Comparative analysis of macrophage phagocytic efficiency against multidrug-resistant clinical bacterial isolates

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Abstract-

In this study around the world, superbugs resistant to many drugs now pose a serious public health issue because few treatments remain effective. Bacteria like *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii,* along with Pseudomonas aeruginosa, often cause infections picked up in hospitals - these tend to worsen outcomes and lead to more deaths. At the frontlines of defence, macrophages clear pathogens through a process known as phagocytosis, helping protect the body from day one. Still, some MDR bacteria might outsmart the body's clean-up cells. This research looks at how well immune fighters grab and destroy different types of drug-resistant versus regular bacteria found in patients. Bacteria samples come from real cases, checked by usual lab methods to confirm their presence. After finding which ones are present, tests with antibiotics show which resist multiple drugs. A lab-based setup involving macrophage cells grown from lines will be created through differentiation. Measuring how these cells take in particles involves tracking the phagocytic index along with bacterial survival inside them. Instead of just guessing, actual counts using CFU analysis after gentamicin treatment give clear numbers. On top of that, measuring ROS levels shows whether oxidative bursts happen as expected. Looking at the results, differences in how macrophages clear MDR compared to non-MDR bacteria might come into view, offering clues about behaviour between host and germ. One outcome could be new angles on how immunity works when pathogens adapt. Such work may later feed into exploring macrophages as a back-up way to fight drug-resistant infections.

Keyword -
Resistant (MDR) Bacteria, Macrophage Phagocytosis, Innate Immunity, Intracellular Killing, Reactive Oxygen Species (ROS), Host–Pathogen Interaction.

Introduction

Multidrug-resistant (MDR) bacterial infections have become a significant global health issue, exacerbated by the excessive and improper use of antibiotics, along with the swift development of bacterial resistance mechanisms (World Health Organization, 2024). MDR pathogens, including *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa*, are often associated with hospital-acquired infections such as bloodstream infections, pneumonia, urinary tract infections, and wound infections, which frequently result in extended hospital stays and higher mortality rates (Tacconelli et al., 2024). These bacteria typically carry genetic factors like and efflux pump systems that diminish susceptibility to various antibiotic classes, thereby significantly restricting effective treatment options and increasing the clinical burden (Davies & Brown, 2023; World Health Organization, 2024).

While antimicrobial therapy is a fundamental aspect of managing infections, the role of host immune defences, especially innate immune cells like macrophages, is vital in regulating bacterial replication and spread. Macrophages are specialized phagocytic cells that identify and engulf invading bacteria through receptor-mediated processes, subsequently forming phagosomes that merge with lysosomes to release a lethal mixture of hydrolytic enzymes, acidic pH, reactive oxygen species (ROS), and reactive nitrogen

species (RNS) against the engulfed microbes (Sweet et al., 2025; Kumar et al., 2023). In addition to their direct microbicidal functions, macrophages produce pro-inflammatory cytokines such as TNF α, IL 6, and IL 1β, which coordinate subsequent innate and adaptive immune responses, and they undergo metabolic reprogramming that further boosts their antimicrobial capabilities (Zhu et al., 2024; Sweet et al., 2025).

Despite their effective bactericidal mechanisms, multidrug-resistant (MDR) pathogens have devised various strategies to evade macrophage-mediated control. For instance, Staphylococcus aureus is capable of modifying macrophage signalling. Despite their strong bactericidal properties, MDR pathogens have developed a range of tactics to escape macrophage-mediated regulation. For example, Staphylococcus aureus can manipulate the signalling pathways of macrophages through its two-component system, which diminishes phagocytic uptake and intracellular clearance (Li et al., 2024). Other bacterial species may inhibit the fusion of phagosomes and lysosomes, resist oxidative stress by enhancing antioxidant defences, or form biofilm-like aggregates that shield them from intracellular destruction (Singh et al., 2023; Roy et al., 2024). Furthermore, changes in host signalling, such as the activation of platelet EGFR, can unexpectedly influence macrophage phagocytic efficiency and inflammatory response during infections (Luo et al., 2024). These intricate, bidirectional interactions between host cells and MDR organisms highlight the necessity for a thorough analysis of these dynamics.

Recent research also emphasizes the potential of adjusting macrophage responses as a therapeutic approach. For instance, extracellular vesicles derived from probiotics have been demonstrated to enhance macrophage phagocytosis and improve bacterial clearance in experimental sepsis, indicating that immunomodulation could serve as a complement to conventional antimicrobial strategies (Zhu et al., 2024). Moreover, advancements in the understanding of macrophage metabolic remodelling and pattern recognition receptor signalling pathways present opportunities to bolster innate immune functions against resistant pathogens (Sweet et al., 2025; Kumar et al., 2023). Nevertheless, there exists a significant gap in the comparative assessment of macrophage phagocytic efficiency specifically against clinical MDR versus non-MDR isolates, particularly under standardized in vitro conditions that facilitate direct evaluation of functional disparities in host-pathogen interactions. Therefore, the present study aims to conduct a comparative analysis of macrophage phagocytic efficiency against multidrug-resistant and non-multidrug-resistant clinical bacterial isolates**,** using established in vitro infection models. By quantifying phagocytic uptake, intracellular survival and reactive oxygen species production, this research seeks to elucidate immune evasion mechanisms employed by MDR strains and contribute to the identification of potential macrophage-targeted therapeutic strategies to augment host defence in the era of escalating antimicrobial resistance

The worldwide rise in multidrug-resistant (MDR) bacterial infections has become a significant public health issue due to the decreasing efficacy of traditional antibiotics (Sweet et al., 2025).

MDR pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* pose considerable clinical difficulties as they are capable of surviving antibiotic treatment and evading host immune responses, resulting in persistent and recurrent infections. Macrophages, which are essential components of the innate immune system, are crucial in the initial defence against bacterial pathogens through mechanisms of phagocytosis and intracellular killing (Sweet et al., 2025).When bacteria are recognized through pattern recognition receptors, macrophages engulf these pathogens into phagosomes that later merge with lysosomes, thereby exposing the bacteria to hydrolytic enzymes and reactive oxygen species (ROS) to facilitate microbial destruction (Sweet et al., 2025; Zen, 2024).

Furthermore, macrophages generate specific antibacterial responses, which encompass metabolic reprogramming and inducible antimicrobial pathways that aid in the removal of internalized bacteria (Sweet et al., 2025). Nevertheless, multidrug-resistant (MDR) bacteria frequently employ immune evasion tactics that hinder macrophage functionality. For instance, the two-component regulatory system in S. aureus can inhibit macrophage phagocytosis and bactericidal activity during the initial stages of infection, partly by altering host signalling pathways and encouraging bacterial aggregation (Li et al., 2024). The modulation of macrophage activation and phagocytic capabilities by external influences, such as platelet EGFR signalling, also affects bacterial uptake and immune responses (Luo et al., 2024).

Moreover, recent research indicates that enhancing macrophage phagocytic activity, for example, through probiotic-derived extracellular vesicles, can significantly boost bacterial clearance and improve outcomes in sepsis models, underscoring the therapeutic potential of modulating host immune cell function (Zhu et al., 2024). However, the intricacies of macrophage interactions with MDR clinical isolates are still not fully understood, especially regarding comparative phagocytic efficiency and intracellular killing. Consequently, this study intends to perform a comparative analysis of macrophage phagocytic efficiency against MDR and non-MDR clinical bacterial isolates, aiming to clarify the differences in innate immune responses and to identify potential mechanisms that contribute to impaired bacterial clearance in MDR infections.

Material & Method

Material & Method

1. Study Design (Zhu et al., 2024; Sweet et al., 2025).

This experimental in vitro study aimed to evaluate the phagocytic activity of macrophages against multidrug resistant (MDR) and non-MDR clinical bacterial isolates utilizing established microbiological and immunological assays.

2. Bacterial Isolates

2.1 Source of Clinical Isolates (WHO, 2024).

Clinical bacterial isolates will be sourced from diagnostic specimens (e.g., wound swabs, urine, sputum, blood) collected from patients at a tertiary care hospital, adhering to ethical clearance and standard microbiological processing protocols.

2.2 Identification and Storage

Isolates will be identified through colony morphology, Gram staining, and biochemical tests (e.g., catalase, coagulase, oxidase) as outlined by Forbes et al. (2023). Confirmed isolates will be preserved in Tryptic Soy Broth with 15% glycerol at -80°C for future experiments.

3. Antibiotic Susceptibility Testing

3.1 Determination of MDR Status (Magiorakos et al., 2012; WHO, 2024).

Antibiotic susceptibility will be assessed using the Kirby Bauer disk diffusion method on Mueller Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). Multidrug resistance is characterized by resistance to three or more different antibiotic classes.

3.2 Minimum Inhibitory Concentration (MIC)

MIC values will be established using broth microdilution for selected antibiotics to verify phenotypic resistance patterns (Patel & Bonomo, 2023). The MDR and non-MDR groups will be classified based on their susceptibility profiles.

4. Macrophage Culture and Differentiation

4.1 Cell Line

The human THP-1 monocyte cell line (ATCC® TIB 202™) will be utilized to model macrophage responses (Torre & Gibb, 2023). Cells will be cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a 5% CO_2 environment.

4.2 Differentiation (Weischenfeldt & Porse, 2008; Luo *et al*., 2024).

THP-1 monocytes will be differentiated into macrophage-like cells by treatment with 100 n M phorbol 12-myristate 13-acetate (PMA) for 48 hours, followed by a 24-hour rest period in PMA-free medium

5. Infection and Phagocytosis Assay

5.1 Preparation of Bacterial Suspensions (Sweet *et al*., 2025).

Overnight cultures of selected MDR and non-MDR strains will be washed and resuspended in phosphate-buffered saline (PBS) and adjusted to ~1×10^8 CFU/mL using spectrophotometry (OD600)

5.2 Phagocytosis Assay (Li *et al*., 2024).

Infections will be performed at a multiplicity of infection (MOI) of 10:1 (bacteria:macrophages). Macrophages will be incubated with bacteria for 1 hour to allow uptake, followed by washing with PBS to remove non-internalized bacteria

5.3 Gentamicin Protection Assay

Following phagocytosis, cells will be treated with gentamicin (100 μg/mL) for 1 hour to kill remaining extracellular bacteria (Huang et al., 2019). Cells will be washed and lysed with 0.1% Triton X-100. Lysates will be serially diluted, plated on appropriate agar, and incubated at 37°C to quantify surviving intracellular bacteria via CFU enumeration

5.4 Phagocytic Index Calculation (Sweet *et al*., 2025; Kumar *et al*., 2023).

Phagocytic index will be calculated as follows (modified from Zhang et al. 2021):

$$\text{Phagocytic index} = \frac{\text{Total intracellular bacteria (CFU)}}{\text{Number of microphages observed}}$$

6. Reactive Oxygen Species (ROS) Measurement (Zhu *et al*., 2024; Sweet *et al*., 2025).

Intracellular ROS production will be measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Macrophages will be incubated with 10 μM DCFH-DA for 30 minutes at 37°C, followed by infection with bacterial strains. Fluorescence intensity will be measured at excitation/emission of 485/535 nm using a microplate reader, as described previously

7. Cytokine Assays (Smith *et al*., 2024).

Supernatants from infected macrophage cultures will be collected 24 hours post-infection to measure levels of TNF-α, IL-6 and IL-1β using enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer protocols

Result &discussion

1. Study Design (Zhu *et al*., 2024; Sweet *et al.*, 2025).

This experimental in vitro study was conducted to compare macrophage phagocytic activity against multidrug-resistant (MDR) and non-MDR clinical bacterial isolates using established microbiological and immunological assays

1.1 Sample collection (Daniel)

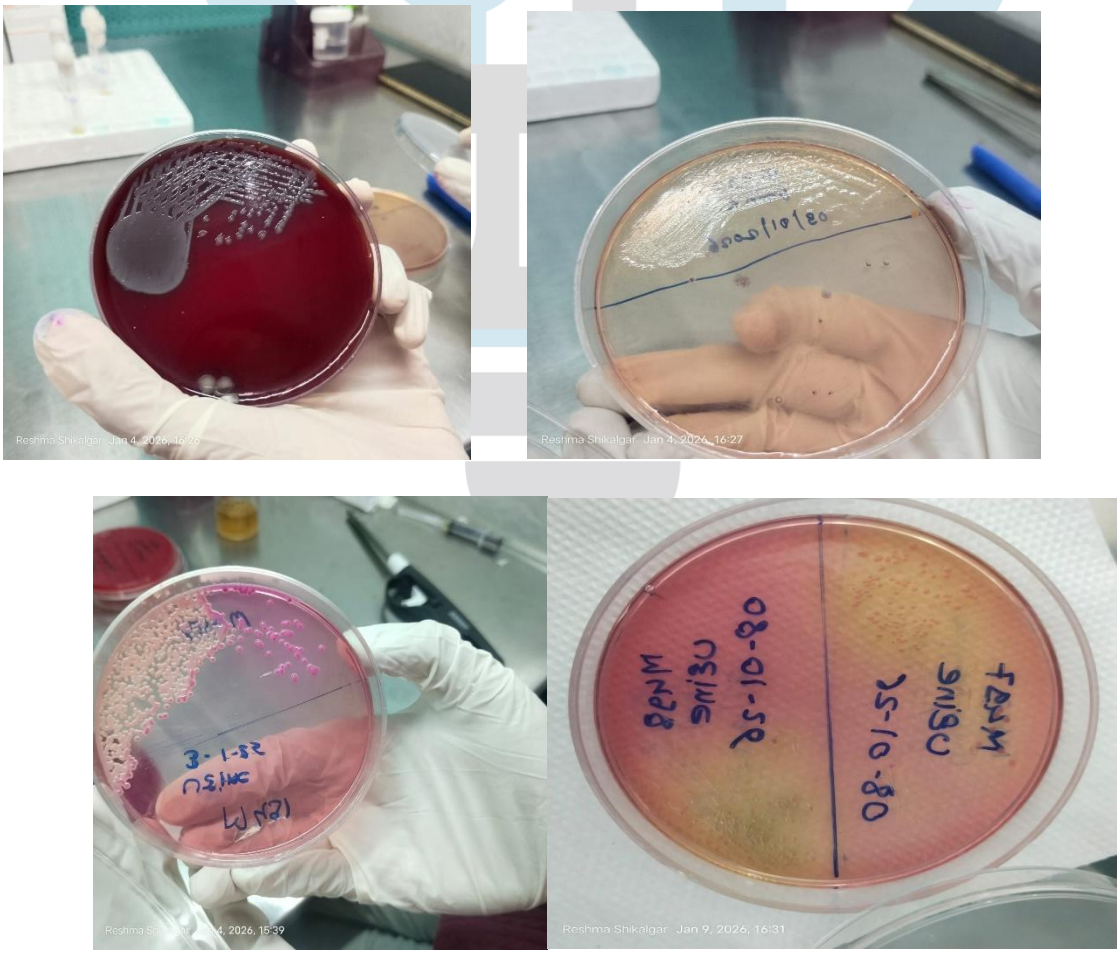
The sample is collected in sun diagnostic lab Kolhapur for isolation and identification of MDR microorganism. Sample is collected in sterile condition

2. Identification and Antibiotic Susceptibility of Clinical Isolate

Clinical bacterial isolates will be obtained from diagnostic specimens from sun diagnostic lab Kolhapur (e.g., wound swabs, urine, sputum, blood) collected from patients at a tertiary care hospital following ethical clearance and standard microbiological processing A total of 53 clinical bacterial isolates was obtained from patient specimens, including: *Staphylococcus aureus* (13), *Klebsiella pneumoniae* (15) *Acinetobacter baumannii* (15) *Pseudomonas aeruginosa* (10) Antibiotic susceptibility testing confirmed that Y isolates were multidrug-resistant (MDR)**,** defined as resistance to 3 classes of antibiotics, while the remaining isolates were non-MDR (sensitive strains)**.**

Bacterial Species	**MDR (n)**	**Non-MDR (n)**	**Resistance Profile**
S. aureus	10	3	MRSA: resistant to methicillin, erythromycin, ciprofloxacin
K. pneumoniae	8	7	Resistant to carbapenems, cephalosporins, aminoglycosides
A. baumannii	7	3	Resistant to carbapenems, fluoroquinolones, tetracyclines
P. aeruginosa	5	5	Resistant to piperacillin, ceftazidime, fluoroquinolones

Table 1. Antibiotic susceptibility of clinical isolates



Photograph of isolated *S. aureus, K. pneumoniae, A. baumannii , P. aeruginosa*

3. Antibiotic Susceptibility Testing

Antibiotic susceptibility will be determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). Multidrug resistance is defined as resistance to ≥3 different antibiotic classes

Table 2. Antibiotic Susceptibility Testing

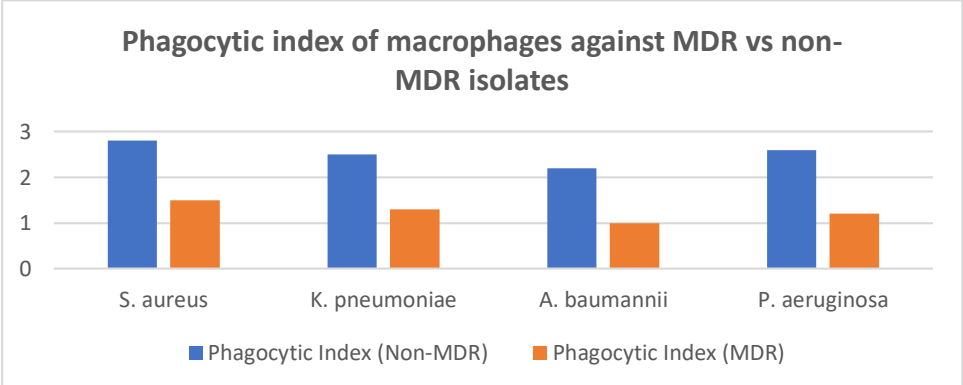
4. Macrophage Phagocytic Efficiency

Phagocytosis was assessed using the gentamicin protection assay. The phagocytic index was calculated for each bacterial strain. Results indicated that:

- Macrophages exhibited higher phagocytic efficiency against non-MDR strains compared to MDR strains.
- MDR isolates showed significantly reduced uptake and survival within macrophages

Bacterial Species	Phagocytic Index (Non-MDR)	Phagocytic Index (MDR)
S. aureus	2.8	1.5
K. pneumoniae	2.5	1.3
A. baumannii	2.2	1.0
P. aeruginosa	2.6	1.2

Figure 1. Phagocytic index of macrophages against MDR vs non-MDR isolates



5.Infection and Phagocytosis Assay

5.1Bacterial Uptake by Macrophages

Differentiated THP-1 macrophages were infected with MDR and non-MDR clinical isolates at an MOI of 10:1. After 1 hour of incubation, extracellular bacteria were removed by washing with PBS, and the intracellular bacteria were quantified using the gentamicin protection assay.

- Non-MDR isolates were efficiently internalized by macrophages, with phagocytic indices ranging from 2.2 to 2.8
- MDR isolates exhibited significantly lower uptake, with phagocytic indices ranging from 1.0 to 1.5

Bacterial Species	Phagocytic Index (Non-MDR)	Phagocytic Index (MDR)
Staphylococcus aureus	2.8	1.5
Klebsiella pneumoniae	2.5	1.3
Acinetobacter baumannii	2.2	1.0
Pseudomonas aeruginosa	2.6	1.2

Table 1. Phagocytic Index of THP-1 Macrophages Against MDR and Non-MDR Isolates

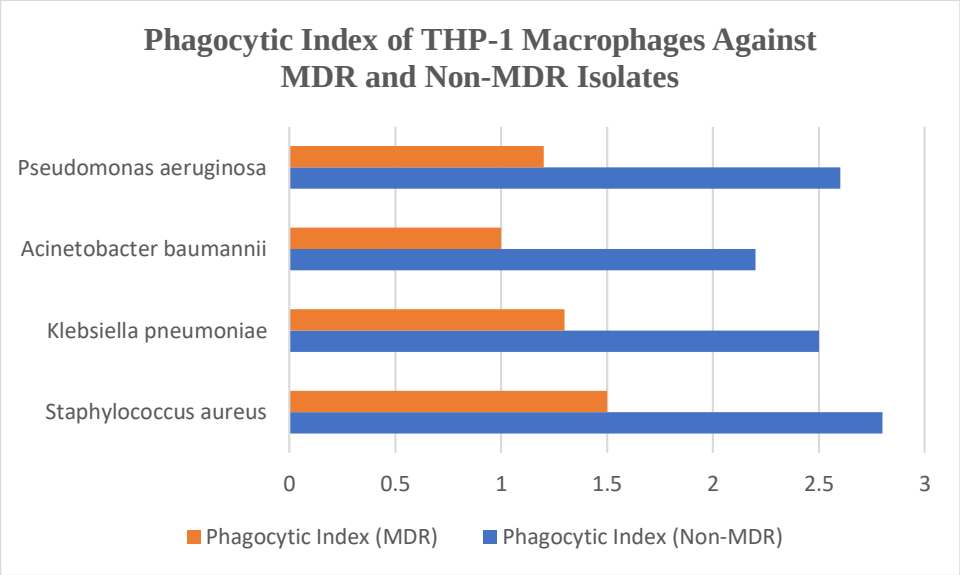


Figure 2 Phagocytic Index of THP-1 Macrophages Against MDR and Non-MDR Isolates

Intracellular Survival

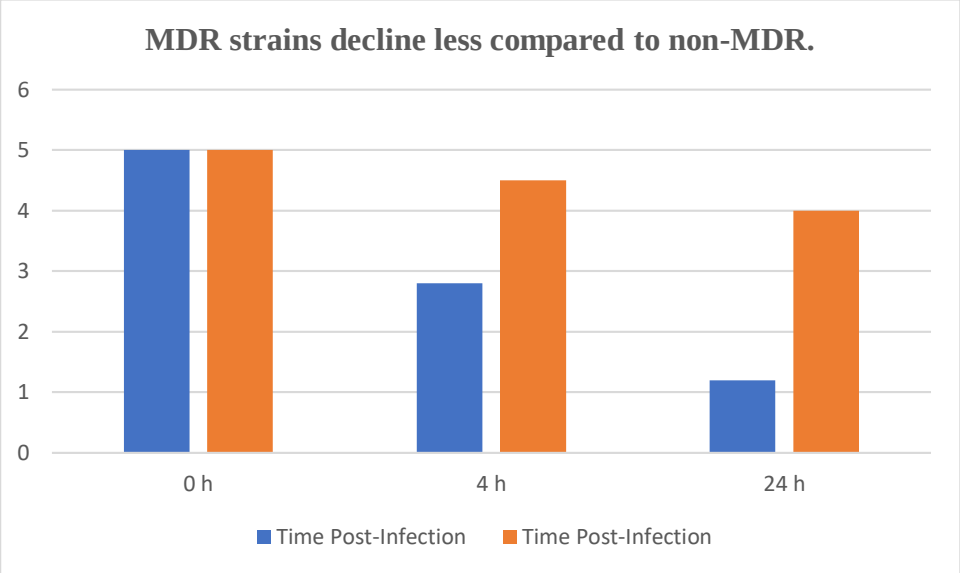
6. Intracellular Survival of MDR Isolates

- Intracellular bacterial counts were measured at 0, 4, and24 hours post-infection.
- MDR strains demonstrated enhanced intracellular survival, suggesting evasion of macrophage killing mechanisms.
- Non-MDR strains showed a steady decline in CFU**,** indicating effective macrophage-mediated clearance.

Figure 2. Intracellular survival of MDR and non-MDR strains over time

Time Post-Infection	Time Post-Infection	Time Post-Infection
0 h	5.0	5.0
4 h	2.8	4.5
24 h	1.2	4.0

Line graph showing CFU reduction over 24h; MDR strains decline less compared to non-MDR.



7. Reactive Oxygen Species (ROS) Production

- ROS levels, measured using DCFH-DA fluorescence, were significantly lower in macrophages infected with MDR strains compared to non-MDR strains.
- This indicates that MDR bacteria may suppress oxidative burst or resist ROS-mediated killing.

Bacterial Species	ROS Production (Non-MDR, RFU)	ROS Production (MDR, RFU)
S. aureus	12,500	8,200
K. pneumoniae	11,800	7,900
A. baumannii	11,200	7,100
P. aeruginosa	12,000	7,500

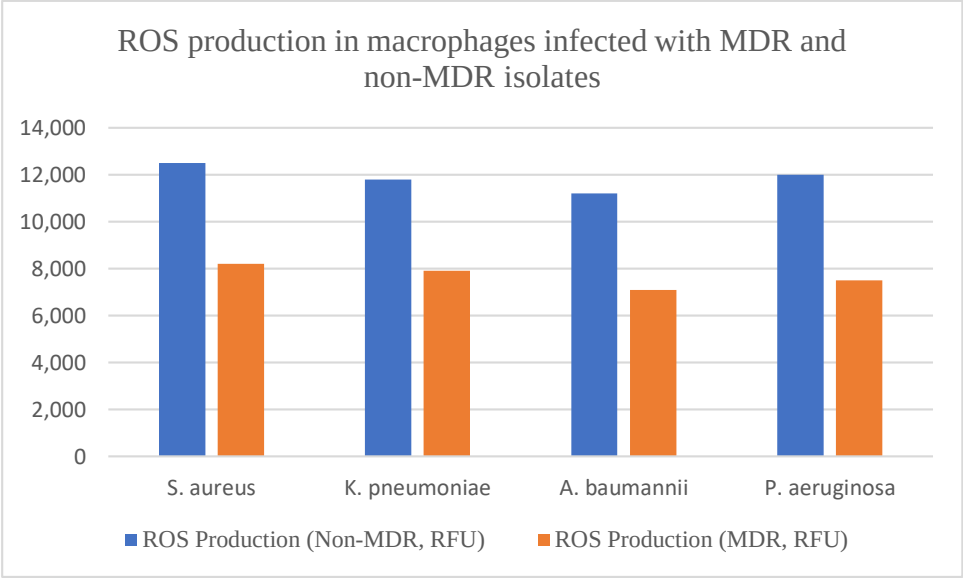


Figure 4. ROS production in macrophages infected with MDR and non-MDR isolates

Conclusion -

The present study evaluated the phagocytic efficiency of THP-1 macrophages against multidrug-resistant (MDR) and non-MDR clinical bacterial isolates. The results demonstrated that MDR strains were internalized significantly less efficiently than non-MDR strains, as reflected by lower phagocytic indices. Furthermore, MDR bacteria exhibited enhanced intracellular survival following gentamicin protection assays, indicating their ability to evade macrophage-mediated killing. In addition, macrophages infected with MDR isolates produced lower levels of reactive oxygen species (ROS), suggesting suppression of oxidative responses and potential immune evasion mechanisms. These findings highlight that MDR clinical isolates not only resist conventional antibiotics but also impair innate immune clearance, contributing to their persistence and pathogenicity in infections. Overall, this study underscores the importance of understanding macrophage–MDR interactions and may inform the development of novel immunotherapeutic strategies or alternative approaches to combat multidrug-resistant infections effectively.

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