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HBL-Like Toxins and Inflammasome Activation in *Bacillus*

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RESEARCH REPORT

HBL-Like Toxins and Inflammasome Activation in *Bacillus*

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We hereby declare that this EP project is from student's own work. The EP Report has been read and presented to i3L's Examination Committee. The EP has been found to be satisfactory and accepted as part of the requirements needed to obtain an i3L bachelor's degree.

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ABSTRACT

The innate immune system is the first line of host defence that can utilise pattern recognition receptors (PRRs) such as NLRP3 to detect and respond towards danger signals. *Bacillus*, particularly *B. cereus*, are food-borne pathogens known to produce hemolysin BL (HBL), a pore-forming toxin that can trigger NLRP3 inflammasome activation. This study investigates NLRP3 activation induced by four other Bacillus species aside from *B. cereus* and the mechanism required by their virulence factors to mediate this effect. To elucidate these, IncuCyte® Cytotoxicity Assay, western blot, ELISA, and LDH assay were performed for two different cell lines: THP-1 and B16F10 cells. Findings suggest that these virulence factors can trigger caspase-1 and gasdermin D (GSDMD) cleavage, resulting in increased pro-inflammatory cytokine IL-1β and IL-18 levels, as well as elevated dead cell percentage in the absence of NLRP3 inhibitor MCC950. Moreover, the role of Litaf and Cdip1 was deemed a necessity for mediating the activity of these virulence factors indicated by the distinct difference in effects on wild-type (WT) and knockout cells.

Keywords: innate immunity, NLRP3, *Bacillus*, MCC950, Litaf, Cdip1

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LIST OF ABBREVIATIONS

ADAM10	A disintegrin and metalloproteinase domain 10
ASC	Apoptosis-associated speck-like
BAP31	B-cell receptor-associated protein 31
Cdip1	Cell death-inducing p53-target protein 1
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMPs	Damage associated molecular patterns
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
GSDMD	Gasdermin D
HD1	Helical domain 1
HD2	Helical domain 2
HBL	Hemolysin BL
HRP	Horseradish peroxidase
IL-18	Interleukin-18
IL-1β	Interleukin-1β
LDH	Lactate dehydrogenase
Litaf	LPS-induced TNF-α factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MyD88	Myeloid differentiation primary response 88
NADH	Nicotinamide adenine dinucleotide
NBD	Nucleotide-binding domain

NHE	Nonhemolytic enterotoxin
NLR	Nucleotide-binding domain and leucine-rich repeat containing
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NLR family pyrin domain-containing protein 3
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PI	Propidium iodide
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
PYD	Pyrin domain
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STAT6 (B)	Signal transducer and activator of transcription 6 (B)
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
WHD	Winged helical domain

I. INTRODUCTION

1.1. Background Information

The innate immune system is the first line of host defence against foreign invaders such as pathogens or disturbances in the cellular homeostasis (Nilsson et al., 2015). This is possible due to the ability of innate immune cells to recognise danger signals, namely pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) from damaged cells by utilising germline-encoded pattern recognition receptors (PRRs) (Rumpret et al., 2021). One of the most well studied PRR is the NLRP3, an intracellular censor that recognises stress signals caused by pathogens such as bacteria (Akbal et al., 2022).

Bacteria represent a significant class of pathogens which are diverse in their survival characteristics and often encountered by the innate immune system (Soni et al., 2024). *Bacillus* are rod-shaped gram negative bacteria that have the ability to form spores, allowing them to survive in harsh environmental conditions (Ghosh, 2023; McKenney et al., 2013). More than 200 species have been discovered in which many of them were reported to cause gastroenteritis, sepsis, and death (Colaco et al., 2021; Porwal et al., 2009). One of the most well studied *Bacillus* species is *Bacillus cereus*, a foodborne bacterium found in undercooked or processed food that has the ability to cause diarrhoea and emesis due to the toxins it possess (Ehling-Schulz et al., 2019; Tewari et al., 2015).

A previous study by Marthur et al. (2019) reported that one of the toxins from *B. cereus* which is the pore-forming toxin hemolysin BL (HBL), has the ability to activate NLRP3 censor. This is due to its ability to form pores on the plasma membrane, allowing the release of intracellular potassium ions that leads to the disruption of ionic balance. The activation of

NLRP3 causes the recruitment of the bipartite adaptor protein ASC and, subsequently, pro-caspase-1, to form the NLRP3 inflammasome (Nagar et al., 2023). The formation of this inflammasome complex results in the cleavage of pro-caspase-1, causing its activation. The active caspase-1 will cause the cleavage of the pore-forming protein gasdermin D (GSDMD) as well as pro-IL-1β and pro-IL-18 into their active forms (Devant & Kagan, 2023). The active GSDMD triggers inflammation-induced cell death, known as pyroptosis, one of which is characterised by the release of cellular contents and pro-inflammatory cytokines via these pores (Yu et al., 2021).

Previously, Man Lab had discovered four *Bacillus* species aside from *B. cereus* that were able to trigger inflammasome activation, in which the screening for censor results indicated that NLRP3 might be the censor that caused this inflammasome complex formation. Furthermore, when Man Lab investigated the virulence factor of these bacteria, they found that the bacterial supernatants of these four *Bacillus* species were found to potentially be proteinaceous in nature due to their inability to cause caspase-1 and GSDMD cleavage after heating or proteinase treatment. Additionally, they observed that anti-HBL of *B. cereus* could abolish the activity of these supernatants to cause inflammasome activation, therefore giving rise to the possibility of their characteristics being HBL-like.

A recent study by Liu et al. (2020) discovered two receptors responsible for the complete formation of HBL as a pore-forming toxin that allows it to punch holes onto the plasma membrane and ultimately trigger NLRP3 inflammasome activation. These receptors are Litaf, as the major receptor, and Cdip1, as the minor. Following their discovery, they generated a CRISPR knockout cell line that does not have these receptors and reported that this cell line had a high resistance towards HBL of *B. cereus*.

1.2. Research Aim

To confirm the occurrence of HBL-like toxins-mediated cell death caused by the four *Bacillus* species, which can be achieved by:

1. Establish the correlation between the activation of the NLRP3 inflammasome and the virulence factors of four specific *Bacillus* species.
2. Decipher the nature of the virulence factors secreted by the four *Bacillus* species.

1.3. Scope of Research

In order to realise these aims, I conducted an in vitro study involving the treatment of different *Bacillus* species on mammalian cells and evaluate the occurrence of HBL-like toxins-mediated cell death based on the changes in cellular morphology, activation of NLRP3 downstream pathway, presence of pro-inflammatory cytokines as the end product of NLRP3-mediated inflammation, and cell death percentage following bacteria treatment.

1.4. Hypothesis

I hypothesise that the four bacterial supernatants cause NLRP3 activation, which leads to caspase-1 and GSDMD cleavage, followed by elevated IL-1β and IL-18 when exposed to THP-1 cells. Additionally, I also speculate the increase in cell death percentage in B16F10 wild type (WT) cells in comparison to the Litaf$^{-/-}$Cdip1$^{-/-}$ cells when treated with the four bacterial supernatants.

II. LITERATURE REVIEW

2.1. The Inflammasome and NLRP3

The inflammasome is a cytoplasmic multiprotein complex that can trigger inflammation and systemic immune response, which is essential for host defence (Zheng et al., 2020). This complex consists of nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing proteins (NLRs), adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and caspase-1 (Kelley et al., 2019). NLRs are receptors, which are majorly expressed in the cytosol, that can sense molecules associated with infection and cellular stress to induce inflammasome activation (Almeida-da-Silva et al., 2023). One of the most studied NLRs is NLRP3 which consists of three domains: the N-terminal pyrin domain (PYD), central NACHT domain, and the C-terminal leucine-rich repeat (LRR). The PYD domain of NLRP3 will interact and recruit ASC, while the LRR is deemed to play a role in self-suppressing NLRP3. On the other hand, the NACHT domain can be further divided into four subdomains: the nucleotide-binding domain (NBD), winged helical domain (WHD), helical domain 1 (HD1), and helical domain 2 (HD2). NBD can bind to ATP which contributes to NLRP3 oligomerisation, while WHD and HD1 facilitate the conformational change that results in the exposure of the PYD domain, allowing ASC to bind to NLRP3 (Que et al., 2024).

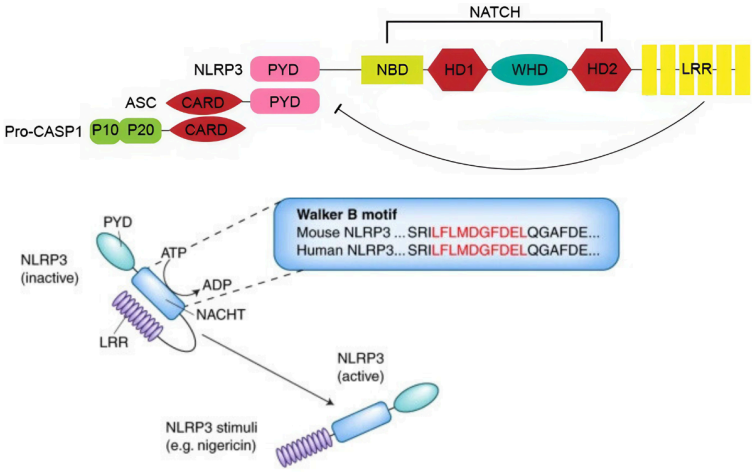

Figure 1. Structure and conformational change of NLRP3 (adapted from Gorka et al., 2019 and Que et al., 2024).

NLRP3 generally requires two signals to induce its activation: priming and activation signal (Chen et al., 2023; Kelley et al., 2019). The priming stimuli is needed as the NLRP3 expression under normal condition is thought to be insufficient to induce NLRP3-mediated inflammasome activation (Blevins et al., 2022; Kelley et al., 2019). Therefore, exposure to toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), are required to activate the transcription factor NF-κB that can increase NLRP3 expression (Kelley et al., 2019). Following this priming step, NLRP3 can be activated by various stimuli although it has yet to be observed interacting directly with the signal (Kelley et al., 2019; Zhan et al., 2023). Mitochondrial dysfunction, ionic imbalance (e.g. K+ efflux), and lysosomal damage have been reported to activate NLRP3 (Kelley et al., 2019).

The activation of NLRP3 ultimately results in pyroptosis, which is termed as pro-inflammatory cell death characterised by cell swelling, membrane blebbing, cytoplasm flattening, and intact nucleus with random DNA fragmentation (Yu et al., 2021). This is because activated NLRP3 will trigger the recruitment of ASC and pro-caspase-1 to form the NLRP3 inflammasome complex that will result in the cleavage and, subsequently, activation of caspase-1 (Que et al., 2024). Active caspase-1 can cleave gasdermin D (GSDMD) to its

active form, its N-terminal domain, which allows it to oligomerise and form pores on the plasma membrane, leading to the release of cellular contents. Additionally, caspase-1 can cleave pro-IL-1β and pro-IL-18, which are pro-inflammatory cytokines, into their active forms and be released from the GSDMD pores (Chen et al., 2023). While cellular contents and these cytokines are released which causes membrane rupture in the process, water will enter the cell causing it to swell and undergo osmotic lysis (Yu et al., 2021).

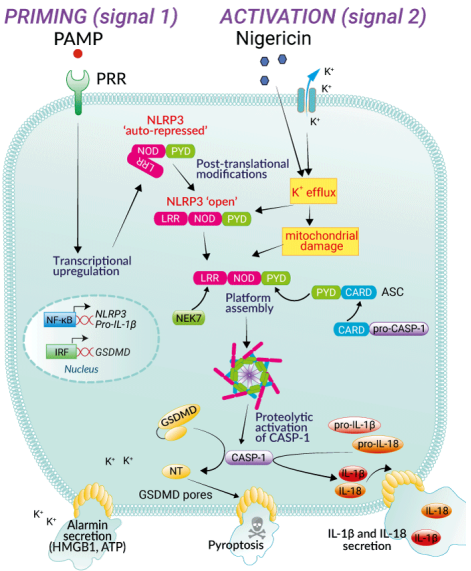

Figure 2. Mechanism of NLRP3-mediated pyroptosis (retrieved from InvivoGen, n.d.).

2.2. *Bacillus cereus* and NLRP3

Bacillus cereus is a species of the *Bacillus* genus, which are gram-positive bacteria with rod-shaped morphology and spore-forming capability (Elshaghabee et al., 2017). *B. cereus* is a foodborne pathogenic bacteria commonly found in cereal, soybean, and meat products (Du et al., 2023). Generally, *B. cereus* has two types of toxins: enterotoxins and emetic toxin cereulide. Enterotoxins (e.g. hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE)) are heat sensitive toxins that require the bacteria to be present and form pores on the intestinal epithelial cells, causing imbalance ion transport as well as release of water and electrolytes. This ultimately causes diarrhoeal symptoms (Dietrich et al., 2021; Messelhäußer

et al., 2018). On the other hand, the emetic toxin cereulide is a heat stable toxin produced during bacterial vegetative growth and interacts with the 5-HT3 serotonin receptors causing stimulation on the vagus nerve, which triggers nausea and vomiting symptoms (Dietrich et al., 2021).

Previous studies reported that the pore-forming toxin HBL, one of the enterotoxins of *B. cereus*, can form pores on the plasma membrane which causes K+ efflux (Prince & Kovac, 2022). Such a phenomenon can be sensed by NLRP3, inducing its activation that ultimately results in pyroptosis (Tuipulotu et al., 2021). This HBL toxin consists of three components: the binding component B, as well as the lytic component L1, and L2 (Zeighami et al., 2020). Previous study by Mathur et al. (2019) reported that HBL could only be functional as a tripartite pore when all the components are assembled, in which its B component is the most crucial among the three as an “anchor” for its complex.

2.3. Litaf and Cdip1 exploited by HBL

The lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-α factor (Litaf) is a transcription factor that upregulates TNF-α expression. It plays a role as one of the downstream proteins of LPS mediated-TLR-4 activation, phosphorylated by MyD88 and induce TNF-α expression together with STAT6 (B) (Zou et al., 2015). In contrast, the Cell death-inducing p53-target protein 1 (Cdip1) is a proapoptotic protein that is low in expression under normal conditions but upregulated when endoplasmic reticulum (ER) stress occurs (Inukai et al., 2024). It can interact with B-cell receptor-associated protein 31 (BAP31) to trigger the initiator caspase-8 cleavage, which ultimately leads to apoptosis (Inukai et al., 2024; Namba et al., 2013). Its structure is an analog of Litaf with C-terminal domain anchoring the protein on the cell membrane (Inukai et al., 2021; Liu et al., 2020). A previous study by Liu et al. (2020) reported that these two receptors contributed to the complete HBL

formation as a tripartite pore by facilitating the binding of the B component of this toxin, thus allowing the recruitment of L1 and L2.

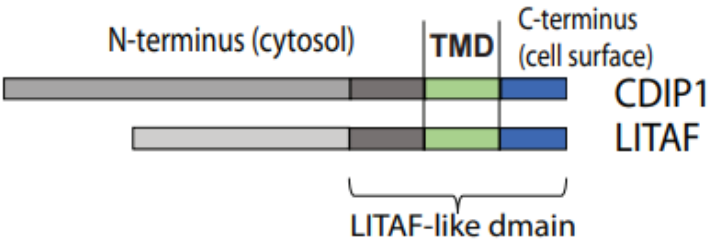

Figure 3. Structure of Litaf and Cdip1 (retrieved from Liu et al., 2020).

2.4. IncuCyte® Cytotoxicity Assay

The IncuCyte® Live-Cell Analysis System is a live-cell imaging that automatically monitors, captures, and analyses cell images (Akere et al., 2024; Siller et al., 2019). The system is placed inside an incubator which ensures controlled culture conditions during real-time monitoring. Equipped with two lasers, it is able to capture fluorescence images and phase contrast, which is acquired using a platform such as IncuCyte® S3 image analysis suite (Bravo et al., 2023; Siller et al., 2019). Fluorescence signals from the captured images are then analysed and quantified using pre-defined imaging masks (Siller et al., 2019).

In the case of IncuCyte® Cytotoxicity Assay, membrane impermeable dye propidium iodide (PI) can be used as it will intercalate with the DNA when membrane damage occurs (Ligasová & Koberna, 2021). IncuCyte® will use a green laser to excite PI at approximately 535 nm, and in return, PI will emit a red colour at approximately 617 nm to be detected by the system (Kulkeaw, 2021). The presence of PI will distinguish the dead cells from live cells, and can be quantified to determine the dead cell percentage (Di Virgilio et al., 2019; Siller et al., 2019).

2.5. Western Blot

Western blot is used to detect and separate proteins based on their molecular weight by undergoing gel electrophoresis, membrane transfer, and antibody incubation (Mahmood & Yang, 2012). In order to perform gel electrophoresis, cells from the samples need to be lysed to release their protein content and ensure they are negatively charged using the addition of RIPA lysis buffer and Laemmli buffer. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is then performed to separate the proteins based on their molecular weight as they migrate towards the positively charged pole. Following this process, the samples are transferred to a membrane, which can be done by three different techniques: usually wet or semi dry transfer (Gavini & Parameshwaran, 2019). Wet transfer is effective to facilitate the transfer of large sized proteins, while semi dry transfer is quicker to be performed than wet transfer and suitable for small molecular weight proteins (Cell Signaling Technology, 2024; Gavini & Parameshwaran, 2019). Once the samples are in the membrane, antibody probing is conducted using a primary antibody that is complementary to the target protein and horseradish peroxidase (HRP) containing-secondary antibody (Pillai-Kastoori et al., 2020). For visualisation, substrate (e.g. enhanced chemiluminescence (ECL)) that can react with the enzyme linked to the secondary antibody is added where the substrate is oxidised, producing a signal that can be detected by an imaging system (e.g. ChemiDoc) (Bass et al., 2017; Kielkopf et al., 2021).

2.6. ELISA

The enzyme-linked immunosorbent assay (ELISA) is based on the concept of antigen-antibody binding and often performed to detect and quantify specific proteins using antibodies that have complementary sequence to the target (Liu et al., 2024; Sakamoto et al., 2018). Generally, there are two types of ELISA: direct and indirect ELISA (Sakamoto et al., 2018). Direct ELISA involves the usage of an enzyme-labelled antibody such as HRP, in which

the antibody will directly bind to the target antigen in the sample, while the enzyme on the antibody will catalyse the oxidation of a chromogenic substrate (e.g. 3,3',5,5'-Tetramethylbenzidine (TMB)) (Guo et al., 2016; Sakamoto et al., 2018). This reaction will result in a colour change that can be detected by a spectrophotometer (Sakamoto et al., 2018). Although the overall process of indirect ELISA is the same as direct ELISA, the difference lies in the usage of another antibody that will firstly bind to the target antigen and the enzyme-linked antibody will bind to the antigen-bound antibody instead of directly binding to the target (Sakamoto et al., 2018). In some procedures, biotin and streptavidin are also incorporated to further ensure specific binding between the antibody and antigen (Liu et al., 2024).

2.7. LDH Assay

The lactate dehydrogenase (LDH) assay is a cytotoxicity assay that can measure the number of dead cells based on the presence of LDH enzyme. This is because LDH is normally found inside the cells and will leak to the extracellular space when the membrane is damaged (Van den Bossche et al., 2020). In this assay, the amount of LDH released can be determined by adding lactate, which will produce pyruvate and the main product and NADH as a byproduct through oxidation (Kaja et al., 2017). NADH can reduce tetrazolium salt to a coloured formazan product that can be measured spectrophotometrically, in which the amount of product is directly proportional to the presence of LDH in the sample (Kumar et al., 2018).

III. MATERIALS & METHODS

3.1. Bacterial Supernatant Collection

The bacterial colonies were picked and mixed in a 15mL tube containing BHI media. The bacteria were incubated overnight at 37°C on the shaker. The tube was centrifuged at 14,000xg for 15 minutes. The supernatant was filtered to a new 15mL tube.

3.2. Cell Culture and Maintenance

THP-1 cells were maintained in complete RPMI media with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) at 37°C with 5% CO_2. The B16F10 cells were maintained in complete Dulbecco's modified eagle media (cDMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) at 37°C with 5% CO_2. Cells were passaged once reached 80-90% confluency.

3.3. IncuCyte® Cytotoxicity Assay

THP-1 cells were seeded in two 12-well plates, each well with 2,000,000 cell density in complete RPMI media. LPS was added to all of the wells while MCC950 was added to half of the total wells alternately. Cells were incubated for two hours at 37°C with 5% CO_2. propidium iodide (PI) was added to all of the wells. Bacterial supernatants, Nigericin, *Salmonella typhimurium* were added to their irrespective wells (Appendix 1). The plates were placed in the IncuCyte® machine. The machine was set to scan for every 10 minutes with a total scan of three hours.

B16F10 cells were seeded in a 24-well plate, each well with 500,000 cell density in cDMEM. Once attached, PI was added to all of the wells. Bacterial supernatants, HBL, and

α-toxin were added to their irrespective wells (Appendix 2). The plates were placed in the IncuCyte® machine. The machine was set to scan for every 10 minutes with a total scan of three hours.

3.4. Western Blot

RIPA lysis buffer and 4x dye were added to the plates containing THP-1 cells post-IncuCyte® Cytotoxicity Assay. Samples were collected to 1.5mL of microcentrifuge tubes and heated at 100^{o}C for 15 minutes. Samples were loaded to the wells of gels for SDS-PAGE and ran for 45 minutes at 100V. PVDF membranes were incubated in methanol for two minutes and transferred to a container with transfer buffer. Gels were moved to containers with transfer buffer. Transfer sandwiches were made with the order of wet pad-PVDF membrane-gel-wet pad and placed on the transfer chamber. Semi dry transfer was performed using the settings of 2.5A; 25V for 20 minutes. Membrane was blocked using 5% skim milk on a shaker for one hour. Primary antibodies for caspase-1 and GSDMD were added and membranes were incubated overnight. Membranes were washed using TBST for 10 minutes on a shaker and repeated four times. Anti-mouse and anti-rabbit secondary antibodies for caspase-1 and GSDMD respectively, were added and incubated on a shaker for one hour. Membranes were washed using TBST for 10 minutes on a shaker and repeated four times. Clarity ECL substrates were added to the membranes in a 1:1 ratio and incubated for two minutes. Membranes were visualised using the ChemiDoc Imaging System.

3.5. ELISA

Microwell strips were washed twice with Wash Buffer. The wells were emptied and tapped on absorbent pad. Sample Diluent was added to all wells. Standard dilutions were added in duplicate to the designated wells. Samples were added in duplicate to the designated wells (Appendix 3). Biotin-Conjugate was added to all wells. Plate was covered

with an adhesive film and incubated at room temperature. Adhesive film was removed and wells were emptied. Microwell strips were washed three times. The wells were emptied and tapped on absorbent pad. Diluted Streptavidin-HRP was added to all wells. Plate was covered with an adhesive film and incubated at room temperature for one hour on a microplate shaker. Adhesive film was removed and wells were emptied. Microwell strips were washed three times. The wells were emptied and tapped on absorbent pad. TMB Substrate Solution was added to all wells. Microwell strips were incubated for approximately 10 minutes at room temperature. Colour development was monitored until the highest standard had developed a dark blue colour (OD of 0.9 – 0.95) using ELISA reader at 620 nm. Stop Solution was added to each well. Absorbance of each microwell was read using a spectrophotometer at 450 nm immediately after Stop Solution was added.

3.6. LDH Assay

PBS was added into each well of the 96-well plate. Sample was added into each well containing PBS excluding the last row for standard (Appendix 4). Standard was added into each well of the last row containing PBS. CytoTox Reagent was added into each sample or standard. Plate was incubated in the dark and checked until its colour changed into a gradation of brown. Stop Solution was added into each well and bubbles were removed. Plate was read at 490 nm within one hour after Stop Solution was added.

3.7. Data Analysis

Data from IncuCyte® Cytotoxicity Assay, ELISA, and LDH assay was analysed and figures were generated using GraphPad Prism version 10.3.1. Western blot images were organised using ImageLab and Microsoft Powerpoint. Statistical analysis was performed using one-tailed Student's t-test with $p<0.05$ considered significant.

IV. RESULTS AND DISCUSSION

4.1. NLRP3 is correlated with the activation of caspase-1 and GSDMD caused by the four bacterial supernatants in THP-1

To evaluate the effect of the four bacterial supernatants on NLRP3-mediated pyroptosis, IncuCyte® cytotoxicity assay was performed with scans every 10 minutes and percentage of PI^+ cells was calculated.

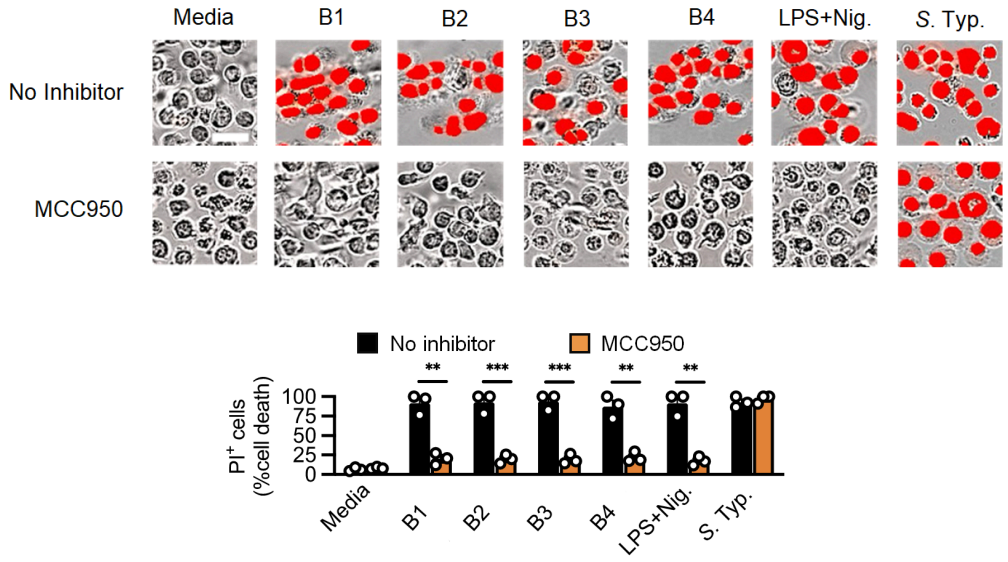

Figure 4. IncuCyte® cytotoxicity assay result of THP-1 cells with and without MCC950 treated with four different bacterial supernatants. (a) IncuCyte® images of THP-1 cells (red spots indicating PI stain). (b) Calculated percentage of PI^+ cells in different sample treatments, *p<0.05 considered statistically significant.

The main condition differed in the presence of the NLRP3 inhibitor MCC950, therefore, cells with and without MCC950 were compared when treated with the bacterial supernatants. The normal THP-1 morphology could be observed in the media condition and no difference could be observed between cells with and without MCC950. However, when cells were exposed to the bacterial supernatants, cells with MCC950 exhibited a significantly

lower number of dead cells compared to cells without the inhibitor, indicating that NLRP3 contributed to the bacterial supernatant-induced cell death. This is also confirmed using Nigericin treatment as the positive control, which showed similar results with cells treated with the supernatants. This is because nigericin is a potassium ionophore that facilitate K+/H+ anti-port across the membrane, which also causes the release of intracellular K+ similar to how HBL activates NLRP3 although LPS priming is required as its effects alone are unable to trigger NLRP3 activation unlike the bacterial toxins that causes sufficient membrane damage to bypass this first signal (Armstrong et al., 2019; Nanda et al., 2021). Additionally, *S.* Typhimurium as the negative control further confirmed this as a similar number of dead cells was observed in both conditions, with or without MCC950. This is due to the fact that *S.* Typhimurium kills the cells regardless of the NLRP3 activation as it requires NLRC4 as the sensor instead to induce inflammasome activation (Andrade & Zamboni, 2020).

In order to further confirm the NLRP3-mediated inflammasome activation, western blot was performed to evaluate the effect of the bacterial supernatants on caspase-1 and GSDMD cleavage which indicate their activation (Devant & Kagant, 2023).

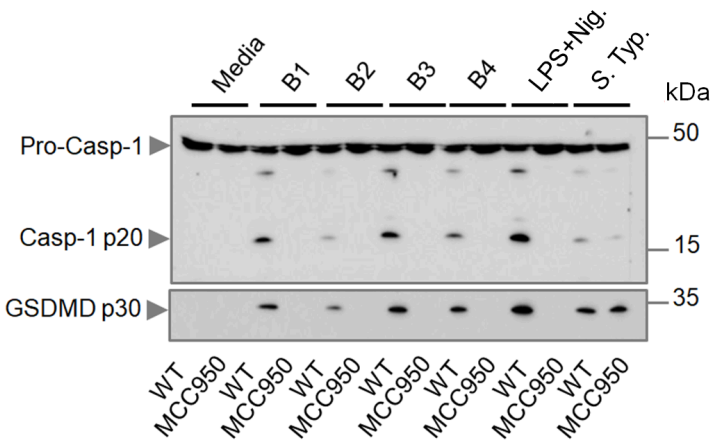

Figure 5. Western blot result of THP-1 with and without MCC950 treated with four different bacterial supernatants.

The cleavage of caspase-1, which also subsequently leads to GSDMD cleavage, are the primary characteristics of inflammasome activation (Kesavardhana & Kanneganti, 2017).

Cells without MCC950 exhibited cleavage of both caspase-1 and GSDMD, while none was observed in the presence of MCC950. This confirms that NLRP3 is required to induce inflammasome activation that triggers both caspase-1 and GSDMD cleavage. This is justified by the same pattern in cells treated with Nigericin, while cleaved bands are present in cells with and without MCC950 when exposed to *S.* Typhimurium. However, to further confirm these results, ELISA was performed to measure IL-1β and IL-18 levels upon exposure towards the supernatants.

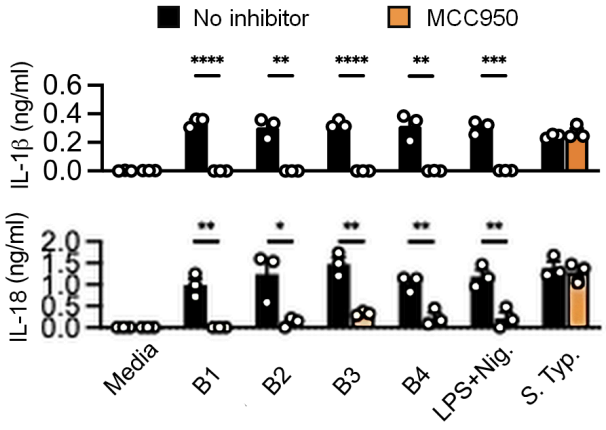

Figure 6. ELISA result of THP-1 with and without MCC950 treated with four different bacterial supernatants, *p<0.05 considered statistically significant.

Consistent with the western blot results, cells without MCC950 exhibited a significantly higher IL-1β and IL-18 levels compared to those with the inhibitor. This is because these pro-inflammatory cytokines are cleaved and subsequently activated by caspase-1. Therefore, when caspase-1 and GSDMD are activated as the downstream event of NLRP3 activation, IL-1β and IL-18 levels will also be increased (Danielski et al., 2020; Yu et al., 2021).

To confirm the overall results, LDH assay was performed to evaluate the difference in the presence of NLRP3 on the bacterial supernatants-induced cell death. In accordance with the other results obtained for this experiment, cells without MCC950 showed a significantly higher percentage of dead cells in comparison to cells with the inhibitor. This is because the

activation of GSDMD would create pores on the plasma membrane, causing the release of cellular contents, including the LDH enzyme, which is the indicator for cell death in this assay (Sborgi et al., 2016; Wang et al.,2022).

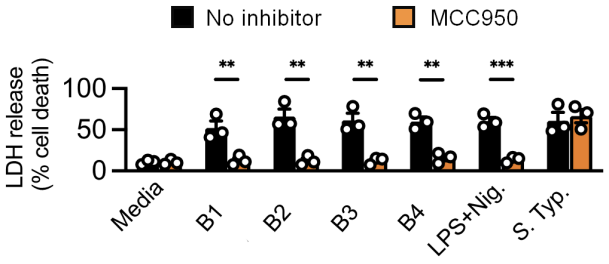

Figure 7. LDH assay result of THP-1 with and without MCC950 treated with four different bacterial supernatants, *p<0.05 considered statistically significant.

4.2. Litaf and Cdip1 are required to induce the effect from HBL-like toxins

To evaluate the role of Litaf and Cdip1 in contributing to the effects caused by the bacterial supernatants, IncuCyte® cytotoxicity assay was performed with the same settings used for the THP-1 experiment.

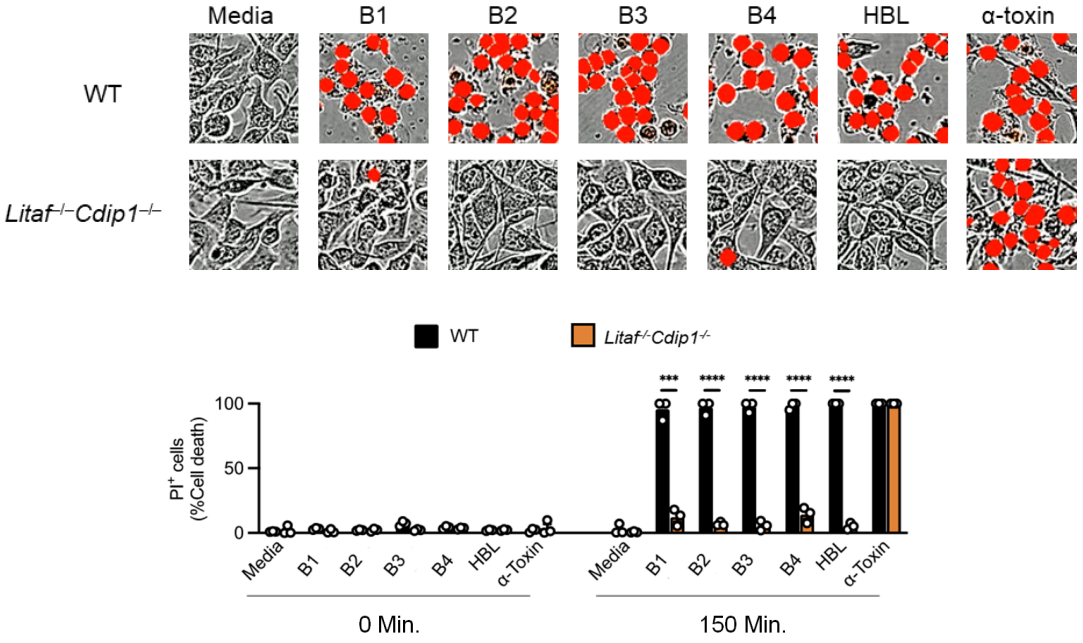

Figure 8. IncuCyte® cytotoxicity assay result of WT and Litaf$^{-/-}$Cdip1$^{-/-}$ B16F10 cells treated with four different bacterial supernatants. (a) IncuCyte® images of WT and Litaf$^{-/-}$Cdip1$^{-/-}$ B16F10 cells (red spots indicating PI stain). (b) Calculated percentage of PI+ cells in different sample treatments, *p<0.05 considered statistically significant.

Liu et al. (2020) previously reported that Litaf and Cdip1 contributed to the complete formation of HBL as a pore forming toxin by facilitating the binding of the B component of the toxin, allowing further recruitment of L1 and L2 to complete the toxin complex, resulting in its functionality. The normal B16F10 morphology could be observed in media condition and no difference was observed between the WT and Litaf$^{-/-}$Cdip1$^{-/-}$ cells. However, WT cells displayed a significantly higher percentage of PI+ cells that indicate dead cells when exposed to the bacterial supernatants, including those exposed to HBL toxin of *B. cereus*. This signifies that the virulence factors of the four different Bacillus species other than *B. cereus* have a high similarity to HBL from *B. cereus* that drive them to also require Litaf and Cdip1 to be present in order to affect the cells. This is also supported by the nonsignificant percentage of dead cells between WT and Litaf$^{-/-}$Cdip1$^{-/-}$ cells when exposed to α-toxin as it does not require these two receptors as it binds to ADAM10, a disintegrin and metalloproteinase binding protein, that allows it to form heptameric pores on the plasma membrane (Bonifacius et al., 2020; von Hoven et al., 2019; von Hoven et al., 2016). Nevertheless, LDH assay was performed to confirm such results.

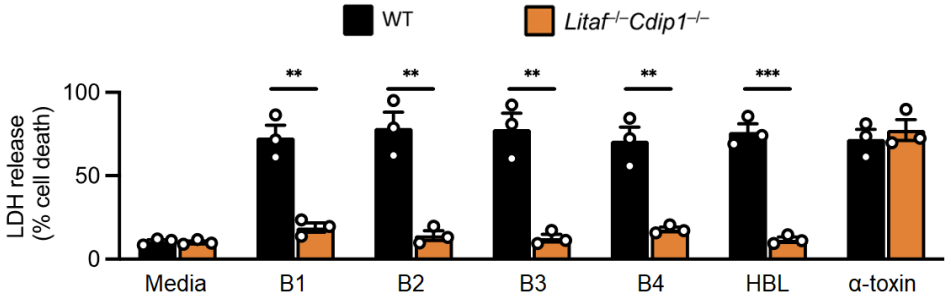

Figure 9. LDH assay result of WT and Litaf$^{-/-}$Cdip1$^{-/-}$ B16F10 cells treated with four different bacterial supernatants, *p<0.05 considered statistically significant.

In alignment with the IncuCyte® cytotoxicity assay results, WT cells exhibited a significantly higher cell death percentage compared to Litaf$^{-/-}$Cdip1$^{-/-}$ cells when treated with

the four bacterial supernatants as well as HBL. This indicates Litaf and Cdip1 are required to facilitate the effect of the virulence factors from these four bacteria in a similar manner as HBL of *B. cereus*. Additionally, nonsignificant percentage of cell death between WT and Litaf$^{-/-}$Cdip1$^{-/-}$ cells was also observed when treated with α-toxin, which is also consistent with the previous results. Such results also verify that these virulence factors of the four different *Bacillus* species are HBL-like toxins.

4.3. Limitations

While this research presents valuable insights in the mechanism of virulence factors from the four *Bacillus* species other than *B. cereus*, it is essential to recognise a limitation that can be considered for future research. The faint bands of cleaved caspase-1 and gasdermin D (GSDMD) in the Western blot result of THP-1 cells treated with *S*. Typhi may be due to the aged bacteria culture, therefore the band intensity can possibly be increased by using the fresh batch of the bacteria. Findings from this study can be further validated using an in vivo model to give a better understanding of the host-pathogen interactions and confirm the physiological relevance from NLRP3-mediated pyroptosis observed.

V. SELF REFLECTION

Throughout this experience working in Si Ming Man's lab, I have acquired and improved a wide range of laboratory techniques that will be crucial for my future career in biomedical research. I have learned how to use an IncuCyte® machine for real-time analysis, which will be useful in performing live-cell imaging. I have significantly improved my western blot technique, which would be invaluable in molecular biology and clinical research. I have re-learned bacterial culture that is one of the basic skills critical in microbiology research. I have also learned to work on suspension cell culture which will be very useful when working in the field of immunology.

Based on my experience in this programme, I believe my strength lies in the ability to adapt in a foreign country quickly enough to work on my projects and learn these new techniques, while enjoying the company of my labmates. I also believe my time management plays a significant role in helping me adapt as organised schedules allowed me to keep up with the activities I needed to do. However, I also realise my attention to detail tends to slow down my work pace due to my anxiety and pressure in a new laboratory environment surrounded by people far more experienced than me. Nevertheless, Si Ming and my labmates were very welcoming and supportive, ready to help me when needed, which allowed me to enjoy my time with them despite the busy tasks I had to do for my project.

i3L played an important role in helping me apply for this program, which is an invaluable chapter in my life. Its values also contributed to this experience of mine. Grit allowed me to approach challenges with perseverance, especially when I had to troubleshoot and redo experiments due to unexpected results. Integrity guided me in maintaining accuracy and detail in data reporting. Additionally, Role-Model motivated me to be dependable and conscientious, which helped me positively respond to the new environment.

The Immunology and Molecular Cellular Biology courses I took back in my third semester majorly contributed to my success during the internship as they provided me with the necessary background knowledge to understand the concept behind my project and analyse the obtained results based on that concept. Aside from that, the laboratory courses from the early semesters introduced me to the basic laboratory rules and techniques required, allowing me to adapt well despite it being an advanced research lab.

The BRIGHT sessions I participated during my time in i3L were crucial in developing my soft skills, particularly in communication and teamwork. They allow me to freely communicate my opinions, ideas, and even questions with confidence to those who are more experienced in the field. Furthermore, these sessions also helped me to effectively collaborate with my mentor, labmates, and supervisor, resulting in a supportive and productive work environment.

I was able to positively contribute to the lab's works by assisting my mentor when working on my project, as well as discussing the results that we already acquired. Furthermore, I also provided assistance to any of my labmates who needed it when I was free. My attention to detail also helped my labmates to avoid any miscalculations and misread in the protocol when conducting their experiments, preventing errors that could possibly arise from small yet critical mistakes.

VI. CONCLUSION

The virulence factors of the four *Bacillus* species other than *B. cereus* could trigger NLRP3 inflammasome activation that results in pyroptosis, indicated by the reduced dead cell percentage, absence of caspase-1 and GSDMD cleavage,lower IL-1β and IL-18 levels, and decreased LDH release in the presence of NLRP3 inhibitor MCC950. Additionally, these virulence factors may act similarly with HBL of B. cereus as they also relied to Litaf and Cdip1 receptors to facilitate their cytotoxic effects, which is evidenced by the reduced cell death in Litaf$^{-/-}$Cdip1$^{-/-}$ cells. Overall, these findings suggest that these virulence factors activate NLRP3 inflammasome via a mechanism similar to HBL of *B. cereus*. However, future studies could consider validating these findings using an in vivo model to further observe the hot-pathogen interactions in a more complex condition.

REFERENCES

Akbal, A., Dernst, A., Lovotti, M., Mangan, M. S., McManus, R. M., & Latz, E. (2022). How location and cellular signaling combine to activate the NLRP3 inflammasome. *Cellular & Molecular Immunology, 19*(11), 1201-1214. https://doi.org/10.1038/s41423-022-00922-w

Akere, M. T., Zajac, K. K., Bretz, J. D., Madhavaram, A. R., Horton, A. C., & Schiefer, I. T. (2024). Real-Time Analysis of Neuronal Cell Cultures for CNS Drug Discovery. *Brain Sciences, 14*(8), 770. https://doi.org/10.3390/brainsci14080770

Almeida-da-Silva, C. L. C., Savio, L. E. B., Coutinho-Silva, R., & Ojcius, D. M. (2023). The role of NOD-like receptors in innate immunity. *Frontiers in Immunology, 14*, 1122586. https://doi.org/10.3389/fimmu.2023.1122586

Andrade, W. A., & Zamboni, D. S. (2020). NLRC4 biology in immunity and inflammation. *Journal of Leukocyte Biology, 108*(4), 1117-1127. https://doi.org/10.1002/JLB.3MR0420-573R

Armstrong, H., Bording-Jorgensen, M., Chan, R., & Wine, E. (2019). Nigericin promotes NLRP3-independent bacterial killing in macrophages. *Frontiers in Immunology, 10*, 2296. https://doi.org/10.3389/fimmu.2019.02296

Bass, J. J., Wilkinson, D. J., Rankin, D., Phillips, B. E., Szewczyk, N. J., Smith, K., & Atherton, P. J. (2017). An overview of technical considerations for Western blotting applications to physiological research. *Scandinavian Journal of Medicine & Science in Sports, 27*(1), 4-25. https://doi.org/10.1111/sms.12702

Blevins, H. M., Xu, Y., Biby, S., & Zhang, S. (2022). The NLRP3 inflammasome pathway: a review of mechanisms and inhibitors for the treatment of inflammatory diseases. *Frontiers in Aging Neuroscience, 14*, 879021. https://doi.org/10.3389/fnagi.2022.879021

Bonifacius, A., Goldmann, O., Floess, S., Holtfreter, S., Robert, P. A., Nordengrün, M., ... & Huehn, J. (2020). Staphylococcus aureus alpha-toxin limits type 1 while fostering type 3 immune responses. *Frontiers in Immunology, 11*, 1579. https://doi.org/10.3389/fimmu.2020.01579

Bravo, D. D., Shi, Y., Sheu, A., Liang, W. C., Lin, W., Wu, Y., ... & Wang, J. (2023). A real-time image-based efferocytosis assay for the Discovery of functionally inhibitory Anti-MerTK antibodies. *The Journal of Immunology, 210*(8), 1166-1176. https://doi.org/10.4049/jimmunol.2200597

Cell Signaling Technology. (2024). *Are you ready to transfer your protein? Wet vs Semi-dry Transfer*. Cell Signaling Technology Inc. https://blog.cellsignal.com/wet-versus-semi-dry-transfer

Chen, Y., Ye, X., Escames, G., Lei, W., Zhang, X., Li, M., ... & Yang, Y. (2023). The NLRP3 inflammasome: contributions to inflammation-related diseases. *Cellular & Molecular Biology Letters, 28*(1), 51. https://doi.org/10.1186/s11658-023-00462-9

Colaco, C. M., Basile, K., Draper, J., & Ferguson, P. E. (2021). Fulminant Bacillus cereus food poisoning with fatal multi-organ failure. *BMJ Case Reports CP, 14*(1), e238716. https://doi.org/10.1136/bcr-2020-238716

Danielski, L. G., Giustina, A. D., Bonfante, S., Barichello, T., & Petronilho, F. (2020). The NLRP3 inflammasome and its role in sepsis development. *Inflammation, 43*, 24-31. https://doi.org/10.1007/s10753-019-01124-9

Devant, P., & Kagan, J. C. (2023). Molecular mechanisms of gasdermin D pore-forming activity. *Nature Immunology, 24*(7), 1064-1075. https://doi.org/10.1038/s41590-023-01526-w

Dietrich, R., Jessberger, N., Ehling-Schulz, M., Märtlbauer, E., & Granum, P. E. (2021). The food poisoning toxins of Bacillus cereus. *Toxins, 13*(2), 98. https://doi.org/10.3390/toxins13020098

Di Virgilio, F., Jiang, L. H., Roger, S., Falzoni, S., Sarti, A. C., Vultaggio-Poma, V., ... & Adinolfi, E. (2019). Structure, function and techniques of investigation of the P2X7 receptor (P2X7R) in

mammalian cells. In *Methods in Enzymology* (Vol. 629, pp. 115-150). Academic Press. https://doi.org/10.1016/bs.mie.2019.07.043

Du, H., Sun, X., Chong, X., Yang, M., Zhu, Z., & Wen, Y. (2023). A review on smart active packaging systems for food preservation: Applications and future trends. *Trends in Food Science & Technology*, 104200. https://doi.org/10.1016/j.tifs.2023.104200

Ehling-Schulz, M., Lereclus, D., & Koehler, T. M. (2019). The Bacillus cereus group: Bacillus species with pathogenic potential. *Microbiology Spectrum, 7*(3), 10-1128. https://doi.org/10.1128/microbiolspec.gpp3-0032-2018

Elshaghabee, F. M., Rokana, N., Gulhane, R. D., Sharma, C., & Panwar, H. (2017). Bacillus as potential probiotics: status, concerns, and future perspectives. *Frontiers in Microbiology, 8*, 1490. https://doi.org/10.3389/fmicb.2017.01490

Gavini, K., & Parameshwaran, K. (2019). Western blot. https://www.ncbi.nlm.nih.gov/books/NBK542290/

Ghosh, A. K. (2023). Functionality of probiotics on the resistance capacity of shrimp against white spot syndrome virus (WSSV). *Fish & Shellfish Immunology*, 108942. https://doi.org/10.1016/j.fsi.2023.108942

Gorka, O., Neuwirt, E., & Groß, O. (2019). Walking over the inflammasome. *Nature Chemical Biology, 15*(6), 552-553. https://doi.org/10.1038/s41589-019-0292-8

Guo, L., Xu, S., Ma, X., Qiu, B., Lin, Z., & Chen, G. (2016). Dual-color plasmonic enzyme-linked immunosorbent assay based on enzyme-mediated etching of Au nanoparticles. *Scientific Reports, 6*(1), 32755. https://doi.org/10.1038/srep32755

Inukai, R., Mori, K., Kuwata, K., Suzuki, C., Maki, M., Takahara, T., & Shibata, H. (2021). The novel ALG-2 target protein CDIP1 promotes cell death by interacting with ESCRT-I and VAPA/B. *International Journal of Molecular Sciences, 22*(3), 1175. https://doi.org/10.3390/ijms22031175

Inukai, R., Mori, K., Maki, M., Takahara, T., & Shibata, H. (2024). Cytoprotective Role of Autophagy in CDIP1 Expression-Induced Apoptosis in MCF-7 Breast Cancer Cells. *International Journal of Molecular Sciences, 25*(12), 6520. https://doi.org/10.3390/ijms25126520

Invitrogen. (2019). *Mouse IL-18 ELISA User Guide*. Thermo Fisher Scientific. Retrieved 19 December 2024, from https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018176_618-3_Mo_IL-18_ELISA_UG.pdf

InvivoGen. (n.d.). *Nigericin*. InvivoGen. Retrieved 31 October 2024, from https://www.invivogen.com/nigericin

Kaja, S., Payne, A. J., Naumchuk, Y., & Koulen, P. (2017). Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes. *Current Protocols in Toxicology, 72*(1), 2-26. https://doi.org/10.1002/cptx.21

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 inflammasome: an overview of mechanisms of activation and regulation. *International Journal of Molecular Sciences, 20*(13), 3328. https://doi.org/10.3390/ijms20133328

Kesavardhana, S., & Kanneganti, T. D. (2017). Mechanisms governing inflammasome activation, assembly and pyroptosis induction. *International Immunology, 29*(5), 201-210. https://doi.org/10.1093/intimm/dxx018

Kielkopf, C. L., Bauer, W., & Urbatsch, I. L. (2021). Analysis of proteins by immunoblotting. *Cold Spring Harbor Protocols, 2021*(12), pdb-prot102251. https://doi.org/10.1101/pdb.prot102251.

Kulkeaw, K. (2021). Progress and challenges in the use of fluorescence-based flow cytometric assays for anti-malarial drug susceptibility tests. *Malaria Journal, 20*(1), 57. https://doi.org/10.1186/s12936-021-03591-8

Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harbor Protocols, 2018*(6), pdb-prot095497. https://doi.org/10.1101/pdb.prot095497

Ligasová, A., & Koberna, K. (2021). DNA dyes—highly sensitive reporters of cell quantification: comparison with other cell quantification methods. *Molecules, 26*(18), 5515. https://doi.org/10.3390/molecules26185515

Liu, Y., Luo, Y., Li, W., Xu, X., Wang, B., Xu, X., ... & Chen, D. (2024). Current analytical strategies for the determination of quinolone residues in milk. *Food Chemistry, 430*, 137072. https://doi.org/10.1016/j.foodchem.2023.137072

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *North American Journal of Medical Sciences, 4*(9), 429. https://doi.org/10.4103/1947-2714.100998

Mathur, A., Feng, S., Hayward, J. A., Ngo, C., Fox, D., Atmosukarto, I. I., ... & Man, S. M. (2019). A multicomponent toxin from Bacillus cereus incites inflammation and shapes host outcome via the NLRP3 inflammasome. *Nature Microbiology, 4*(2), 362-374. https://doi.org/10.1038/s41564-018-0318-0

Messelhäußer, U., & Ehling-Schulz, M. (2018). *Bacillus cereus*—a multifaceted opportunistic pathogen. *Current Clinical Microbiology Reports, 5*, 120-125. https://doi.org/10.1007/s40588-018-0095-9

McKenney, P. T., Driks, A., & Eichenberger, P. (2013). The Bacillus subtilis endospore: assembly and functions of the multilayered coat. *Nature Reviews Microbiology, 11*(1), 33-44. https://doi.org/10.1038/nrmicro2921

Nagar, A., Bharadwaj, R., Shaikh, M. O. F., & Roy, A. (2023). What are NLRP3-ASC specks? an experimental progress of 22 years of inflammasome research. *Frontiers in Immunology, 14*, 1188864. https://doi.org/10.3389/fimmu.2023.1188864

Namba, T., Tian, F., Chu, K., Hwang, S. Y., Yoon, K. W., Byun, S., ... & Lee, S. W. (2013). CDIP1-BAP31 complex transduces apoptotic signals from endoplasmic reticulum to mitochondria under endoplasmic reticulum stress. *Cell Reports, 5*(2), 331-339. https://doi.org/10.1016/j.celrep.2013.09.020

Nanda, S. K., Prescott, A. R., Figueras-Vadillo, C., & Cohen, P. (2021). IKKβ is required for the formation of the NLRP3 inflammasome. *EMBO Reports, 22*(10), e50743. https://doi.org/10.15252/embr.202050743

Nilsson, J., Gonçalves, I., & Edsfeldt, A. (2015). Chronic inflammation and atherosclerosis. In *Early Vascular Aging (EVA)* (pp. 157-167). Academic Press. https://doi.org/10.1016/B978-0-12-801387-8.00016-8

Pillai-Kastoori, L., Heaton, S., Shiflett, S. D., Roberts, A. C., Solache, A., & Schutz-Geschwender, A. R. (2020). Antibody validation for Western blot: By the user, for the user. *Journal of Biological Chemistry, 295*(4), 926-939. https://doi.org/10.1074/jbc.RA119.010472

Porwal, S., Lal, S., Cheema, S., & Kalia, V. C. (2009). Phylogeny in aid of the present and novel microbial lineages: diversity in Bacillus. *PLoS One, 4*(2), e4438. https://doi.org/10.1371/journal.pone.0004438

Prince, C., & Kovac, J. (2022). Regulation of enterotoxins associated with Bacillus cereus sensu lato toxicoinfection. *Applied and Environmental Microbiology, 88*(13), e00405-22. https://doi.org/10.1128/aem.00405-22

Que, X., Zheng, S., Song, Q., Pei, H., & Zhang, P. (2024). Fantastic voyage: The journey of NLRP3 inflammasome activation. *Genes & Diseases, 11*(2), 819-829. https://doi.org/10.1016/j.gendis.2023.01.009

Rumpret, M., von Richthofen, H. J., Peperzak, V., & Meyaard, L. (2021). Inhibitory pattern recognition receptors. *Journal of Experimental Medicine, 219*(1), e20211463. https://doi.org/10.1084/jem.20211463

Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., & Morimoto, S. (2018). Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *Journal of Natural Medicines, 72*, 32-42. https://doi.org/10.1007/s11418-017-1144-z

Sborgi, L., Rühl, S., Mulvihill, E., Pipercevic, J., Heilig, R., Stahlberg, H., ... & Hiller, S. (2016). GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. *The EMBO Journal, 35*(16), 1766-1778. https://doi.org/10.15252/embj.201694696

Siller, I. G., Enders, A., Steinwedel, T., Epping, N. M., Kirsch, M., Lavrentieva, A., ... & Bahnemann, J. (2019). Real-time live-cell imaging technology enables high-throughput screening to verify in vitro biocompatibility of 3D printed materials. *Materials, 12*(13), 2125. https://doi.org/10.3390/ma12132125

Soni, J., Sinha, S., & Pandey, R. (2024). Understanding bacterial pathogenicity: a closer look at the journey of harmful microbes. *Frontiers in Microbiology, 15*, 1370818.

Tewari, A., & Abdullah, S. (2015). Bacillus cereus food poisoning: international and Indian perspective. *Journal of Food Science and Technology, 52*, 2500-2511. https://doi.org/10.1007/s13197-014-1344-4

Tuipulotu, D. E., Mathur, A., Ngo, C., & Man, S. M. (2021). Bacillus cereus: epidemiology, virulence factors, and host–pathogen interactions. *Trends in Microbiology, 29*(5), 458-471. https://doi.org/10.1016/j.tim.2020.09.003

Van den Bossche, S., Vandeplassche, E., Ostyn, L., Coenye, T., & Crabbé, A. (2020). Bacterial interference with lactate dehydrogenase assay leads to an underestimation of cytotoxicity. *Frontiers in Cellular and Infection Microbiology, 10*, 494. https://doi.org/10.3389/fcimb.2020.00494

von Hoven, G., Qin, Q., Neukirch, C., Husmann, M., & Hellmann, N. (2019). Staphylococcus aureus α-toxin: small pore, large consequences. *Biological Chemistry, 400*(10), 1261-1276. https://doi.org/10.1515/hsz-2018-0472

von Hoven, G., Rivas, A. J., Neukirch, C., Klein, S., Hamm, C., Qin, Q., ... & Husmann, M. (2016). Dissecting the role of ADAM10 as a mediator of Staphylococcus aureus α-toxin action. *Biochemical Journal, 473*(13), 1929-1940. https://doi.org/10.1042/BCJ20160062

Wang, S., Liu, Y., Zhang, L., & Sun, Z. (2022). Methods for monitoring cancer cell pyroptosis. *Cancer Biology & Medicine, 19*(4), 398. https://doi.org/10.20892/j.issn.2095-3941.2021.0504

Yu, P., Zhang, X., Liu, N., Tang, L., Peng, C., & Chen, X. (2021). Pyroptosis: mechanisms and diseases. *Signal Transduction and Targeted Therapy, 6*(1), 128. https://doi.org/10.1038/s41392-021-00507-5

Zeighami, H., Nejad-Dost, G., Parsadanians, A., Daneshamouz, S., & Haghi, F. (2020). Frequency of hemolysin BL and non-hemolytic enterotoxin complex genes of Bacillus cereus in raw and cooked meat samples in Zanjan, Iran. *Toxicology Reports, 7*, 89-92. https://doi.org/10.1016/j.toxrep.2019.12.006

Zhan, X., Li, Q., Xu, G., Xiao, X., & Bai, Z. (2023). The mechanism of NLRP3 inflammasome activation and its pharmacological inhibitors. *Frontiers in Immunology, 13*, 1109938. https://doi.org/10.3389/fimmu.2022.1109938

Zheng, D., Liwinski, T., & Elinav, E. (2020). Inflammasome activation and regulation: toward a better understanding of complex mechanisms. *Cell Discovery, 6*(1), 36. https://doi.org/10.3389/fimmu.2023.1122586

Zou, J., Guo, P., Lv, N., & Huang, D. (2015). Lipopolysaccharide-induced tumor necrosis factor-α factor enhances inflammation and is associated with cancer. *Molecular Medicine Reports, 12*(5), 6399-6404. https://doi.org/10.3892/mmr.2015.4243

APPENDICES

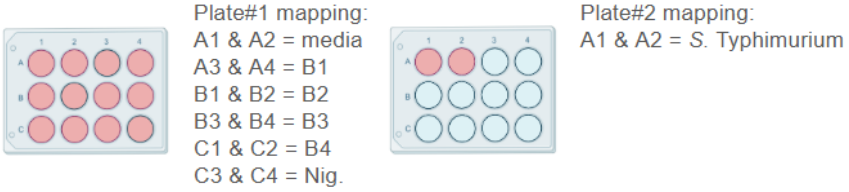

Figure A1. Plate mapping for THP-1 cells treated with bacterial supernatants and controls.

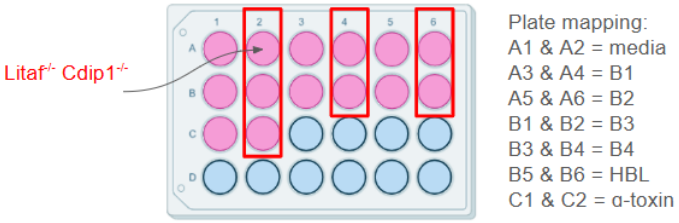

Figure A2. Plate mapping for WT and KO B16F10 cells treated with bacterial supernatants and controls.

	1	2	3	4
A	Standard 1 2000.0 pg/mL	Standard 1 2000.0 pg/mL	Sample 1	Sample 1
B	Standard 2 1000.0 pg/mL	Standard 2 1000.0 pg/mL	Sample 2	Sample 2
C	Standard 3 500.0 pg/mL	Standard 3 500.0 pg/mL	Sample 3	Sample 3
D	Standard 4 250.0 pg/mL	Standard 4 250.0 pg/mL	Sample 4	Sample 4
E	Standard 5 125.0 pg/mL	Standard 5 125.0 pg/mL	Sample 5	Sample 5
F	Standard 6 62.5 pg/mL	Standard 6 62.5 pg/mL	Sample 6	Sample 6
G	Standard 7 31.3 pg/mL	Standard 7 31.3 pg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

Figure A3. Plate mapping for ELISA based on Invitrogen Mouse ELISA IL-18 User Guide (retrieved from Invitrogen, 2019).

Samp1	Samp2	Samp3	Samp4	Samp5	Samp6	Samp7	Samp8	Samp9	Samp10	Samp11	Samp12
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------	------	------	------	------	------	------	------	------	------	------	------
------	------	------	------	------	------	------	------	------	------	Samp83	Samp84
Strd1	Strd2	Strd3	Strd4	Strd5	PBS	Strd1	Strd2	Strd3	Strd4	Strd5	PBS

Figure A4. Plate mapping for LDH assay based on Man Lab LDH Assay Protocol.