RESEARCH ARTICLE

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

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***Campylobacter jejuni* is a leading cause of gastroenteritis worldwide. Solar disinfected (SODIS) water can reduce diarrheal 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 (1) heat and chemically attenuated (1% formalin at 60°C for one hour), (2) exposure to solar ultraviolet radiation (SUVR) for 0, 4, and 8 hours, and (3) non-exposure to SUVR. The *C. jejuni* samples were used to infect macrophage RAW264.7 cells, and its intracellular growth was assessed using the gentamicin protection assay. Cytotoxicity was assessed by using the Lactate Dehydrogenase Assay (LDH). Apoptosis of the treated macrophages was analyzed 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, the solar irradiation of *C. jejuni* eliminates its metabolic activity and reduces its ability to induce cytotoxicity and apoptosis in the macrophages.**

Keywords: apoptosis; *Campylobacter jejuni*; macrophages; solar disinfection.

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Introduction

Campylobacter species are the most common cause of bacterial gastroenteritis in humans [1]. *Campylobacter* spp. are present in contaminated food products such as raw meat, milk, and water [2]. As a waterborne pathogen, solar disinfection (SODIS) treatment of water could curb this pathogen's spread since consumption of solar irradiated water has been associated with reduced diarrheal cases [3]. Previous studies demonstrated the effectiveness of natural sunlight to inactivate *C. jejuni* [4]. 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 recommended for reducing gastroenteritis caused by *C. jejuni* infection [4].

Ingested *C. jejuni* colonizes the colon's intestinal tract, 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 [5, 6]. 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*) [7-9]. In addition, when monocytes and macrophages engulf *C. jejuni*, it activates the nucleotide-binding oligomerization domain 1 (NOD1) [10-13]. NOD1 encodes an intracellular multi-domain scaffolding protein that consists of caspase activation and a recruitment domain (CARD) [14]. Cellular infection is accompanied by the secretion of numerous pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and IL-1β [15-17].

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 [18]. 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* [19]. 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 [20].

Host cell death has conservatively been divided into two distinct morphological and biochemical processes known as apoptosis and necrosis [21]. Apoptosis is an energy-dependent process that plays a primary role in eliminating cells during development and homeostasis [22]. It plays a role in the pathogenesis of several enteric microorganisms [23-25]. Morphologically, apoptotic cells shrink and at least initially maintain the integrity of their plasma membrane [26]. In contrast, necrotic cell death occurs in response to many 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 [26].

Microbial virulence factors such as bacterial replication and host cell cytotoxicity have significant consequences on the host's innate and adaptive responses. 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.

Material and methods

Bacterial culture preparation

Campylobacter jejuni ATCC® 33560™ (Thermo Fischer Scientific, Waltham, MA, USA) were inoculated on Chocolate Blood Agar Plates (Thermo Fischer Scientific, Waltham, MA, USA) 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, USA). A single colony from the incubated plate was transferred to Mueller Hinton broth (Merck, Modderfontein, South Africa) and incubated at 42°C for 48 h without shaking. The *C. jejuni* was harvested by centrifugation at 4,000 g 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.

Samples preparation

Aliquots of 15 mL of the bacterial cell suspension were shaken for 15 seconds and exposed to solar irradiation in 25 mL tissue culture flasks under atmospheric conditions. Previous studies have shown that Ultraviolet-A rays (wavelengths, 315 to 400 nm) cause indirect damage to DNA, proteins, and lipids through reactive oxygen intermediates (ROS) [27], and ultraviolet-B rays (wavelengths, 290 to 315 nm) causes direct damage to the DNA by inducing the formation of DNA photoproducts [28]. Control flasks with the same mixture were exposed to similar atmospheric conditions except for solar ultraviolet radiation (SUVR) by enclosing the samples in an opaque black ventilated box [29]. The flasks were then placed on the top of aluminum 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 8.00 am to 4.00 pm. The amount of solar ultra-violet irradiation (UVA radiation) was measured at 30 min intervals and captured by the Lutron 340A UV Light Meter (Lutron Electronics Company, Coopersburg, PA, USA). The non-solar and solar irradiated samples were enumerated at time-points 0, 4, and 8 h using the Miles and Misra drop method [30] under microaerophilic conditions on Brilliance Campycount plates (Thermo Fischer Scientific, Waltham, MA, USA), at 42°C for 48 h. 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.

Metabolic activity of *C. jejuni*

The heat attenuated, 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, USA) that incorporates a colorimetric growth indicator to detect 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 13,000 g 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 [31].

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, USA) supplemented with 5% fetal 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*.

Intracellular growth assays

Twenty-four well tissue culture plates were seeded with $1x10^5$ macrophages/mL and incubated for 24 h. Thereafter, the pre-seeded macrophage monolayers were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, London, UK) and then co-incubated with *C. jejuni* that was: (1) heat-chemically attenuated

Equation 1:

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

(HA), (2) non-irradiated (NS), and (3) solar irradiated (SI) for 4 and 8 h at a multiplicity of infection (MOI) of 1:10 (macrophage : *C. jejuni*). The plates were incubated for 12 h to allow for adhesion and invasion of the bacteria. The monolayers were then 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 [16]. 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 [30].

Cytotoxicity assay

After infection of the macrophages with HA, NS, and SI *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, USA) 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 the 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, USA). The cytotoxicity was calculated as shown in equation 1.

Apoptosis assay

An apoptotic assay was carried out after 3 and 24 h post-infection (PI) for macrophages treated with HA, NS, SI4, and SI8. The live, necrotic, mid, and late apoptosis RAW264.7 cells were captured by Guava EasyCyte 8HT flow cytometry (Merck/Millipore, Molsheim, France) using the MultiCaspase Sulforhodamine (SR) kit (Merck/Millipore, Molsheim, France) 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), incubated at room temperature for 10 min, and analyzed by flow cytometry. The positive control for apoptosis in each experiment was represented by treating the macrophages with 50 µg/mL of Melphalan.

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 (GraphPad Software, San Diego, CA, USA). A two-tailed p-value of <0.05 was considered to be statistically significant.

Results

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; 8:00 am) to 47.4 ± 0.8 W/m^2 (t = 240 min; 12:00 pm) and then decreased to 17.6 ± 0.7 W/m^2 at the end of the experiment (t = 480; 4:00 pm) (Figure 1).

No viable counts were observed in all the samples of *C. jejuni* that were HA, NS, SI4, and SI8

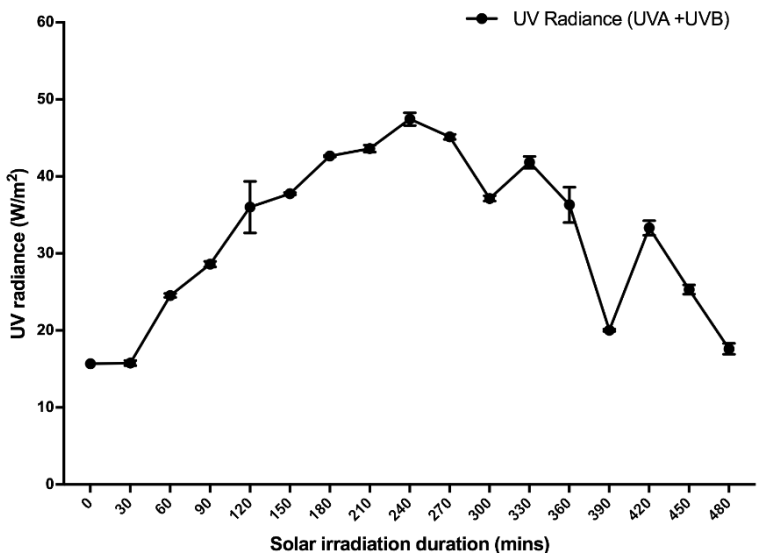

Figure 1. UV-A irradiance during solar irradiation. Standard error bars for three readings at each time point are indicated.

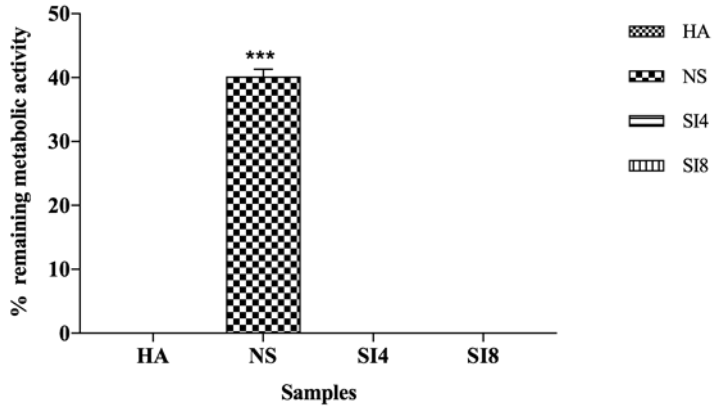

Figure 2. Metabolic activity of *C. jejuni* that were treated according to the following conditions: (1) heat and chemical treatment (HA), (2) Non-solar irradiated (NS), (3) solar irradiated for a duration of 4 h (SI4) and 8 h (SI8). Standard error bars are indicated. Significant differences between the treatments are indicated on the graph where a p-value < 0.05 was considered significant (***).

treated. Metabolic assays showed that there was activity in the HA and the SI4 and SI8 treatments (Figure 2). However, the 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 NS and SI *C. jejuni* samples (Figure 2).

Intracellular growth assays

No intracellular growth was observed for macrophages treated with the HA, NS, and SI treated *C. jejuni*.

Cytotoxicity analysis of macrophages infected with *C. jejuni*

At 3 h post-infection (PI), cytotoxicity levels of the macrophages treated with HA, NS, and SI *C. jejuni* were very low (<10%). The highest percentage cytotoxicity was observed in macrophages treated with NS *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%) (Figure 3). After 24 h of PI, a significant increase in cytotoxic activity was noted in all the samples (p<0.001) (Figure 3). The highest cytotoxic level (30.28% ± 0.05%) was

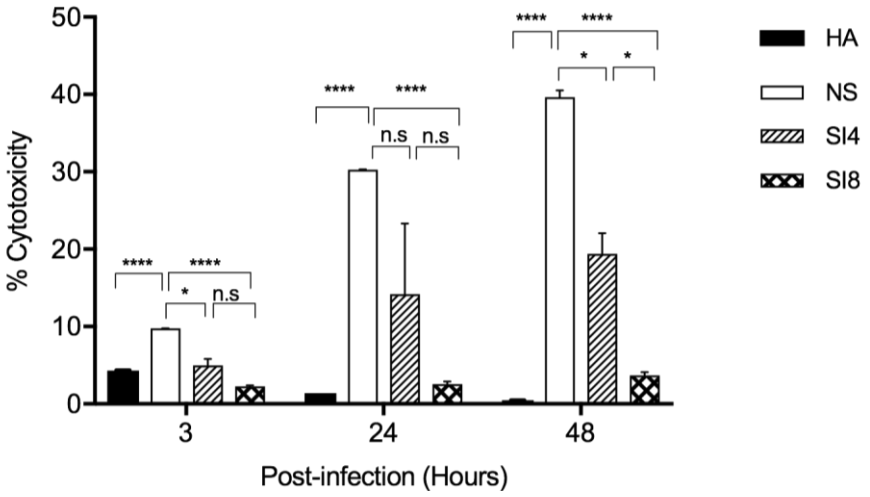

Figure 3. Host cytotoxicity assay (LDH) after infection with (1) heat/chemical attenuated (HA), (2) non-solar irradiated (NS), and (3) solar irradiated (SI) *C. jejuni*. LDH in the supernatants of infected and uninfected macrophages was sampled and measured at 3, 24, and 48 h after infection at various multiplicity of infection (MOI) of 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.

observed in macrophages treated with NS *C. jejuni*. A significant decrease in cytotoxicity was noted in macrophages treated with HA *C. jejuni* (1.41% ± 0 .00%) (p<0.001). The least cytotoxicity (2.57% ± 0.32%) occurred in the SI8 samples, whereas the SI4 samples had cytotoxicity levels of 14.18% ± 9.14%.

After 48 h of PI, an increase in cytotoxicity levels was observed in all the macrophages except for those co-cultured with HA *C. jejuni*. The highest cytotoxicity level of the macrophages was observed with the NS controls with a cytotoxicity level of 39.66% ± 0.87%, and the lowest occurred in the HA *C. jejuni* (0.45% ± 0.037%). Between the SI samples, the macrophages treated with SI4 exhibited significantly higher cytotoxicity levels compared to those SI8 (p=0.01). Moreover, the macrophages treated with the SI as well as the HA inactivated samples produced significantly lower levels of LDH in comparison to their controls (Figure 3)

Apoptotic assays

The potential for the HA, NS, and SI treated *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 in the number of live macrophages for all treatment groups in comparison to the untreated macrophages (p<0.001) (Figure 4). The highest percentage of live cells was observed in macrophages treated with *C. jejuni* that was SI4 (84.70% ± 2.05%). The lowest viability was noted in macrophages treated with NS treated *C. jejuni* and was 71.80% ± 1.70% (Figure 4). A significant increase in necrotic cells was noted in macrophages treated with HA, NS, SI4, and SI8 samples of *C. jejuni* (p<0.001) 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 HA and SI8 (Figure 4). However, a significant increase in mid-apoptotic cells was observed in macrophages treated with SI8 (p=0.031).

During late infection (24 h of PI), the HA, NS, and SI treated *C. jejuni* showed a significant increase in the percentage of apoptotic cell death and a drastic decrease in necrotic cell death (p<0.001). Macrophages treated with HA treated *C. jejuni*

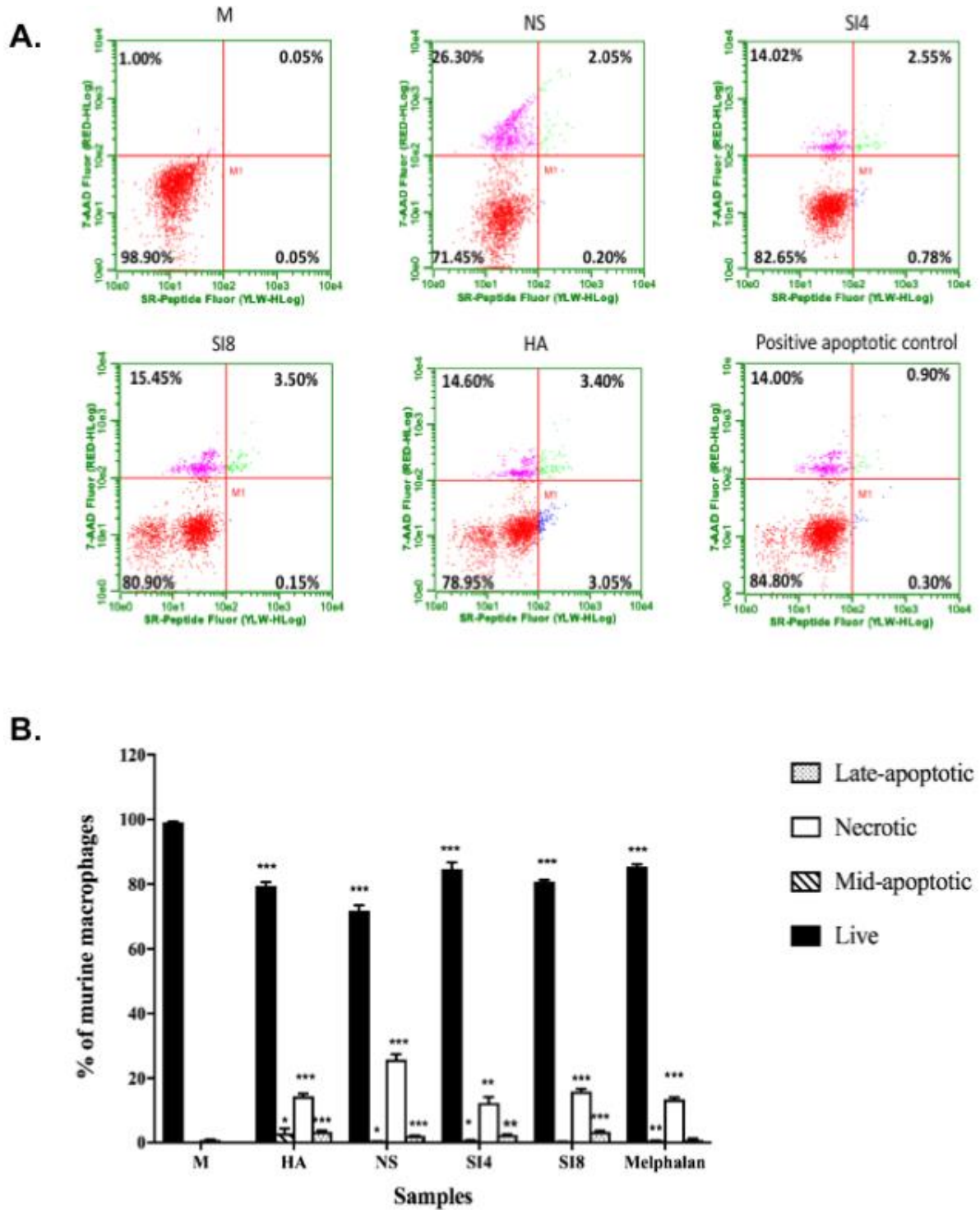

Figure 4. (A) Representative fluorescence plot of RAW264.7 cells at 3 h of PI *C. jejuni* that were (1) untreated macrophages (M), (2) non-solar irradiated at 0 h (NS), (3) solar irradiated samples after 4 (SI4) and 8 (SI8) h, (4) heat and chemical attenuation (HA), and (5) positive control macrophages 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.

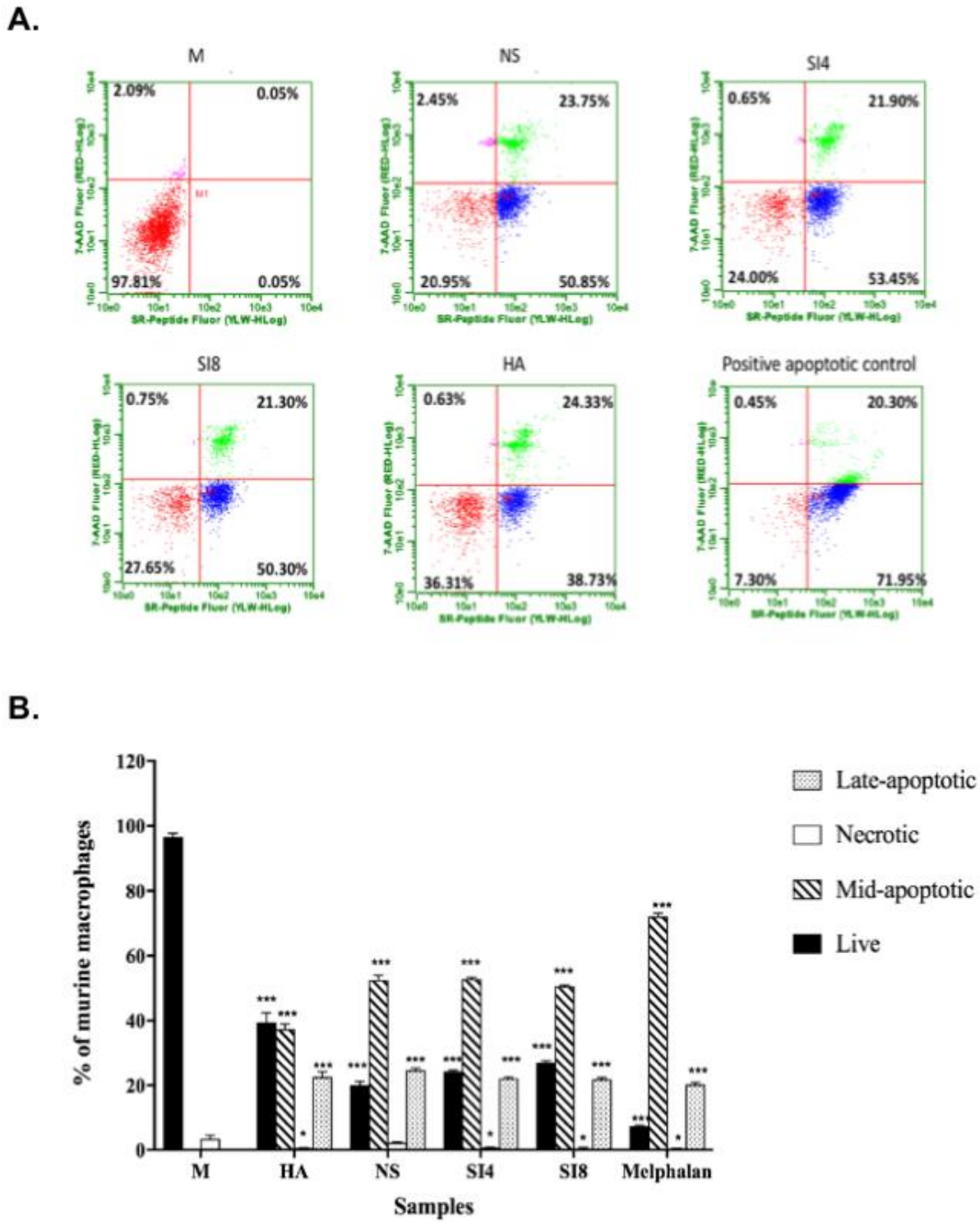

Figure 5. Flow cytometry analysis. **(A)** Representative fluorescence plot RAW264.7 of macrophages following 24 h of stimulation with *C. jejuni* that had been (1) media alone (M) (2) heat and chemical attenuation (HA), (3) non-solar irradiated at 0 h (NS), (4) solar irradiated samples after 4 (SI4) and 8 (SI8) h, and (5) 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.

had the highest viability (39.42% ± 3.00%). The macrophages treated with 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 SI8 treated *C. jejuni* had higher rates of viability than that of SI4 (Figure 5).

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 in late-apoptotic cells were also observed in all the samples ($p<0.001$); 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 ($p<0.001$) (Figure 5).

In the 48 h of PI samples, no cell counts were observed for the positive apoptotic control (Malphalan), and some of the samples treated with NS *C. jejuni*. Therefore, those results were not included for comparative reasons.

Discussion

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) (Figure 1) led to enhanced cellular damage and consequent inactivation of the bacteria. The SODIS efficiency was probably enhanced by the aluminum foil through the return of UV-A and short-wavelength visible radiation through the containers [33].

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 [34]. Other factors, including temperature [35], reactive oxygen species (ROS), and growth conditions, could have also influenced the VBNC state of *C. jejuni* [36]. The SODIS process involves exposure of samples to atmospheric temperatures 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 [36]. 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 defense proteins such as *SoxRS*, *OxyR*, and *RpoA* [37]. 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 favorable conditions [38, 39]. Therefore, the viability of the cells was determined by assessing the metabolic activity of HA, NS, and SI treated *C. jejuni* using Alamar Blue. The NS treated *C. jejuni* retained its metabolic activity, whereas HA and SI treated *C. jejuni* showed no metabolic activity (Figure 2).

This study showed that while NS treated *C. jejuni* bacteria are metabolically active, they were non-culturable. It appears that they acquired a metabolically active-but-non-culturable (ABNC) state [40]. The ABNC state seems to support the long-term survival of bacteria under unfavorable 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

unfavorable conditions that are not conducive to 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 [40].

The loss of metabolic activity in the SI treated *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, which could negatively affect the structural and enzymatic proteins within the cells [41]. Vital cellular functions such as transcription and translation, respiration, ATP synthesis, catalase, molecular chaperone functions, amino acid synthesis, and degradation are all affected by UV-A irradiation. With the loss of catalase activity, the cells lose their defense against ROS, making them more susceptible to oxidative stress. Additionally, the damage to translational proteins decreases the cells' ability to self-repair [42].

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 [38, 43].

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

As expected, HA and SI treated *C. jejuni* did not multiply in the macrophages suggesting that the bacteria were denatured in both treatments. However, the NS treated *C. jejuni* also did not exhibit any intracellular growth in the macrophages even after 3, 24, and 48 h of post-infection. This may be 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 [44]. 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 [16, 45]. The survival of intracellular bacteria within phagocytic cells presents a way to evade the host immune defenses and allows the proliferation and dissemination of bacteria throughout the host [46]. However, once the bacteria are inside the macrophages, they must be able to survive the unfavorable conditions present in the host's cell, such as oxidative products (e.g., ROS), minimal nutrients, and unfavorable pH conditions [47].

In this study, *Campylobacter jejuni* samples 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.

Cytotoxicity analysis of macrophages infected with *C. jejuni*

Figure 3 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 h post-infection (Figure 3). The greater cytotoxic effects exhibited by the NS treated *C. jejuni* may result from 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 [12, 49]. These toxins have specific effects on different processes in eukaryotic cells; for instance, some toxins interfere with intracellular signaling by interacting with particular proteins in various signaling cascades and others such as the CDT interfere with the cell cycle [50].

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 cytotoxicity levels than the 8 h-solar irradiated sample (Figure 3); this may perhaps be due to the higher level of protein damage caused by prolonged SI [42]. This finding suggests that SI 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 HA, SI4 and SI8, and NS *C. jejuni* produced low levels of LDH (Figure 3). This indicates that the macrophages were undergoing some form of necrotic cell death [51].

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 HA, NS, and SI4 and SI8 treated *C. jejuni* showed a significant increase ($p<0.001$) in necrotic cells during early infection (3 h of PI) (Figure 4A and 4B). Macrophages treated with NS *C. jejuni* had the highest proportion of necrotic cells (Figure 4A and 4B), whereas treatments involving SI 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 HA and SI treated *C. jejuni*. Necrosis is characterized by increased membrane permeability, which allows the 7-AAD dye to permeate and intercalate to DNA [52, 53].

Infection of macrophages for 24 h showed a decrease in the proportion of necrotic cells (Figure 5A and 5B). This may be due to efferocytosis. When macrophages die, they are engulfed and digested by other macrophages via the process of efferocytosis [54]. Once a macrophage has engulfed a damaged macrophage through efferocytosis, it undergoes programmed cell death (apoptosis) thus eliminating inflammatory conditions in the body [26]. The highest and lowest apoptotic cells were 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 cells recognizing self-antigens, and cytotoxic killing [55]. Since apoptotic cells initially maintain their membrane integrity, they do not release their intracellular contents rapidly; thus, pro-inflammatory signals are not released (immunosuppressive).

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 regulating cell death.

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