are delivered to the lysosomes through the autophagic pathway (Appelqvist *et al.*, 2013). The significance of lysosomes in the immune responses of macrophages led us to explore the proteins connected with lysosome function in macrophages infected with solar irradiated *C. jejuni*. Our results showed that several DEPs were upregulated in the lysosomal compartment of macrophages infected with 8h-solar irradiated *C. jejuni*. Some of the DEPs that were upregulated in SI8-treated macrophages were acid hydrolases such as tripeptidyl peptidase (TPP1), arylsulfatase (ARS) and acetylglucosamine-6-sulfatase (GNS). The acid hydrolases (Cooper, 2000) are active in acidic environments (approximately pH 5). The TPP1 enzymes are lysosomal proteases that have a tripeptidyl exopeptidase activity with an optimum pH of 4-5. They belong to the group of serine peptidases (sedolisines) and are present in bacteria and mammals (Wlodawer *et al.*, 2001; Comellas-Bigler *et al.*, 2002). The TPP1 enzyme, removes tripeptides from the N-terminus of

proteins during protein degradation in the lysosome (Tian *et al.*, 2006). Sulfatase enzymes, namely, ARS and GNS enzymes, were also upregulated in this study. The acidic environment inside the lysosome is regulated by the enzyme v-ATPase (ATP-ev) (Nishi & Forgac, 2002; Forgac, 2007; Mindell, 2012). The V-ATPase is a multimeric enzyme structure that carries protons into the phagosome lumen at the expense of cytosolic ATP (Marshansky & Futai, 2008). The enzyme was upregulated in macrophages treated with SI4 and SI8-treated macrophages.

The lysosomal compartment plays a crucial role in cellular homeostasis, energy metabolism, cell clearance, cell signalling, innate immunity, calcium signalling, and apoptosis (Boya, 2012; Settembre *et al.*, 2013). Lysosomes in macrophages play a significant part in innate immune and adaptive immune responses in the body (Murray & Wynn, 2011) because lysosome proteolysis generally generates peptides that bind to the major histocompatibility complex (MHC) molecules (Murray & Wynn, 2011). During the adaptive immune response, antigenic peptides are used to present crucial pathogen information to the T lymphocyte system (Watts, 2012). Thus, we postulate that lysosomes may play a role in the signalling pathways involved in both innate and adaptive immune responses macrophages activated by solar irradiated *C. jejuni.*

7.5 Conclusion

In conclusion, in this work, RAW264.7 macrophage cells were used as a model to investigate the host immune responses to solar irradiated *C. jejuni*. SWATH-MS data revealed that the coordinate up-regulation of MHC-I processing pathways occurred at 24 h p.i. and provides insights into the possible molecular mechanisms of immune protection that may occur in users of SODIS-treated water. It is likely, that solar irradiated *C. jejuni* bacterial proteins undergo proteasomal degradation and the resulting peptides are transported to the endoplasmic reticulum (ER) and loaded onto MHC-I molecules. Peptide loading results in class I complexes consolidation and transit to the cell surface where antigens can be scanned by circulating CD8 + T cells. Additionally, solar irradiated *C. jejuni* also undergoes degradation in the phagosome. The phagosome has the potential to create antigens that can be expressed on the cell surface of macrophages to stimulate different lymphocytes and induce appropriate immune responses, thus, connecting the innate to adaptive immunity and this could also have health benefits to the consumer of SODIS-treated water. The successful application of SWATH-MS technology in the host-interaction model in this work established a new experimental platform

for investigating possible host immune responses to solar irradiated pathogens, and this may have a potential role in the development of peptide-based vaccines.

Chapter 8 An *in-vitro* infection model to assess the proteins expressed during the interaction of solar irradiated and heat-attenuated *S.* Typhimurium with murine macrophages

Abstract

Salmonellosis, caused by *Salmonella enterica* serovar Typhimurium, is a prevalent gastrointestinal disease in developing countries. It is a waterborne disease that can be prevented by solar disinfection (SODIS) of unsafe drinking water. It is known that *S.* Typhimurium can be inactivated in contaminated water using solar ultraviolet radiation (SUVR). The consumption of SODIS-treated water has resulted in a reduction of diarrheal incidences in areas where this treatment method has been implemented to make potable water. Little is known about the immunological profile of the host response to solar irradiated *S.* Typhimurium. In this study, we utilised a SWATH-mass spectrometry-based proteomics approach to investigate and compare global proteomic changes in macrophages treated with LPS, non-solar irradiated, and solar irradiated *S.* Typhimurium. A total of 11 peptides were identified at 1% FDR in macrophages treated with heat/ chemically attenuated *S.* Typhimurium (HA). Gene ontology analysis revealed that two of the downregulated-regulated proteins (*Ifitm*3 and *Stat*1) were involved in the type 1 interferon signalling pathway. The two upregulated proteins, *Hmox1* and *Sqstm1,* are usually overexpressed in alternatively activated macrophages (M2 phenotype). However, there were no significant differentially expressed proteins (DEPs) detected in macrophages treated with lipopolysaccharides, non-solar irradiated, and solar irradiated *S.* Typhimurium perhaps due to an overestimation of protein quantity before SWATH-MS analysis. Thus, it is recommended to carry out protein quantification and normalisation post-hydrophilic interaction liquid chromatography (HILIC) clean-up and digestion before SWATH-MS analysis.

Keywords: *Salmonella* Typhimurium, proteomics, solar disinfection (SODIS), macrophages, activation, interaction

8.1 Introduction

The unavailability of sufficient potable water in resource-poor communities is an important health concern worldwide. The implementation of cheap home water treatment methods such as solar disinfection (SODIS) has made significant strides towards the reduction in the number of people without access to improved water sources (Millenium Development Goals, 2015). The use of SODIS to treat microbiologically contaminated water has reduced the risk of acquiring waterborne diseases among its users in over 30 countries worldwide (Eawag, 2014). SODIS involves exposing microbiologically contaminated water in glass or polyethylene terephthalate (PET) bottles to the sun for an approximate period of 6-8 hours, depending on the weather (McGuigan *et al.*, 2012).

The consumption of SODIS treated water could mitigate the spread of zoonotic pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) that is transmitted through drinking contaminated water. The non-typhoidal serovar (NTS) causes self-limiting gastroenteritis in humans, which is commonly zoonotic in origin (Zhang *et al.*, 2003; de Jong *et al.*, 2012). Solar disinfection was shown to be effective in inactivating the organism, possibly by cell death, in contaminated water (Smith *et al.*, 2000; Winfield & Groisman, 2003; Berney *et al.*, 2006). In general, cellular death of bacteria in water results in the release of antigenic materials such as glycopeptides, lipopolysaccharides, lipopeptides, and deoxyribonucleic acids (Bessler *et al.*, 1997). Thus, the availability of antigenic determinants in SODIS water might initiate an immune response in people drinking SODIS water. Prolonged exposure of water to solar irradiation during SODIS might result in minimum modifications or extreme modifications of antigenic epitopes. These antigenic epitopes may result in the inhibition of a prompt immune response. The alternative is that SODIS may induce intermediate modifications of antigenic material of solar irradiated bacteria and induce an immune response in users of SODIS-treated water (Ssemakalu *et al.*, 2014).

Macrophages have a defensive function against pathogens such as bacteria and play an important role in the body's homeostatic maintenance by removing internal waste materials and repairing tissue. Moreover, macrophages can present antigens to T cells and act as cell-mediated immunity effectors; it is known that they inhibit the development of infectious diseases, cancers, and chronic inflammatory diseases such as arteriosclerosis. Mills *et al.* (2000) categorised macrophages as M1 (classically activated macrophages) and M2 (alternatively activated macrophages) based on their roles (Mills *et al.*, 2000). The definition of M1 and M2 macrophage polarity is derived from the pre-genomic era were a few markers were considered to establish variances and similarities in macrophage responses to stimuli. However, updated knowledge of cytokine signaling now exists due to the emergence of transcriptomic and proteomic technologies. Proteomic analysis reveals a far more complicated picture and challenge to the current classical M1 and M2 grouping of macrophage activation

(Martinez & Gordon, 2014). Additionally, macrophages play a crucial in the adaptive immune system by acting as antigen-presenting cells (APCs) along with other signalling molecules / factors needed in the successful adaptive immune response to infection (Kumar, 2019). Thus investigating host-cell interactions between a SODIS-treated pathogen and macrophages may shed some light on how the macrophages react to solar irradiated pathogens.

Proteomics has been regularly used to analyse the reaction of cells and organisms to a changing environment, including growth under different culture conditions and diverse food sources, temperature, nutrients, oxygen, osmotic stress, and toxins (Zai *et al.*, 2017). There are limited studies that have used quantitative proteomics to assess the impact of solar irradiation on microorganisms and their immunogenic potential in a host. It has been reported that the minimum requirement for antigens to stimulate the development of antibodies in the immune system is a molecular weight of 10 kDa (Lindahl *et al.*, 2005). Since the proteomic profiles of pathogens are altered (Bosshard *et al.*, 2010b), this could affect the host cell interactions of irradiated pathogens. Therefore, it is crucial to gain a better insight into the functional interface that exists between solar irradiated *S.* Typhimurium and macrophages.

Salmonella Typhimurium-infected macrophages are often used to investigate the molecular mechanisms underlying the interactions between macrophage and intracellular pathogens. Once macrophages engulf *S.* Typhimurium, the pathogen resides in a modified phagosome known as the Salmonella-containing vacuole (SCV) in which virulent *S.* Typhimurium strains can control SCV biogenesis. The type III secretory system (T3SS) of *Salmonella* pathogenicity island 2 is involved in controlling SCV biogenesis in macrophages. The T3SS apparatus is a needle-like structure that connects the cytoplasms of *S.* Typhimurium and host macrophage cells; this configuration permits direct transportation of bacterial effector proteins into the cytosol of host cells (Marcus *et al.*, 2000; Kuhle *et al.*, 2004). The primary functions of bacterial effector proteins are to block the fusion of SCV with the cellular compartments possessing antibacterial activities, e.g., lysosomes and vesicles containing functional phagocyte NADPH oxidase or inducible nitric oxide synthase (iNOS). By preventing the delivery of antibacterial proteins and peptides into the SCV, *S.* Typhimurium pathogens can avoid macrophage defense mechanisms, and this enables the pathogen to proliferate inside the SCV (Vazquez-Torres & Fang, 2001; Vazquez-Torres *et al.*, 2001; Holden, 2002; Chakravortty & Hensel, 2003; Knodler & Steele-Mortimer, 2003)

The precise molecular mechanisms involved in the different stages of infection, i.e., adhesion, invasion, and replication, are still poorly understood. The intricate interaction between host cells and pathogens involves several hundred to thousands of proteins on both sides (Hartlova *et al.*, 2011; Zhang & Ge, 2011). The elucidation of the molecular mechanisms in host-pathogen interactions is crucial for the control and treatment of infectious diseases worldwide.

Within the last decade, mass spectrometry (MS)- based proteomics has become a practical approach to better elucidate complex and dynamic host-pathogen interactions at the protein level (Yang *et al.*, 2015). Most of the studies have previously focused on the characterization of individual bacterial virulence factors and their interacting host targets by using traditional methods such as genetic and biochemical pathways. These methods have contributed tremendously to the current body of knowledge with regards to infection biology. However, such studies alone are insufficient to explain the complex nature of host-pathogen interactions (Walduck *et al.*, 2004).

Several studies have been carried out on the *Salmonella* proteome (Adkins *et al.*, 2006; Ansong *et al.*, 2008; Shi *et al.*, 2009). Though proteomic studies of bacteria cultured in bacteriological media have been extensively studied, analysis of their protein expression within infected host cells is limited (Schmidt & Völker, 2011). Proteomic measurements are a challenge because limited amounts of bacterial proteins are present together with an abundance of host cell components (Schmidt & Völker, 2011; Sengupta & Alam, 2011). Currently, there are a few studies that have reported the *Salmonella* proteome upon interaction with the host cells. For instance, GFP-expressing *S.* Typhimurium was isolated by FACS from mouse tissues, and 370 *Salmonella* proteins from typhoid fever models were identified. Most of the characterized proteins were metabolic enzymes, a majority of which were found to be non-essential for *Salmonella* virulence (Becker *et al.*, 2006). In another study*, S.* Typhimurium was isolated from RAW264.7 murine macrophages by centrifugation and protein expression levels of the pathogen were analysed at various time points. In total, 315 proteins were examined, and 39 proteins were significantly altered. Nonetheless, the study showed a somewhat limited proteome coverage, given the fact that the *Salmonella* genome encodes approximately 4500 proteins (Shi *et al.*, 2006).

The host proteome is much more complicated than that of bacterial pathogens because there is a greater abundance of proteins present with higher dynamic ranges. Therefore, only a few studies have reported on the host proteome after infection. Instead, host cell transcriptome profiling has been more widely practiced in host-pathogen interactions (Jenner & Young, 2005). A time-course proteomic study of RAW264.7 macrophages upon *S.* Typhimurium infection was carried out . The results showed a global proteomic response to *S.* Typhimurium infection. They also postulated that upon infection *S.* Typhimurium increases the abundance of the mitochondrial SOD2 and decreases the amount of SNX6, most likely via *S.* Typhimurium virulence factor sopB (Shi *et al.*, 2009).

Despite the advances made in elucidating macrophage-*S.* Typhimurium interactions, it is still unclear as to how macrophages respond to infection by solar-irradiated *S.* Typhimurium. Therefore, this study assessed the way in which macrophages react to infection by solar-irradiated *S.* Typhimurium by using a global proteomic approach.

8.2 Methodology

8.2.1 Growth and cultivation of *S.* Typhimurium

In all the experiments, *Salmonella enterica* subsp. *enterica serovar* Typhimurium (ATCC® 29629™) was used from cryo-cultures that was streaked onto Luria Bertani (LB) agar plates. For batch cultivation, LB broth was autoclaved and diluted to one-third of its original strength. Erlenmeyer flasks containing 30 ml of diluted LB were inoculated with a single colony and incubated at 37°C with vigorous shaking until the cells reached exponential growth (OD_{546} between 0.1 and 0.2). The cultures were diluted to an OD_{546} of 0.002 into 150 ml of pre-warmed diluted LB in a 1000 ml Erlenmeyer flask and shaken overnight for 18 h until the stationary phase was reached. The stationary phase was determined by taking five consecutive OD_{546} readings (Bosshard *et al.*, 2010).

8.2.2 Sample preparation of *S.* Typhimurium prior to SUVR exposure and enumeration after exposure

The cultured *S.* Typhimurium bacterial cells were harvested by centrifugation at 16 000 xg for 15 min and washed thrice with autoclaved still mineral water. The pellet was suspended in sterile mineral water up to an OD_{546} of 0.1. The cell suspensions were incubated for 1 h at 37°C to allow the cells to adapt to the mineral water. Aliquots of 15 ml of the cell suspension were exposed to SUVR light in 25 cm^3 tissue culture flasks. For each of the experiments, controls were exposed to similar atmospheric conditions except to SUVR by enclosing the samples in an opaque black ventilated box (Ssemakalu, 2010a). Samples were enumerated by the Miles and Misra method (Miles *et al.*, 1938) and collected for proteomic analysis at different SUVR exposure time points of 2, 4, 6, and 8 h.

The SODIS experiments were carried out on the roof of the laboratory at the Vaal University of Technology in South Africa (26°42'39.1"S 27°51'46.2"E -26.710858, 27.862820) from 8.00am-4.00pm.

8.2.3 Infection of macrophage cells

RAW 264.7 murine macrophage cells were obtained from Cellonax and were grown in Dulbecco,s Modified Eagle's Medium (DMEM) (Gibco), with 10% fetal bovine serum (Invitrogen) and 1% Pen/Strep (Invitrogen). The macrophages were seeded to a density of 5×10^4 cells/well in a medium containing 10% fetal bovine serum, 2mM glutamine, 100 µg/ml antibiotic) in a 6-well culture plate which was kept for 48 hrs in a CO_2 incubator. The wells were washed thrice with phosphate buffer solution (Invitrogen) and treated with *S.*Typhimurium at an infection ratio of 1:10. The infected macrophages were incubated for 3 hr in a CO_2 incubator at 37ºC. After 3 h, the plates were washed to remove the extracellular bacterial cells. The wells were replenished with a medium containing gentamicin (20 µg/ml) to prevent extracellular replication of bacteria (Singhal *et al.*, 2012). At 24 h p.i. the cells were

carefully scraped and transferred to 2 ml microcentrifuge tubes. The tubes were centrifuged at 300 g for 5 min, and the culture media was removed. The pellet was gently re-suspended in pre-chilled PBS buffer (2 ml per 500,000 cells). The cells were washed by centrifugation at 300 g for 5 min and removing the supernatant. The washing step using chilled PBS was repeated 5 times, and the last resuspension was aliquoted into batches of 100,000-500,000 cells/ml. The suspensions were centrifuged at 300xg for 5 min, and excess PBS was removed without disturbing the pellet. The cells were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

8.2.4 Protein extraction and trypsin digestion

Same as section 7.2.6

8.2.5 Sample Preparation: Protein Extraction, Reduction, and Alkylation

Same as section 7.2.6.1

8.2.6 HILIC: Automated Protein Clean-up and Digestion

Same as section 7.2.6.2

8.2.7 LC-MS data acquisition

Same as section 7.2.7

8.2.8 Protein identification and spectral library building

Same as section 7.2.8

8.2.9 SWATH-MS processing

Same as section 7.2.9

8.2.10 Protein regulation profiling

Same as section 7.2.10

8.2.11 Functional annotation of DEPs

Same as section 7.2.11

8.3 Results

SWATH-LC-MS/MS was used to analyse how LPS, non-solar irradiated, and solar irradiated *S.* Typhimurium affect the proteome in macrophages. Only the proteins with at least 2-fold change (FC) (log2FC ≥ 1) and a false discovery rate (FDR) of less than 0.01 (FDR ≤ 0.01) were considered for further analysis. The differentially expressed proteins (DEPs) of treated macrophages were compared with those of the non-treated macrophage controls. In total, 11 differentially expressed proteins (DEPs) met these criteria in macrophages treated with heat-attenuated *S.* Typhimurium (HA). However, no significant DEPs were detected in other treatments. The list of the proteins that were significantly expressed in HA-treated macrophages is shown in Table 9.

Table 9 Differentially expressed proteins in RAW264.7 macrophages treated with heat attenuated *S.* Typhimurium.

UNIPROT ID	Gene Name
Q91ZV0	melanoma inhibitory activity 2(Mia2)
Q9CQW9	interferon-induced transmembrane protein 3(Ifitm3)
Q921H8	acetyl-Coenzyme A acyltransferase 1A(Acaa1a)
P17563	selenium binding protein 1(Selenbp1)
P42225	signal transducer and activator of transcription 1(Stat1)
P11370	Friend virus susceptibility 4(Fv4)
Q9D8C4	interferon-induced protein 35(Ifi35)
Q64337	sequestosome 1(Sqstm1)
Q91VD9	NADH dehydrogenase (ubiquinone) Fe-S protein 1(Ndufs1)
Q8CG47	structural maintenance of chromosomes 4(Smc4)
P14901	heme oxygenase 1(Hmox1)

The response of RAW264.7 macrophages to heat attenuated *S.* Typhimurium is shown in Figure 67. The following six proteins were downregulated: 2(Mia2), 3(Ifitm3), 1A(Acaa1a), 1(Selenbp1), 1(Stat1) and, 4(Fv4) while the following DEPs were significantly up-regulated 35(Ifi35), 1(Sqstm1), 1(Ndufs1), 4(Smc4) and, 1(Hmox1).

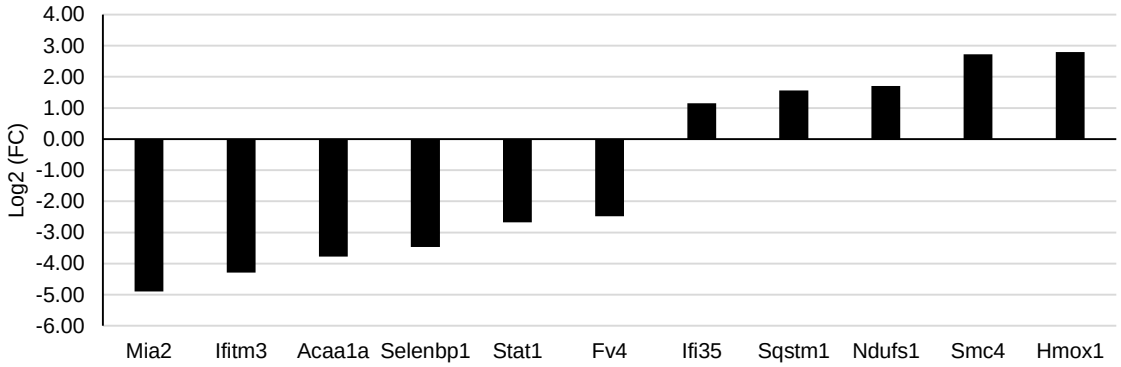


Figure 67 The change fold of differentially expressed proteins in macrophages treated with heat-attenuated *S.* Typhimurium (HA). The protein abundance after infection was compared to the non-treated macrophages.

The interaction network between the differentially expressed proteins (DEPs) in HA-treated macrophages was significant with a p-value = 0.00397 (Fig. 68), indicating that the proteins are at least partially biologically connected. Two significant clusters were formed using MCL clustering. The first cluster represented a type 1 interferon signalling pathway, and the second cluster was functionally enriched for exploiting mitochondrial and metabolic homeostasis (Fig. 68).

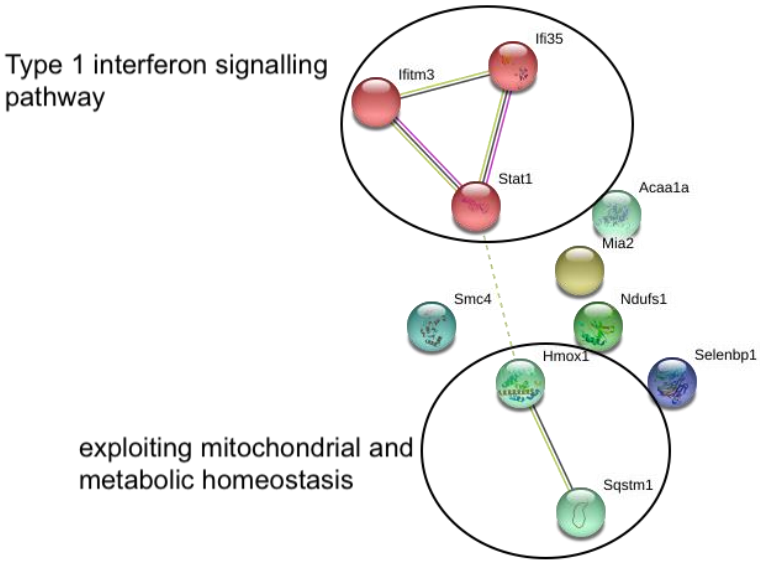


Figure 68 Protein analysis of significant (abs. $log_2FC \geq 1$; adj. p-value ≤ 0.01) differentially expressed proteins (DEPs) in HA-treated macrophages vs. untreated macrophages. STRING analysis was carried out with minimum required interaction score set to "medium confidence 0.400," and only query proteins were included and, MCL clustering was performed relative to the confidence score of the interaction. GO analysis was performed on the resulting clusters.

The biological process ontology for macrophages treated with heat attenuated *S.* Typhimurium (HA) showed 6 **non-significant** enriched terms (p-adjust value > 0.05) using the Benjamin-Hochberg procedure. The six terms were positive for regulation of smooth muscle cell proliferation (GO:0048661), apoptotic process (GO:0006915), response to hydrogen peroxide (GO:0042542), positive regulation of interferon-beta (GO:0035456) and, type I interferon signaling pathway (GO:0060337) (Fig. 69).

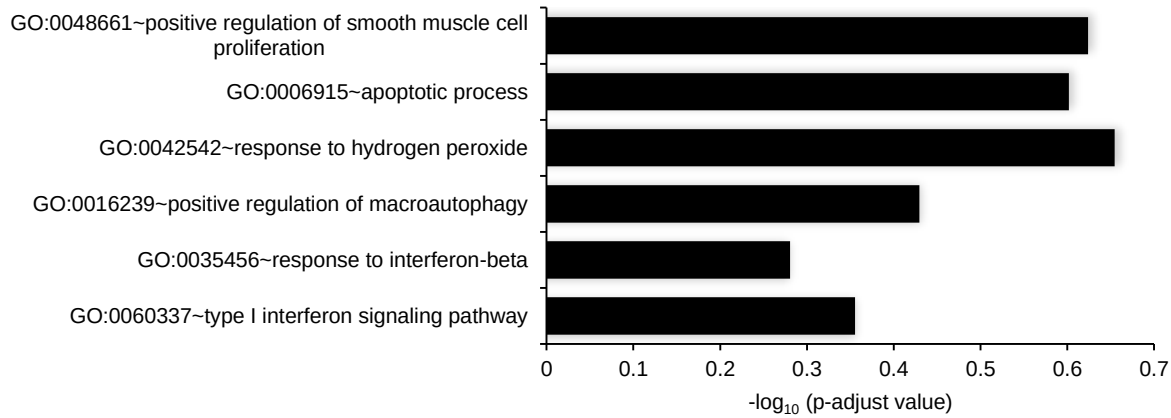


Figure 69 Gene Ontology (GO) enrichment analysis of differentially expressed proteins (DEPs) was classified by their biological functions and arranged according to their statistical significance ($-log_{10}$p-adjust value on the x-axis) from DAVID.

8.4 Discussion

In this study, no significant DEPs were detected in the macrophages treated with LPS, non-solar irradiated, and solar irradiated *S.* Typhimurium*.* This may be due perhaps to the over-estimation of protein quantity before the HILIC clean-up and trypsin digestion that ultimately resulted in significant under-loading for the SWATH analysis. It was likely that the amount of protein was over-estimated using the 2D-Quant kit. The GE Healthcare's 2-D Quant Kit (Uppsala, Sweden) procedure operates by quantitatively precipitating proteins while leaving substances that might interfere with the quantification reaction behind in solution. The assay is based on the specific protein binding of copper ions. In a copper-containing solution, precipitated proteins are resuspended, and unbound copper is analysed using a colorimetric agent. However, there are several total protein quantitation techniques that may be considered for future experiments; these may include traditional techniques such as the 280 nm UV absorbance measurement, Bicinchoninic acid (BCA) and Bradford assays, as well as alternative methods such as Lowry methods and other novel assays produced by commercial suppliers, often offering a well-designed kit for each assay type (Johnson, 2012). The Thermo ScientificTM Pierce^{TM} BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection and total protein quantitation that is being considered for future quantitation analysis. This technique combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the extremely sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid (Smith *et al.*, 1985). This assay's purple-coloured response product consists of the chelation of two BCA molecules with one cuprous ion. This water-soluble complex has a strong 562 nm absorbance that is almost linear with increased protein levels over a wide working range (20-2000 µg / mL). The BCA technique is not a real endpoint technique; that is, it continues to create a complete colour. However, the pace of ongoing colour development is slow enough after incubation to allow a large number of samples to be tested together (Smith *et al.*, 1985). Accurate quantitation of protein is crucial in mass spectrometry. Therefore, it is recommended to carry out peptide quantification pre- and post-HILIC clean-up and digestion. Post- quantification of protein after HILIC will allow for the adjustment and normalization of the protein loads before SWATH-MS analysis.

However, DEPs were detected in macrophages treated with heat attenuated *S.* Typhimurium (Table 9). The main protein that was downregulated by heat attenuated *S.* Typhimurium was interferon-induced transmembrane protein 3 (*Ifitm*3). This protein plays a vital role in the type 1 interferon signaling pathway (Figs 68, 69). Interferon-γ is an important cytokine crucial for immune cell responses against infectious microbes, and individuals with loss of IFN-γ or its receptor show greater susceptibility to less virulent pathogens (Ottenhoff *et al.*, 1998).

The interferon (IFN)-induced transmembrane protein (*Ifitm*) genes consist of a family of genes encoding the following proteins: Ifitm1, 2, 3, 5, 6, 7, and 10 in mice and IFITM1, 2, 3, 5, and 10 in humans (Hickford *et al.*, 2012; Zhang *et al.*, 2012) and, *Ifitm3* was found to be the most potent antiviral *Ifitm* protein in cell culture (Schoggins *et al.*, 2011; Zhang *et al.*, 2012). However, *Ifitm3* does not restrict all pathogens such *as Salmonella enterica* serovar Typhimurium, *Citrobacter rodentium*, *Mycobacterium tuberculosis, or Plasmodium berghei* (Everitt *et al.*, 2013). Another protein that was downregulated by heat attenuated *S.* Typhimurium was the *Stat1* protein. *Stat1* has been known to activate the expression of several pro-inflammatory genes such as nitric oxide synthetase (iNOS) and also mediates IFN signalling (Shaheen & Broxmeyer, 2017). In another study, *Sta*t1−/− mice were shown to be highly susceptible to *Listeria monocytogens* and viral infections (Meraz *et al.*, 1996). Thus, IFN-γ and its downstream signaling molecules in the host are essential in the regulation of immune responses, especially against infectious diseases (Chandrasekar *et al.*, 2013). Thus, the downregulation of the DEPs may inhibit the expression of IFN-γ, and this correlates with findings in a previous study, which showed that IFN-γ was undetectable in RAW264.7 macrophage cells treated with heat-attenuated *S.* Typhimurium (Chihomvu *et al.*, Unpublished-b).

In this study, we identified *Sqstm1* and *Hmox1* as significantly upregulated proteins following treatment of RAW264.7 murine macrophages with heat attenuated *S.* Typhimurium (Fig. 67). The two DEPs formed the second cluster in the network (Fig. 68). Heme oxygenase (*Hmox1*) is the rate-limiting microsomal enzyme that catalyzes heme degradation (Maines, 1997). A previous report has previously shown that *Homx1* has been involved in the modulation of several immunological events in macrophages such as inflammasome activation, bactericide, and efferocytosis (Kim & Lee, 2013). Moreover, it was postulated that high expression levels of *Hmox1* drive the phenotypic shift of macrophages to the anti-inflammatory phenotype (M2) (Naito *et al.*, 2014). Thus, they concluded that *Hmox1* expression may become a marker protein of M2 macrophages, which might reflect an intracellular redox status, and Hmox1 induction in macrophages may be regarded as a potential therapeutic approach to immunomodulation in many important human diseases (Naito *et al.*, 2014).

Another protein that was upregulated was *Sqstm1*. The Sqstm1 protein has also been implicated in the regulation of anti-inflammatory response in macrophages (Mylka *et al.*, 2018). They reported a novel mechanism by which *CpdA* mitigates macrophage inflammation via the transcriptional upregulation of the autophagy receptor *Sqstm1* protein (Mylka *et al.*, 2018).

8.5 Conclusion

In conclusion, no significant differentially expressed proteins were detected in the macrophages treated with LPS, non-solar irradiated, and solar irradiated S*.* Typhimurium*.* This

may be due to the low protein concentrations in the samples after HILIC clean-up and digestion. It is recommended to carry out protein quantification and normalisation post-HILIC clean-up and digestion. However, the study was able to analyse DEPs in macrophages treated with heat-attenuated *S.* Typhimurium. The macrophages may have adapted an anti-inflammatory M2 phenotype because the IFN-γ signalling pathway was downregulated, and this may have contributed to the non-expression of the chemokine IFN-γ in RAW264.7 cells (Chihomvu *et al.*, Unpublished-b). Moreover, proteins such as *Hmox1* and *Sqstm1* that were upregulated are usually overexpressed in M2 macrophages.

Chapter 9 Conclusion and recommendations

9.1 Conclusion

The use of inexpensive home water treatment methods such as solar disinfection (SODIS) to treat microbiologically contaminated water has reduced the cases of diarrhoeal diseases especially in areas where SODIS is being implemented. The consumption of SODIS treated water could mitigate the spread of waterborne zoonotic pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Campylobacter.* These intracellular organisms behave quite differently when exposed to SUVR; *S.* Typhimurium is resistant whereas *C. jejuni* is highly susceptible to solar disinfection. Therefore, these bacteria are good models for comparing how susceptible and more resistant organisms may potentially impact the immune system of host organisms as shown in the Thesis Summary (Fig. 70).

SODIS treatment of microbiologically unsafe water usually results in bacterial cell death that produces antigenic materials such as lipopolysaccharides, lipopeptides, and deoxyribonucleic acids. These antigenic determinants in SODIS water may elicit an immune response while the water can act as an adjuvant. Thus, investigating host-cell responses between the SODIS-treated pathogens and immune cells such as macrophages may shed some light on how they respond to solar irradiated pathogens.

The aim of this study was to investigate the effect of solar irradiated *S.* Typhimurium and *C, jejuni* on the proliferation and activation of macrophages

In order to achieve the aim of the study, the following objectives were set:

To characterise the effects of solar irradiated *S.* Typhimurium and *C. jejuni* on intracellular growth, cytotoxicity and modulation of cell death on macrophages.

To investigate the chemokine and cytokine profiles of macrophages infected with solar irradiated *S.* Typhimurium and *C. jejuni*.

To analyse the host-cell interactions of macrophages infected with solar-irradiated and non-solar irradiated *S.* Typhimurium and *C. jejuni* using a proteomic approach.

The first objective was to characterise the effects of solar irradiation on the viability of *S.* Typhimurium and *C. jejuni.* This study showed that solar irradiation inactivates *S.* Typhimurium after 4 h and as a result the virulence properties of the organism, especially the intracellular survival of the pathogen is eliminated. Therefore, SODIS treatment of water contaminated with *S.* Typhimurium may be safe for drinking. However, non-solar irradiated *C. jejuni* entered a VBNC state. Bacteria that enter the VBNC state pose a significant threat to public health, mainly due to the difficulty in detecting the bacteria and their potential to resuscitate in the host's body. Thus the metabolic activity of both the non-solar and solar irradiated *C. jejuni* was determined using the Alamar Blue metabolic assay. It was found that non-solar irradiated

C. jejuni remains viable and metabolically active whereas solar irradiated *C. jejuni* are non-viable and metabolically inactive (Fig. 70).

The second objective investigated the potential of resuscitation of solar irradiated *S.* Typhimurium and *C. jejuni* within macrophages (intracellular growth). The results showed that SODIS treatment of both *S.* Typhimurium and *C. jejuni* eliminates the possibility of resuscitation within the host cell, even after 48 h. Intracellular growth and replication is an important virulence trait in intracellular bacteria because they need to proliferate and multiply in order to elicit sickness in host organisms. Intracellular pathogens are usually invasive pathogens capable of replicating within host cells as well as enhancing host cell survival to prolonged infection. The intracellular growth of bacteria inside the host cells is notably the leading cause of the severity of gastroenteritis.

The third objective investigated the cytotoxicity and modulation of cell death of solar irradiated *S.* Typhimurium and *C. jejuni* on macrophages. Solar radiation of *S.* Typhimurium and *C. jejuni* reduced the cytotoxic and cytopathic effects in RAW 264.7 macrophages when compared to the non-solar radiated control. Moreover, the solar irradiated *S.* Typhimurium were shown to elicit pyroptotic cell death; this is usually associated with pro-inflammatory responses in host cells. However, solar irradiated *C. jejuni* elicited apoptotic cell death in macrophages which usually is immunosuppressive. However, the extent of pyroptotic and apoptotic cell death was less than the necrosis and apoptosis inflicted by non-solar irradiated *S.* Typhimurium and *C. jejuni*, respectively (Fig. 70).

The fourth objective evaluated the chemokine and cytokine profiles of macrophages infected with solar irradiated *S.* Typhimurium and *C. jejuni*. This study showed that both non-solar and solar irradiated *S.* Typhimurium suppressed the expression of iNOS in macrophages, whereas heat/chemical attenuated *S.* Typhimurium elicited high levels of iNOS. The chemokine and cytokine profiles showed that solar irradiated *S.* Typhimurium may elicit a pro-inflammatory response whereas heat attenuated samples may be immunosuppressive. The pro-inflammatory immune response elicited by solar inactivated *S.* Typhimurium was almost as strong as that of the live *S.* Typhimurium. These findings are significant in vaccine development because if solar irradiated *S.* Typhimurium is able to elicit a strong immune response in host organisms, it means that immunity may last longer. The profile of cytokines and chemokines secreted by macrophages in response to solar irradiated *S.* Typhimurium is an M1 phenotype which results in a pro-inflammatory immune response (Fig. 70).

The profile of cytokines and chemokines secreted by macrophages in response to solar irradiated *C. jejuni* suggests that it is most likely be a pro-inflammatory immune response during early infection characterised by high expression levels of IL-1ß, IL-15, TNF-alpha, and IP-10. However, during late infection, the pro-inflammatory responses in macrophages treated

with solar irradiated *C. jejuni* are reduced; this implies a shift to a potential anti-inflammatory response during late infection periods.

The final objective was to analyse the proteome of the macrophages infected with solar-irradiated and non-solar irradiated *S.* Typhimurium and *C. jejuni.* The gene ontology analysis revealed that most of the up-regulated proteins in macrophages treated with solar irradiated *C. jejuni* were involved in oxidation-reduction processes, endoplasmic reticulum stress, transport, antigen processing and presentation of exogenous peptide antigens via MHC class I (TAP-dependant) and ATP-biosynthetic processes. KEGG-pathways also revealed that some of the up-regulated proteins were involved in lysosomal and phagosome pathways. The results revealed a better understanding of the global proteome of macrophages infected with solar irradiated *C. jejuni*. The identification of proteins involved in the immune response to solar irradiated *C. jejuni* may facilitate the discovery of novel therapeutic targets and effective vaccines against Camypylobacteriosis in both animals and humans. However, there were no significant differentially expressed proteins detected in macrophages treated with LPS, non-solar irradiated, and solar irradiated S*.* Typhimurium because of potential overestimation of proteins before HILIC clean-up and digestion. Thus, it is recommended to carry out protein quantification and normalisation post-HILIC clean-up. However, we managed to analyse DEPs in macrophages treated with heat-attenuated *S.* Typhimurium*.* The macrophages seem to have adopted an anti-inflammatory M2 phenotype because the IFN-γ signalling pathway was downregulated, and this may have contributed to non-expression of the chemokine IFN-γ in RAW264.7 cells (Chihomvu *et al.*, Unpublished-b). Moreover, proteins such as *Hmox1* and *Sqstm1* are usually overexpressed in M2 macrophages.

In summary, solar irradiated *C. jejuni* (susceptible organism) elicits a milder form of cell death in macrophages, i.e., apoptosis, and this could have led to an anti-inflammatory response during late infection as shown by the chemokine and cytokine profiles. The proteomic analysis also revealed that proteasome activity in macrophages was also downregulated and this could lead to macrophages acquiring the anti-inflammatory phenotype (M1 phenotype). Moreover, our results showed that DEPs involved in host glycolysis activity and pentose phosphate pathway were downregulated at 24 h post-infection, and this is also characteristic of M2 macrophages.

However, solar irradiated *S.* Typhimurium (resistant organism) inflicted pyroptotic cell death which is characterised by membrane leakage of macrophage cellular contents. Pyroptosis is usually associated with pro-inflammatory responses, and this correlates very well with the high expression levels of pro-inflammatory chemokines and cytokines of the macrophages during

early and late infection. Thus, it can be concluded that solar irradiated *S.* Typhimurium induces the M1 phenotype in macrophages.

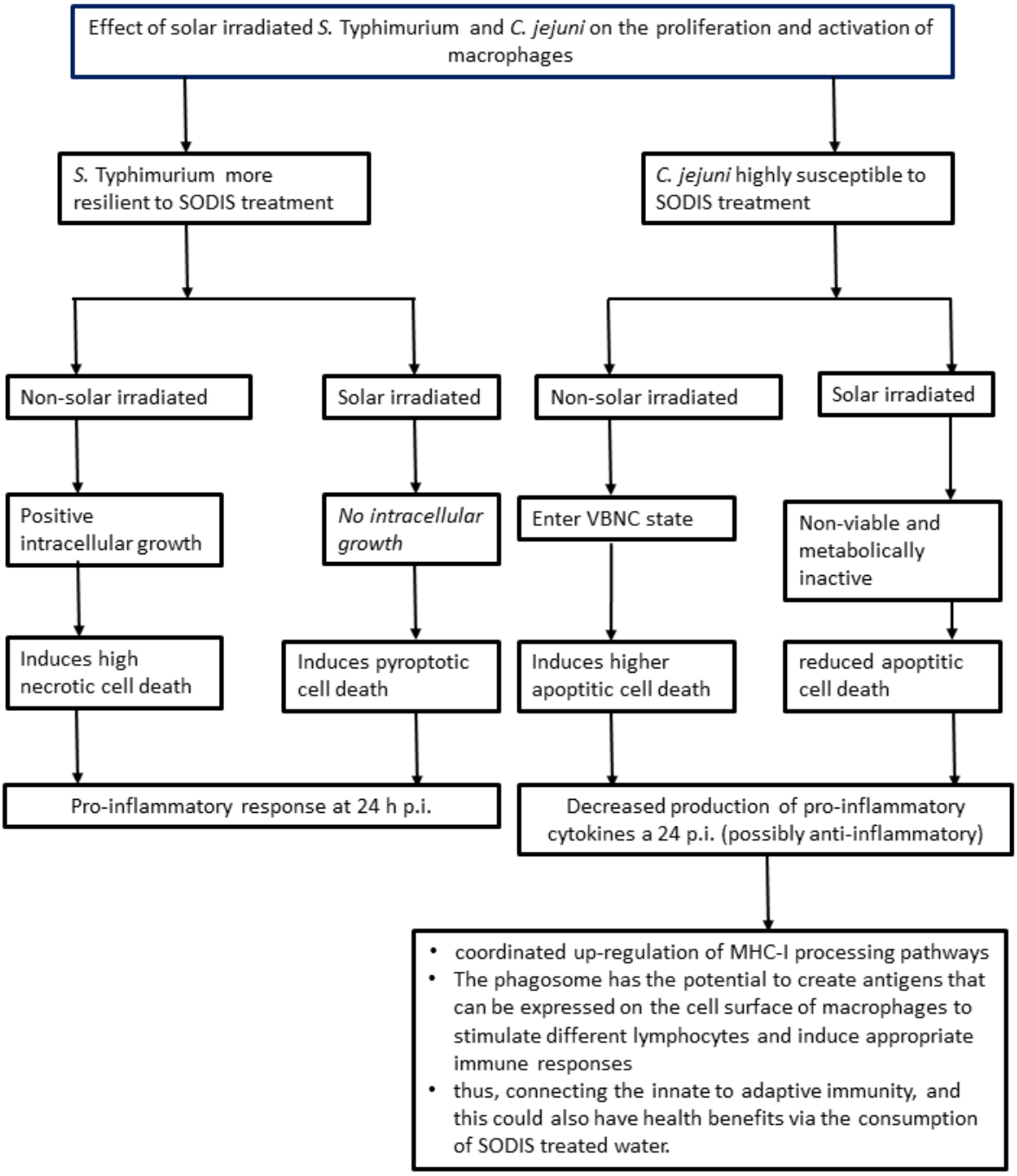


Figure 70 Thesis summary

The findings of this work established a new experimental platform for investigating possible host immune responses to solar irradiated pathogens, and this may be used for future vaccine-development. Future studies will likely provide further insights into the development of highly

immunogenic vaccines against *C. jejuni* and *C. jejuni*. Solar derived vaccines may offer a viable, alternative, sustainable, and strategic therapeutic intervention in infectious diseases.

9.2 Recommendations

Solar disinfection has been successful in reducing diarrehoal cases in areas where SODIS is being used. Thus, investigating the effects of different SUVR intensities, temperatures and dissolved oxygen levels is crucial to obtain the optimum conditions for SODIS disinfection in the context of vaccine development. Solar irradiated *S.* Typhimurium and *C. jejuni* may be considered as vaccine candidates against salmonellosis and campylobacteriosis infection. In this, study we have shown that different solar irradiated strains have different activation profiles in macrophages. Therefore, it will be important to carry out further studies on several waterborne pathogens such as *Vibrio cholerae* O1 (cholera causing bacteria) and *Shigella* species because they are major waterborne pathogens in Africa.

Moreover, it is also imperative to see how SODIS-treated bacteria are taken up by macrophages using fluorescence microscopy. The solar irradiated bacteria may be labelled with a fluorescent dye that can be detected in macrophages.

Additionally, how the innate immunomodulation by solar irradiated pathogens is functionally linked to adaptive immunity remains unexplored. Therefore, *in vivo* studies are essential for providing a foundation for new vaccine development.

This research also addressed the effects of solar irradiated pathogens on cytotoxicity and cell death. However, further research is necessary to determine the exact mechanisms of cellular death.

Examining the co-culture conditions *in vitro* and ascertaining the exact mechanism of cross-talk between T lymphocytes and infected macrophages could shed light on future vaccine development efforts.

This study of interactions between host and pathogen has provided insights into host defenses and solar irradiated pathogen-specific manipulations of intracellular pathogens such *as C. jejuni* that should have practical applications. Learning the activation process for macrophage and solar irradiated bacterial components that elicit a positive immune response will be useful in the development of vaccines and cytokine therapies that targetedly involve the innate immune system. The information in this study should provide a basis of studying the immunological effects of other solar irradiated infectious agents, such as fungi, viruses, and parasites on users of SODIS-treated water.

Based on the findings of this research we approve the treatment of water with SODIS, it may potentially offer immune protection in users of SODIS-treated water. The the up regulation of MHC-I processing pathways in he proteomics studies have provided insights into the possible molecular mechanisms of immune protection that may occur.

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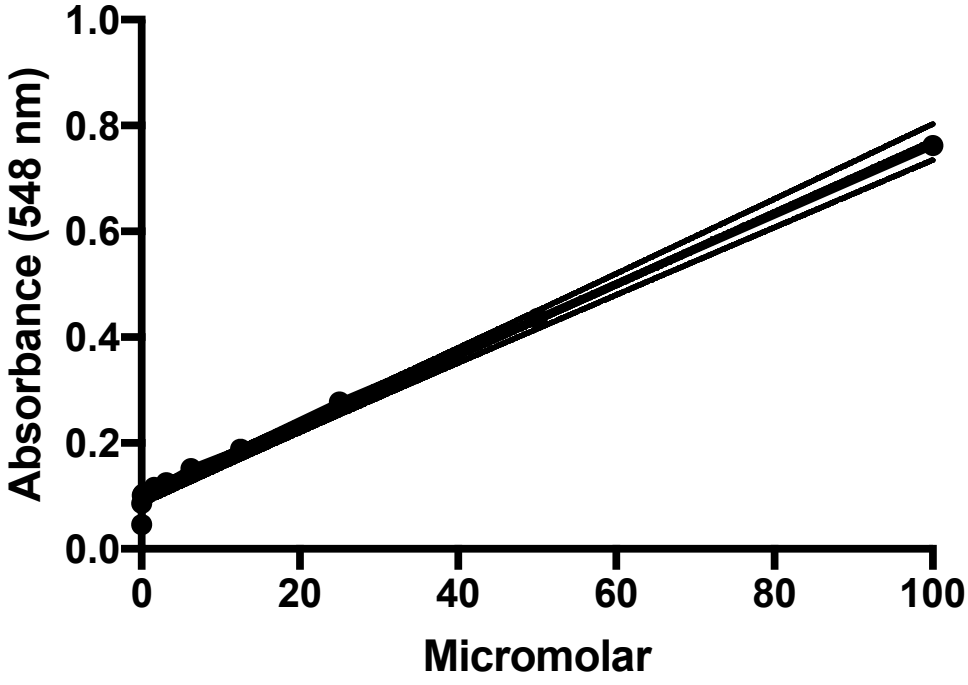
APPENDIX A: STANDARD CURVE FOR NITRIC OXIDE DETERMINATION

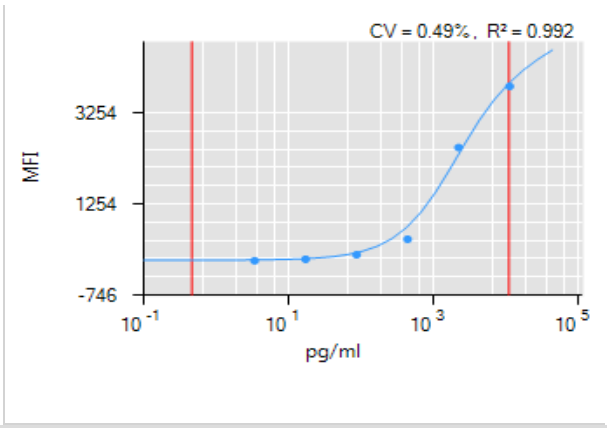


APPENDIX B: CHEMOKINE, CYTOKINE AND GROWTH FACTOR STANDARD CURVES (LUMINEX)

5-P.linear.weighted(8.09, 6115.30, 0.39, 4071.30, 0.95)

DC=(0.46, 11097) Chi = 19.12%

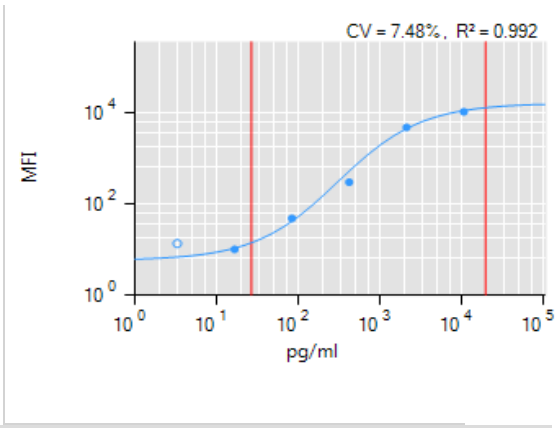
GCSF



4-P.log.weighted(1.74, 9.59, 0.86, 5.70)

DC=(26.64, 19647) Chi = 17.20%

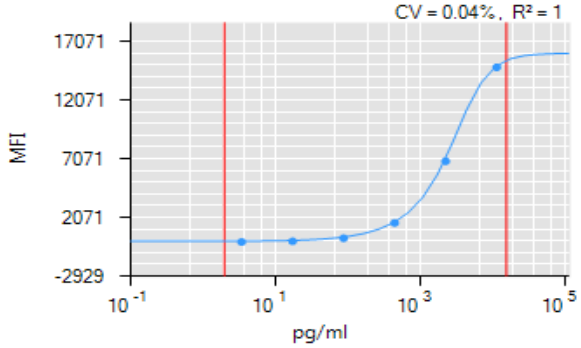
GM-CSF



5-P.linear.weighted(28.70, 15974.05, 2.02, 2392.93, 1.07)

DC=(1.97, 15475) Chi = 2.18%

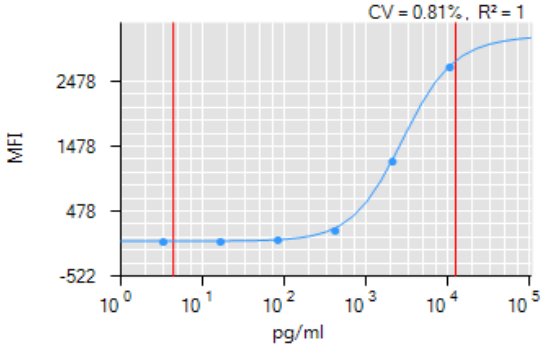
IFNG



5-P.linear.weighted(16.01, 4652.66, 1.43, 5775.90, 0.01)

DC=(4.38, 12541) Chi = 11.70%

IL-1A



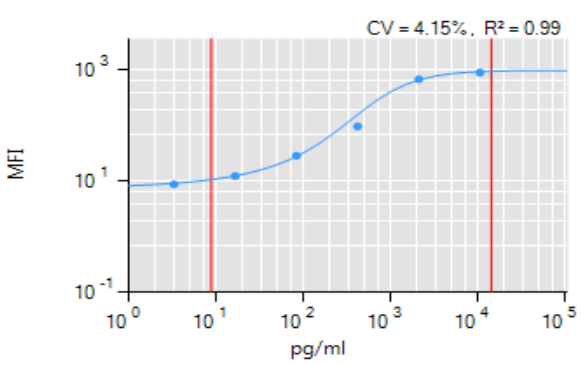
5-P.log(1.96, 6.77, 0.68, 5.53, 1.31)

DC=(8.83, 14361) Chi = 15.94%

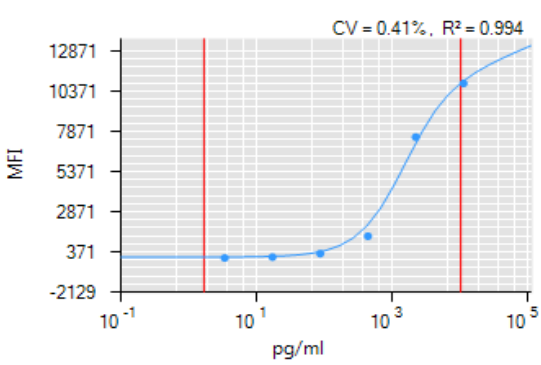
5-P.linear.weighted(10.46, 16491.60, 0.33, 3029.35, 1.11)

DC=(1.68, 10338) Chi = 16.54%

IL-1B



IL-2



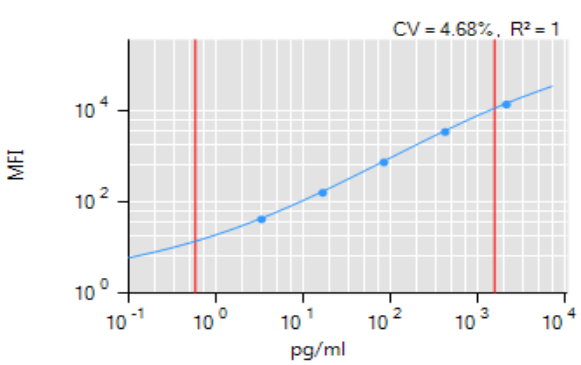
4-P.log(0.34, 13.02, 0.30, 4.48)

DC=(0.58, 1558) Chi = 0.43%

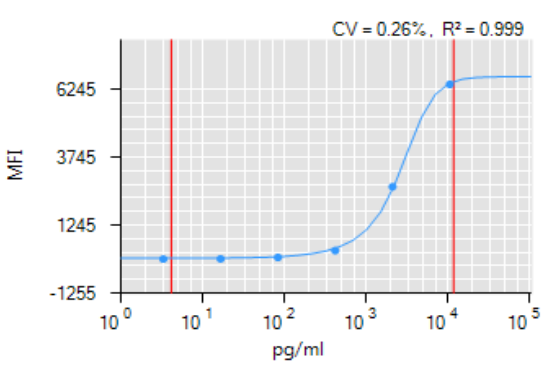
5-P.linear.weighted(10.75, 6595.76, 2.85, 2644.20, 1.21)

DC=(4.14, 11822) Chi = 7.17%

IL-4



IL-5



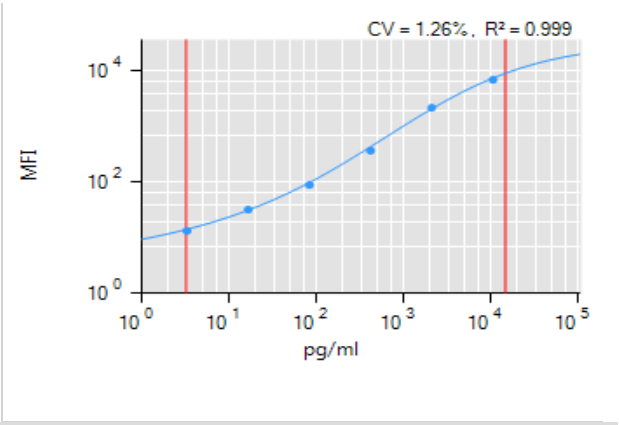
5-P.log(1.09, 10.28, 0.31, 5.70, 0.53)

DC=(3.22, 14725) Chi = 8.68%

5-P.log(1.93, 7.85, 0.39, 4.82, 0.69)

DC=(2.76, 12402) Chi = 9.65%

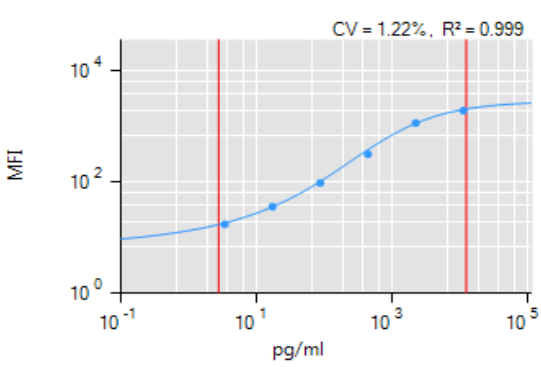
IL-6



4-P.log.weighted(2.11, 8.38, 0.98, 6.71)

DC=(2.49, 21200) Chi = 51.36%

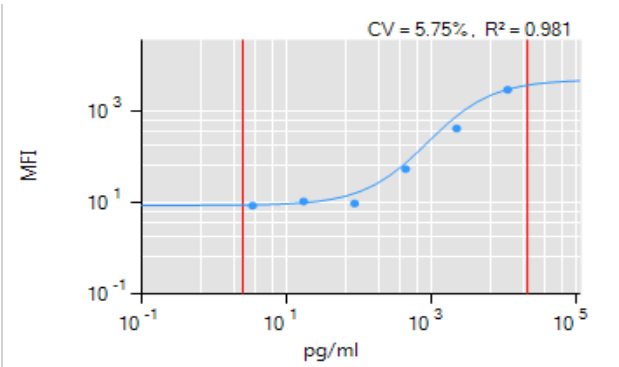
IL-7



5-P.log.weighted(-0.40, 13.01, 0.21, 6.70, 0.35)

DC=(3.53, 18774) Chi = 22.60%

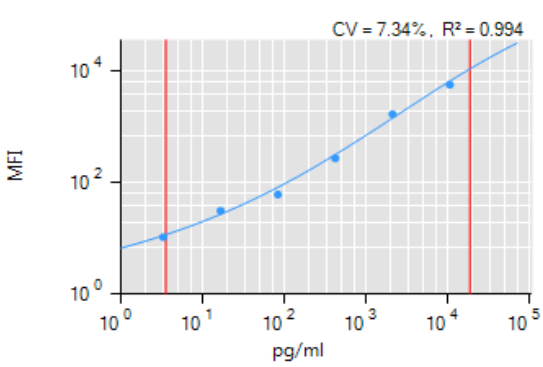
IL-9



5-P.log(0.75, 10.88, 0.27, 5.28, 0.45)

DC=(3.33, 11432) Chi = 6.67%

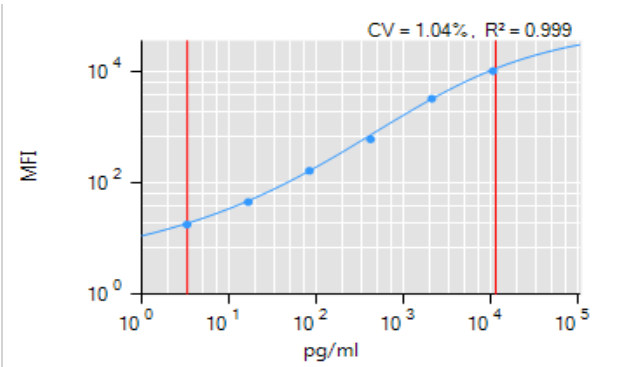
IL-10



5-P.log(1.51, 18.31, 0.07, 9.33, 0.46)

DC=(3.03, 21099) Chi = 4.42%

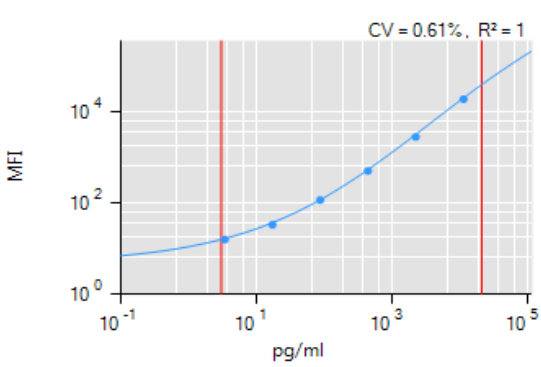
IL-12P40



5-P.log(1.89, 8.14, 0.50, 4.82, 0.96)

DC=(1.66, 12264) Chi = 22.25%

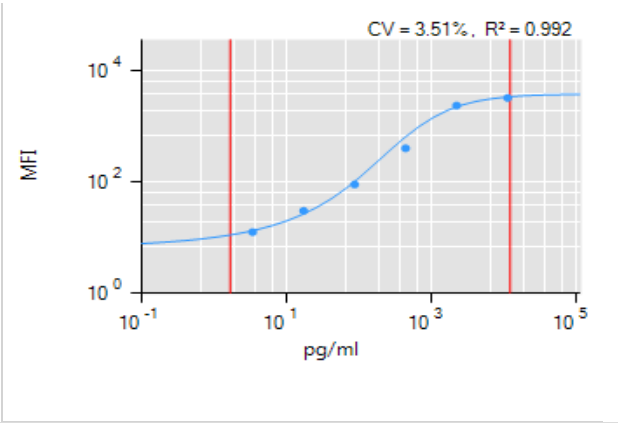
IL-12P70



4-P.log(1.58, 7.58, 0.68, 6.17)

DC=(62.97, 21213) Chi = 4.03%

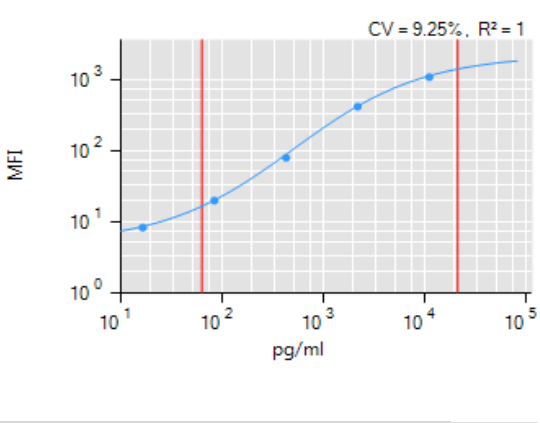
IL-13



5-P.log(2.63, 9.73, 0.43, 4.16, 0.89)

DC=(2.87, 11978) Chi = 29.62%

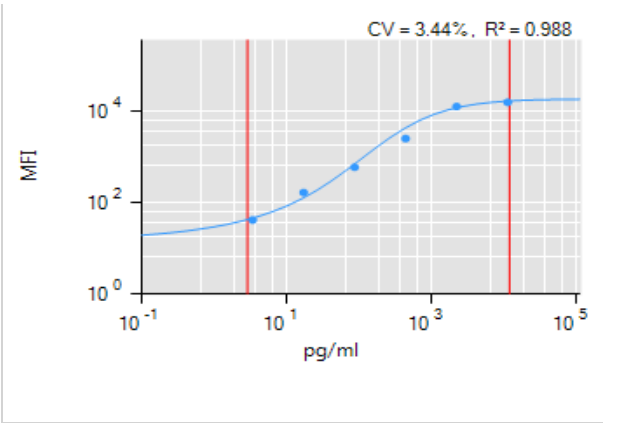
IL-15



5-P.log(3.36, 8.66, 0.61, 5.22, 1.18)

DC=(2.86, 11152) Chi = 16.63%

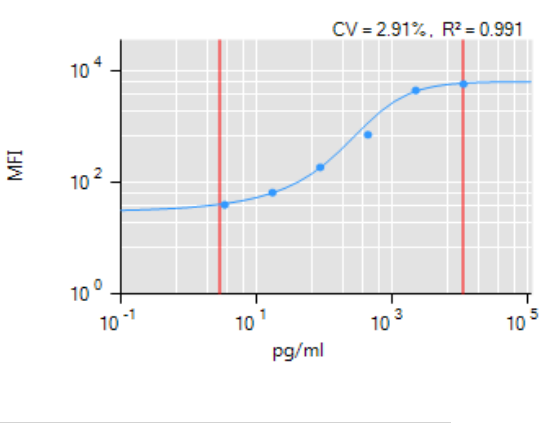
IL-17



5-P.linear.weighted(25.72, 7838.05, 0.22, 2211.65, 1.19)

DC=(2.65, 10995) Chi = 13.67%

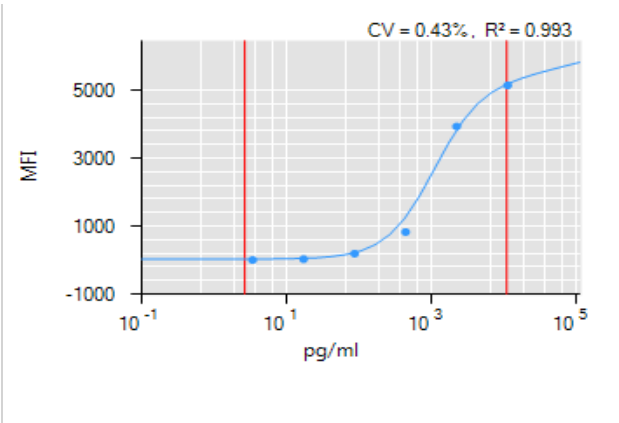
IP-10



5-P.log(2.17, 9.01, 0.69, 5.90, 1.10)

DC=(1.78, 21445) Chi = 1.29%

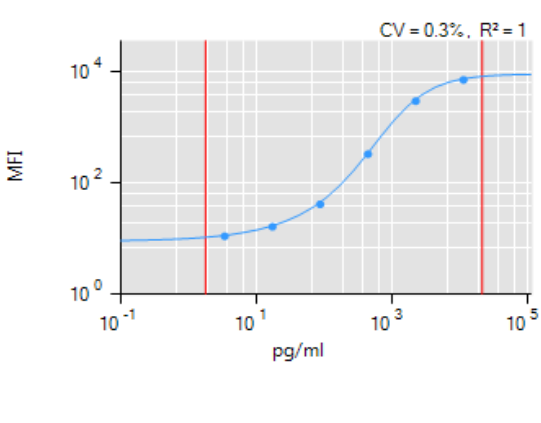
KC



5-P.log(1.91, 9.12, 0.47, 5.13, 0.88)

DC=(2.74, 12790) Chi = 17.53%

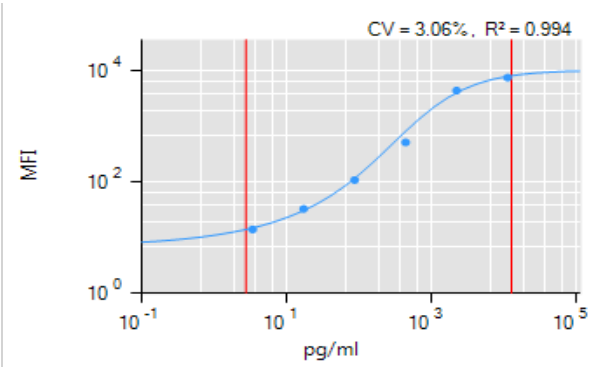
MCP-1



4-P.log(1.79, 10.11, 0.61, 4.92)

DC=(2.21, 1263) Chi = 4.47%

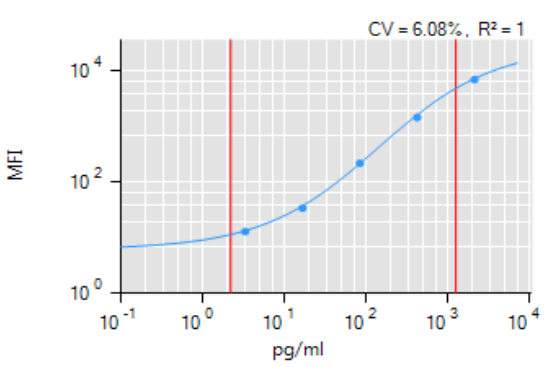
MIP-1A



5-P.log.weighted(2.12, 8.60, 0.58, 6.51, 1.17)

DC=(2.90, 15230) Chi = 22.39%

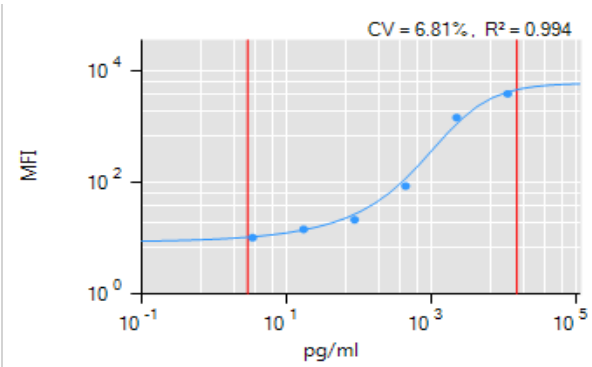
MIP-1B



4-P.log.weighted(-109.95, 11.90, 0.11, -24.13)

DC=(1.59, 400.00) Chi = 19.22%

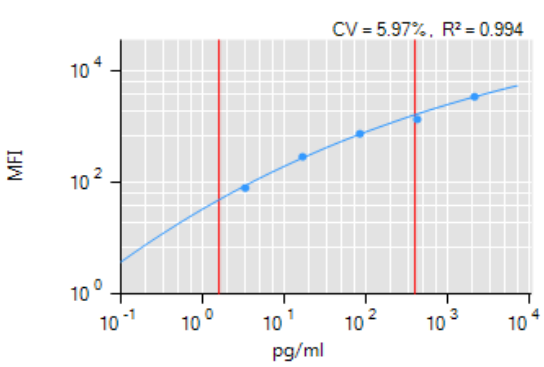
MIP-2



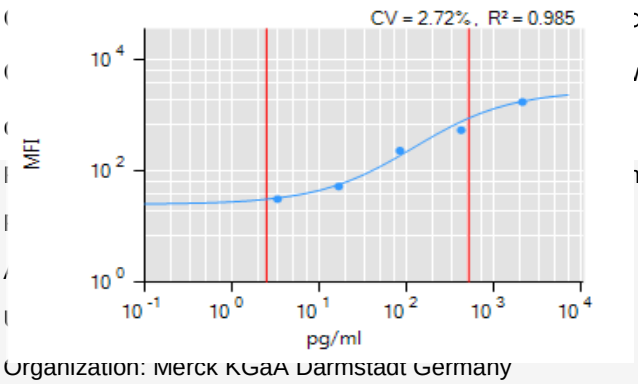
4-P.log.weighted(3.18, 7.84, 0.79, 4.77)

DC=(2.49, 518.77) Chi = 23.10%

RANTES



TNFA



d curve replicates at each dilution level.

veen observed concentrations with expected

n december 2017.csv

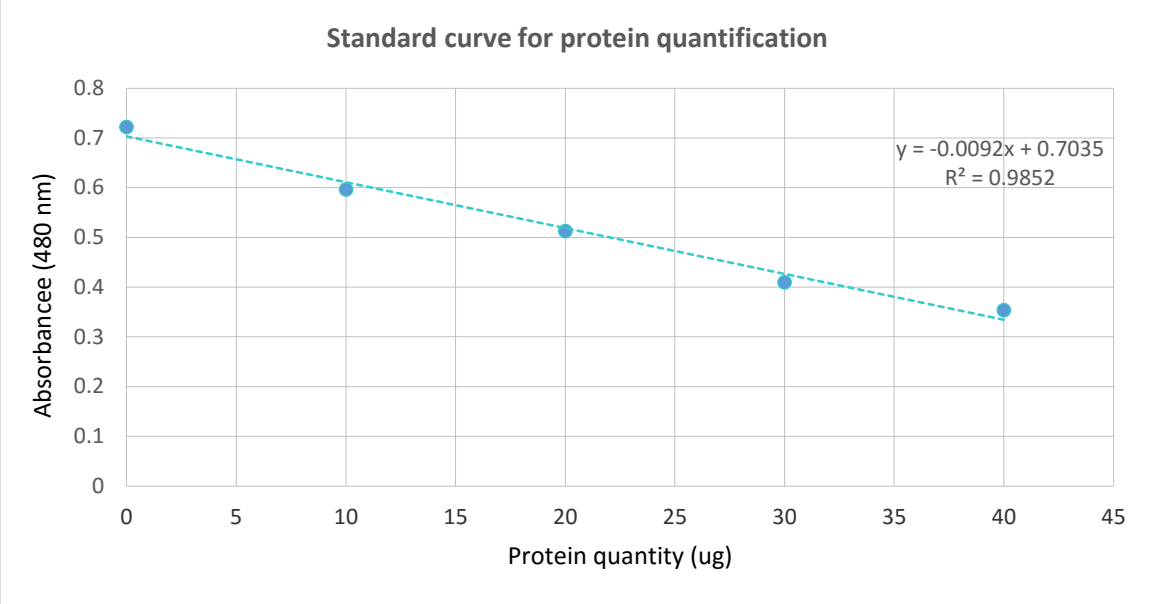
Organization: Merck KGaA Darmstadt Germany

Creation Time: 12/12/2017 09:15:26 Central Standard Time

Signature:

Date:

APPENDIX C-STANDARD CURVES FOR PROTEIN QUANTIFICATION


Standard curve for protein quantification
Absorbancee (480 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
Protein quantity (ug)
y = -0.0092x + 0.7035
R² = 0.9852