# Role of *fnr* in glycolysis of macrophages infected with *Salmonella* Typhimurium

# Thesis

**Submitted to the**
**DEEMED UNIVERSITY**
**ICAR-Indian Veterinary Research Institute**
**Izatnagar - 243 122 (U.P.), India**

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**Roll No. M-6267**

**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

## Master of Veterinary Science
**(Animal Biochemistry)**

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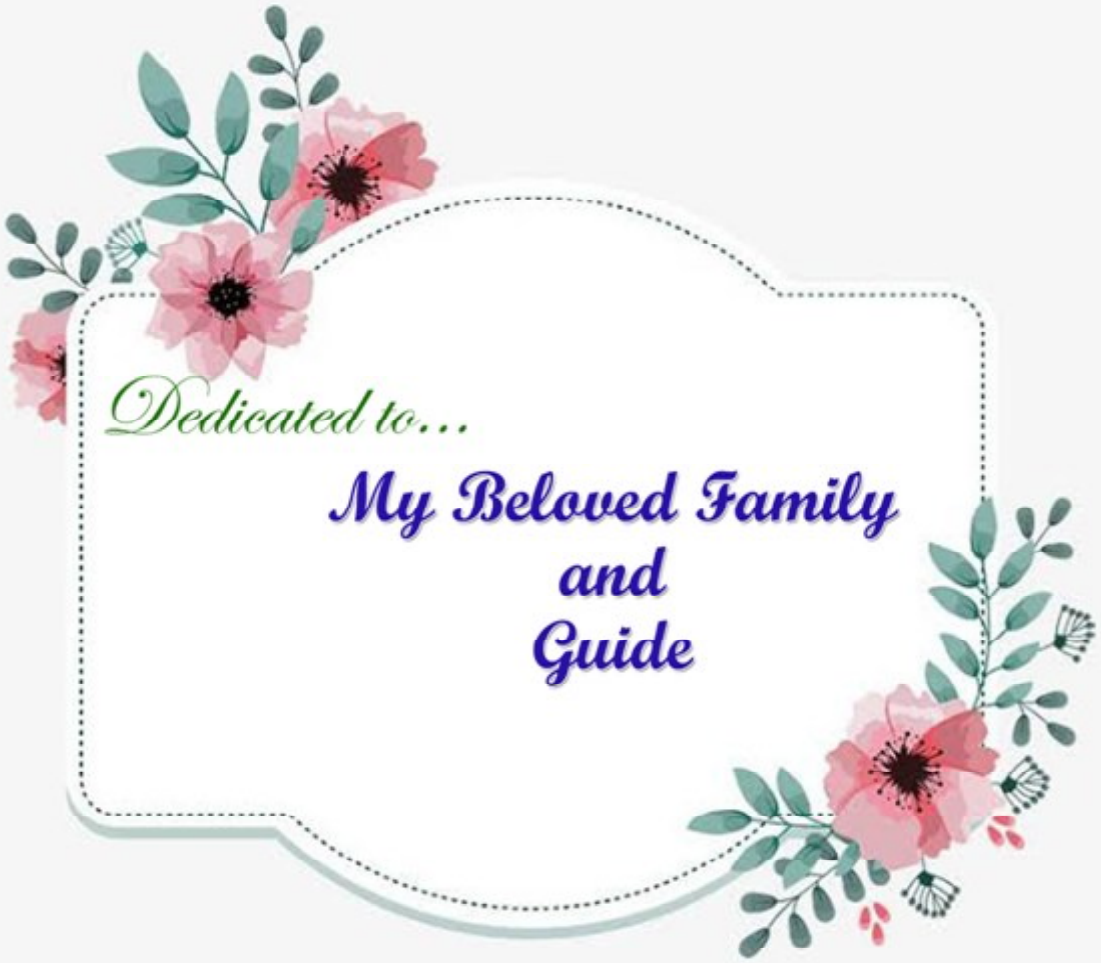
Dedicated to…
My Beloved Family
and
Guide

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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**Dated:** 21/09/2023

# *Certificate*

*This is to be certified that the research work embodied in this thesis entitled* **"Role of *fnr* in glycolysis of macrophages infected with *Salmonella* Typhimurium"** *submitted by* **Dr. V.V. Sreenila, Roll No. M-6267,** *for the award of* **Master of Veterinary Science** *Degree in* **Animal Biochemistry** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

*It is further certified that* **Dr. V.V. Sreenila, Roll No. M-6267,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

**(AJAY KUMAR)**
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# Certificate

*We the undersigned members of Advisory Committee of* **Dr. V.V. Sreenila, Roll No. M-6267,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Animal Biochemistry,** *agree that the thesis entitled* **"Role of *fnr* in glycolysis of macrophages infected with *Salmonella* Typhimurium"** *may be submitted in partial fulfilment of the requirement for the degree.*

*We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of* **Master of Veterinary Science** *Degree of this Institute.*

*It is further certified that the candidate has completed all the prescribed requirements governing the award of* **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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V.V.Sm

***Date:*** 20/09/2023 ***(V.V. Sreenila)***

***Place:*** *ICAR-IVRI, Izatnagar*

# ABBREVIATIONS

| | | |
|---|---|---|
| °C | : | Degree centigrade |
| ≤ | : | Lesser Than |
| ≥ | : | Greater Than |
| % | : | Percentage |
| µg | : | Microgram |
| µL | : | Microliter |
| p C | : | Degree Celsius |
| 2- NBDG | : | 2-(*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-Yl)Amino)-2-Deoxyglucose |
| ArcA | : | Aerobic Respiration Control |
| ATP | : | Adenosine Triphosphate |
| BMDM | : | Bone Marrow-Derived Macrophages |
| bp | : | Base Pairs |
| CARI | : | Central Avian Research Institute |
| CD4 | : | Cluster Of Differentiation 4 |
| cDNA | : | Complementary DNA |
| CFU | : | Colony Forming Units |
| $CO_2$ | : | Carbon Dioxide |
| CS | : | Citrate Synthase |
| DNA | : | Deoxy Ribonucleic Acid |
| $dNTP_S$ | : | Deoxy Nucleoside Triphosphate |
| DW | : | Distilled Water |
| E. coli | : | Escherichia Coli |
| EDTA | : | Ethylene Diamine Tetra-Acetic Acid |
| EMP | : | Embden-Meyerhof-Parnas |
| ENO-1 | : | Enolase-1 |
| FAO | : | Food And Agriculture Organization |
| FBS | : | Fetal Bovine Serum |
| Fig | : | Figure |
| fnr | : | Fumarate And Nitrate Reductase |
| GLUT-1 | : | Glucose Transporter-1 |
| GSK-3 | : | Glycogen Synthase Kinase-3 |
| $H_2O$ | : | Water |
| HEA | : | Hektoen Enteric Agar |
| HIV | : | Human Immuno Deficiency Virus |

| | | |
|---|---|---|
| HK | : | Hetrokinase-2 |
| hpi | : | Hours Post-Infection |
| hrs | : | Hours |
| IL | : | Interleukin |
| iNTS | : | Invasive Nontyphoidal Salmonella |
| LB | : | Luria Bertani |
| LPS | : | Lipopolysaccharides |
| mg | : | Milligram |
| min | : | Minutes |
| ml | : | Milliliter |
| mM | : | Millimole |
| MM | : | Master Mix |
| MOI | : | Multiplicity Of Infection |
| Mol. Wt | : | Molecular Weight |
| mRNA | : | Messenger Ribonucleic Acid |
| NADH | : | Nicotinamide Adenine Dinucleotide |
| NADPH | : | Nicotinamide Adenine Dinucleotide Phosphate |
| NarL | : | Nitrate Reductase |
| NFW | : | Nuclease-Free Water |
| ng | : | Nanogram |
| nm | : | Nanomole |
| NO | : | Nitric Oxide |
| NTS | : | Nontyphoidal Salmonella |
| O2 | : | Oxygen |
| OD | : | Optical Density |
| OXPHOS | : | Oxidative Phosphorylation |
| P. I | : | Post Infection |
| PBMC | : | Peripheral Blood Mononuclear Cells |
| PBS | : | Phosphate Buffered Saline |
| PCR | : | Polymerase Chain Reaction |
| PDK-1 | : | Pyruvate Dehydrogenase Kinase-1 |
| PM | : | Peritoneal Macrophages |
| Pmol | : | Picomole |
| PTEN | : | Phosphatase And Tensin Homolog |
| RBC | : | Red Blood Cells |
| RNA | : | Ribonucleic Acid |
| ROS | : | Reactive Oxygen Species |
| RPM | : | Revolution Per Minute |

| | | |
|---|---|---|
| RPMI | : | Roswell Park Memorial Institute |
| RT | : | Reverse Transcriptase |
| SCV | : | Salmonella Containing Vacuole |
| SDS | : | Sodium Dodecyl Sulfate |
| sec | : | Second |
| SEM | : | Standard Error Mean |
| SLC | : | Solute Carrier |
| SPI | : | Salmonella Pathogenicity Island |
| STM | : | S*almonella* Typhimurium |
| TAE | : | Tris Acetate -Edta |
| Taq | : | Thermus Aquaticus |
| TCA | : | Tricarboxylic Acid |
| TLR | : | Toll-Like Receptors |
| UV | : | Ultra -Violet |
| WT | : | Wild Type |

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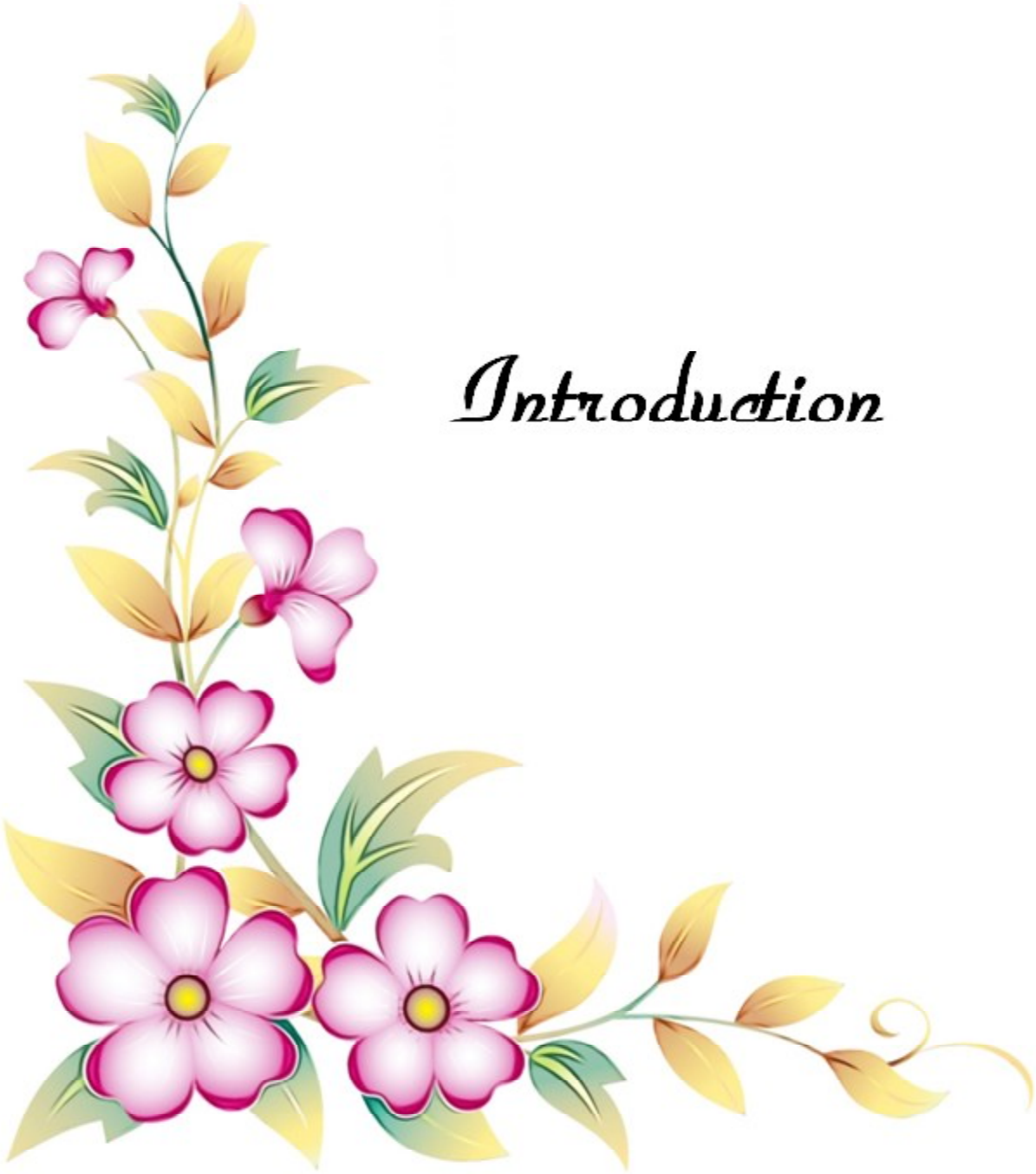


# Introduction

# 1 INTRODUCTION

The genus *Salmonella* from the Enterobacteriaceae family has a large population of medically important pathogens (Su *et al*., 2007). Millions of lives are lost to *Salmonella* infection each year. Salmonellosis is a serious public health problem across the world. Infections produced by non-typhoidal *Salmonella* serovars, in addition to typhoidal *Salmonellae*, are associated with significant morbidity and mortality, resulting in large economic losses (Kumar *et al*., 2016). Public health issues stem from the fact that *Salmonella* may induce self-limiting gastroenteritis and that invasive infections kill humans at significant rates. *Salmonella* contains two species (*Salmonella bongori and Salmonella enterica*) and 2,579 serovars (Grimont and Weill, 2007). *Salmonella enterica* is made up of 1,531 serovars that are mostly connected with human illnesses, which are usually acquired by the ingestion of contaminated food (Crum-Cianflone, 2008). *Salmonella* is linked to a number of enteric disorders like typhoidal fever and non-typhoidal salmonellosis.

Non-typhoidal infections are caused by a range of different *Salmonella* serovars together classified as non-typhoidal *Salmonella*e (NTS). Non-typhoidal *Salmonella* serovars lead to 93.8 million human infections and 1,55,000 fatalities per year (Li, 2022). These serovars are responsible for 38% of foodborne infections, 35% of hospitalizations, and 28% of fatalities related to foodborne illnesses (Green *et al*., 2001). Furthermore, non-typhoidal *Salmonella*e have a diverse reservoir and are widespread. NTS distribution varies greatly by time and region, and the host range is extensive. NTS are frequently found in wild and domestic animals, including chickens, pigs, cattle, cats, dogs, birds, and reptiles, according to the World Health

Organization.

Despite the fact that many animals are *Salmonella* reservoirs and among them, poultry and poultry products are the main causes of salmonellosis in people (Herawati *et al*., 2022). Therefore, it is alarming that reservoirs might potentially transmit disease to people and other animals. The main pathways of dissemination include activities that involve the handling of animals and uncooked meat products. Unsanitary techniques during animal slaughter, as well as the handling and transportation of raw meat, enhance the probability of NTS contamination of meat products (Walia *et al*., 2017). NTS survival and transmission have also been observed through water bodies and the environment (Nair *et al*., 2018). The introduction of novel or less prevalent NTS serovars linked to human infection might represent a threat to India's healthcare system. As a result, regional and national level surveillance is required in India to adopt effective control methods and protect community health (Kumar *et al*., 2016).

Diarrhea induced by *Salmonella* species has a global impact on the health of humans, contributing to significant yearly morbidity and death (Aljahdali *et al*., 2020) and necessitating the development of novel treatment techniques for effective management. In most of the cases, patients become free from infections because of treatment (Keintz and Marcelin., 2022). However, 3-5% of individuals become chronic carriers, with persistent infection in the gallbladder (Li., 2022). Antibiotic usage in animals raised for food caused the emergence and spread of multidrug-resistant (MDR) bacteria throughout the food chain (Kimera *et al*., 2020; Ma *et al*., 2021). Chronic carriers can sporadically shed the germs through their feces and urine for the remainder of their lives (Eng *et al*., 2015).

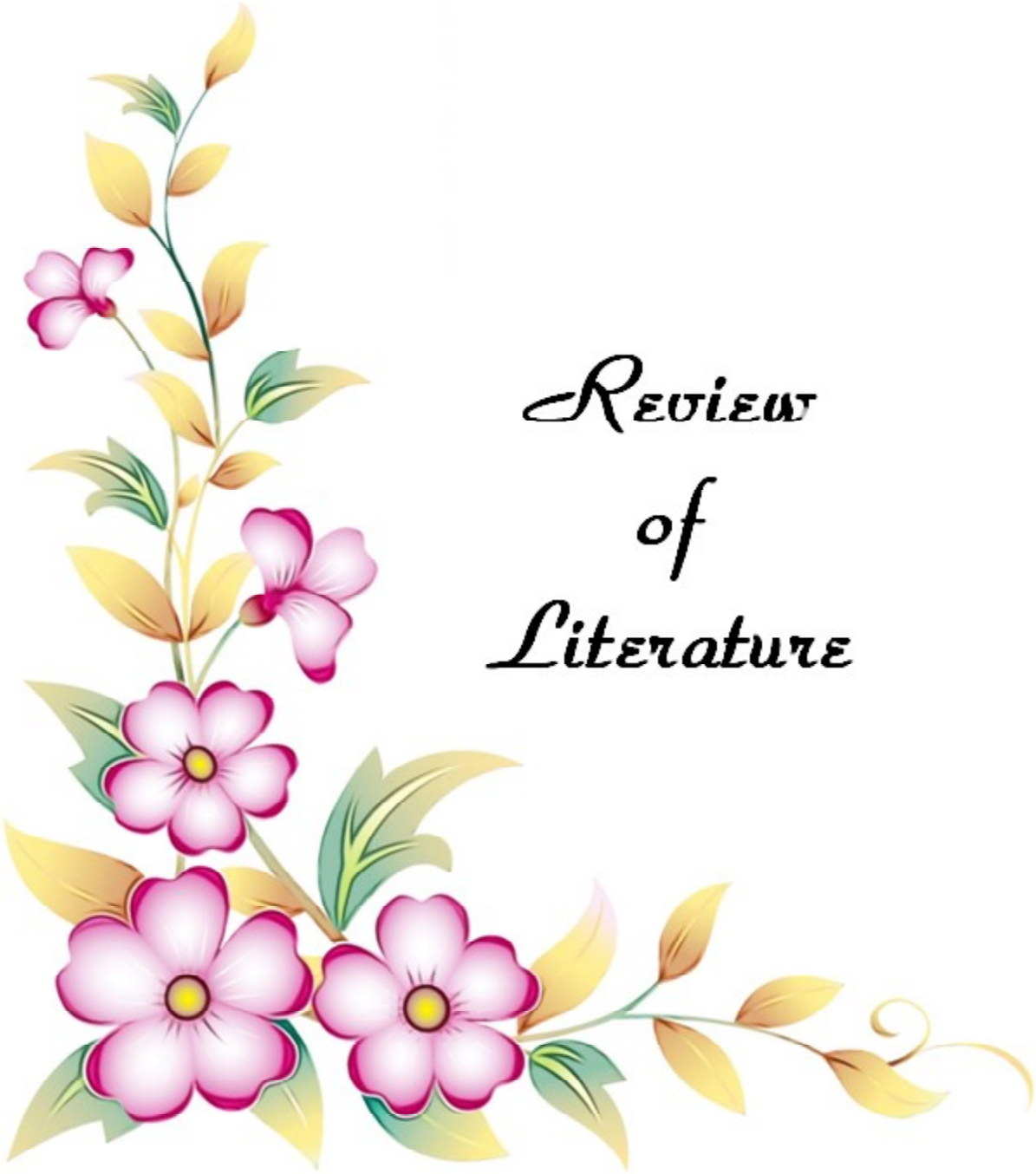
During infection, it invades a variety of cell types, including dendritic cells, epithelial cells, and macrophages. *Salmonella* enters the host's gastrointestinal tract during infection and colonizes the intestine (LaRock *et al*., 2015). It engages with several unfavorable environmental conditions throughout the passage, including low oxygen tension in the intestine and extremely acidic conditions in the stomach, as well as increased bile salt concentrations and metabolites released by commensal bacteria (Alvarez-Ordonez *et al*., 2011). However, *Salmonella* is endowed with a variety of adaptive mechanisms to get around these obstacles

and survive inside the host. The adaption to low oxygen settings is mediated by important anaerobic regulators like *FNR*, ArcA, and *NarL* (Behera *et al*., 2020).

Salmonella is carried to different organs of the body through infection in phagocytic cells. It compromises the phagocytic activity of these cells by producing antimicrobial molecules and also affects the metabolic activity of these cells for their own survival and replication. My lab has developed an *fnr* deletion mutant of *Salmonella* Typhimurium and has seen the role of this gene in bacterial colonization both in the intestine of mouse as well as inside macrophages. *FNR* is a transcriptional regulator and controls the activity of more than hundreds of genes of *Salmonella* Typhimurium. Therefore, the following objective is hypothesized to see the role of *FNR* in glucose metabolism inside poultry macrophages.

## OBJECTIVE

**To Study Glycolytic Changes in macrophages infected with *fnr* deleted *Salmonella* Typhimurium**



# Review of Literature

# 2 REVIEW OF LITERATURE

## 2.1 *Salmonella*- Taxonomy & Classification

Theobald Smith initially identified and isolated *Salmonella* in pigs with classical swine disease in 1855 (Eng *et al*., 2015). The bacterium was given the name in honor of Smith's colleague and American pathologist, Dr. Daniel Elmer Salmon (Ajmera and Shabbir, 2022). The bacterium was initially named "*Bacillus choleraesuis*," but Lignieres altered it to "*Salmonella choleraesuis*" in 1900. *Salmonella*'s nomenclature is contentious and still changing. WHO Collaborating Centre's suggested nomenclatural scheme for *Salmonella* is now used by the CDC (Guyassa and Dima, 2022). Based on variations in their 16S rRNA sequence analyses, this method divides the genus *Salmonella* into two species: *Salmonella enterica* (type species) and *Salmonella bongori* (Guyassa and Dima, 2022).

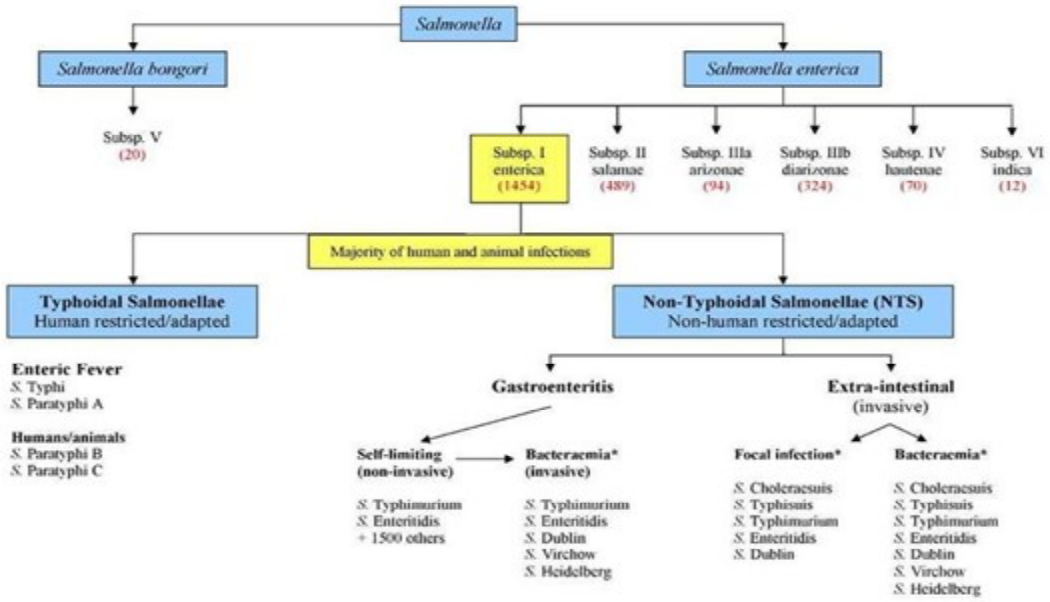



**Fig2.1: Classification of the genus *Salmonella* (Akyala and Alsam, 2015)**

### 2.1.1 *Salmonella enterica*

Animals and humans can contract *Salmonella enterica* infection by consuming contaminated foodstuff or drinking water (Islam *et al*., 2023) which is an important pathogen for both species. The persistence of infection is increased by bacteria's ability to adapt to a variety of adverse conditions in the environment or the host (Chakroun *et al*., 2017). Both the systemic (typhoid fever) group and the non-typhoidal Salmonellae group are significant subgroups of *Salmonella enterica* that are known to infect humans. *S.* Typhi, a serovar that has evolved to live in humans, is the reason for typhoid fever (Ajmera and Shabbir, 2022). Gastroenteritis, which only rarely progresses to an invasive infection, is typically linked to non-typhoidal Salmonellae. On contrary to the frequent association of *S.*Typhimurium with gastroenteritis in humans, susceptible mice become ill with a systemic illness resembling typhoid (Worley, 2023).

The gastroenteritis-causing non-typhoidal intracellular *S.* Typhimurium can infect a variety of hosts (Scharte *et al*., 2022). It causes infection and death in both children and adults around the world. Diarrhea, vomiting, and cramps in the abdomen are among the signs of the infection (Bangera *et al*., 2018). When it affects immunosuppressed or ill hosts, non-typhoidal salmonellosis can be invasive and cause bacteremia, sepsis, and focal infections like meningitis. In healthy individuals, it presents as self-limited gastroenteritis. Vulnerable groups include patients who are immunosuppressed due to HIV infection, cancer, steroid use, sickle cell disease, diabetes, ongoing renal or liver disease, old age, and newly conceived children (Bangera *et al*., 2022). NTS can cause invasive infections as the virulence genes are scattered in plasmids and chromosomes (Ikhimiukor *et al*., 2022).

## 2.2 Transmission

*S.*Typhimurium is transmitted by contaminated food, usually by poultry meat or eggs (Kipper *et al*., 2022). Some farm practices have inadvertently increased the carriage of *S.* Typhimurium by farm animals. For example, the use of protein supplements in animal feed to increase weight gain has helped to increase the likelihood of colonization of the animal's intestinal tract. During slaughter, the bacteria from the intestine of a colonized animal can easily contaminate

the carcass and enter the food supply (Nair *et al*., 2018). Long-term storage of *Salmonella*-contaminated eggs gives the bacteria an opportunity to multiply at room temperature; however, this growth is not accompanied by alterations in the flavor or appearance of the white or yolk. Anyone who likes raw eggs (e.g., in traditional Caesar salad dressing) or undercooked eggs (e.g., in egg-thickened sauces) is well advised to refrigerate eggs immediately after purchase. Farmers and the food industry are making every effort to reduce the contamination level, but there will never be a 100% guarantee of *Salmonella-free* food.

## 2.3 Virulence Factors

*Salmonella* contains multiple pathogen signatures and bacterial effectors, including LPS, flagellin, adhesins, lipoproteins, CpG-DNA, acid tolerance response, invasion of mucosal cells (ruffling), survival in inactivated macrophages (Broz *et al*., 2012).

## 2.4 Pathogenesis

A number of enteric pathogens invade cultured mammalian cells by triggering actin rearrangements that ultimately result in the formation of pseudopods (Mylvaganam *et al*., 2021) which engulf the bacteria. *S.* Typhimurium forces host cells to engulf it, but the process appears somewhat different from that seen in other enteric pathogens (Garai *et al*., 2012). The binding of *S.* Typhimurium to cultured cells causes a change in the appearance of the surface of the host cell that resembles the splash of a droplet of liquid hitting a solid surface. Ruffling and internalization are accompanied by extensive actin rearrangements in the vicinity of the invading bacteria (Takaya *et al*., 2002). After the bacteria are engulfed in a vesicle, the filaments of actin in that region return to normal (Drecktrah *et al.,* 2006). The pathogenicity islands also encode effector proteins, which are secreted by the T3SS (Type-3 Secretion System) and translocated into epithelial cells on the intestine's mucosal surface (Gunn *et al*., 2014). *S.* Typhimurium takes advantage of inflammation to aid in its own colonization (Deriu *et al*., 2013).

Anaerobic conditions promote bacterial adhesion and epithelial cell invasion more than aerobic conditions do (Lee and Falkow., 1990). This species' virulence may be significantly influenced by the mechanisms or genes controlling its adaptation to anaerobic environments

(Fink *et al*., 2007). Intracellular pathogens have two ways of surviving: either they decide to live in the host cell's cytoplasm or create a self-contained niche in the form of a vacuole (Garai *et al*., 2012). *Salmonella* decides to form an intracellular vacuole known as a "*Salmonella* containing vacuole (SCV)," which is the most common choice for bacteria (Wang *et al*., 2021). Bacterial-mediated endocytosis is the process by which epithelial cells engulf *S.* Typhimurium, which is then eventually contained in a membrane-bound vesicle known as a macropinosome or SCV (Fabrega and Vila., 2013). The SCV then uses the host cell's microtubule network to juxtapose itself to the nucleus, where it acquires nutrition from the Golgi apparatus(Patel *et al*., 2014).

In order for the bacteria to survive in a membrane-bound structure, these effectors must interact with the host machinery, modify the vacuole, and modulate intracellular processes (Yu *et al*., 2004). A stage of *Salmonella*'s infectious cycle involves the bacteria escaping from their initial intracellular niche and returning to the gut lumen (Knodler *et al*., 2010). Through the use of additional virulence factors, this extracellular phase within the host results in particular disease pathologies and gives the pathogen a survival advantage. Sepsis is traditionally thought to be a frequent result of facultative intracellular bacterial dissemination (Silva, 2012). Consequently, pathogenesis depends on both the disseminative extracellular phase and the replicative intracellular phase.

## 2.5 Symptoms

Symptoms of gastroenteritis appear within 6 to 24 hrs after ingestion of contaminated food or fluids and last as long as a week. These symptoms are followed by abdominal pain, diarrhea, and sometimes fever. The pain and diarrhea severity vary widely from one person to another, ranging from mild pain and barely detectable diarrhea to pain resembling appendicitis and severe, even bloody, diarrhea (Chen *et al*., 2013). After the symptoms subside, the infected person will continue to excrete bacteria for 3 months. In a small percentage of cases (1 to 3%), an infected person sheds the bacteria for over a year (chronic carrier) (Gunn *et al*., 2014). Most cases of gastroenteritis occur in children <5 years; symptoms tend to be most severe in this age group (Chen *et al*., 2013).

## 2.6 Treatment

Global health is gravely concerned by the drug resistance of intracellular infections like STM, which highlights the urgent need to create new, more potent therapies for these germs.

Infections with *S.*Typhimurium are often self-limiting, and there is little proof that taking antibiotics would significantly reduce how long patients will have symptoms. Antibiotic treatment is therefore inappropriate for gastroenteritis situations that are not complex (Onwuezobe *et al*., 2012). Patients who are all proceed to invasive illnesses, such as newborns, HIV patients, people over 50 with atherosclerosis, cardiovascular disease, severe joint pathology, and people using immunosuppressive medications, should be given antibiotic treatment (Shane *et al*., 2017). Antibiotics should be administered to patients who develop iNTS (Fierer *et al*., 2022). Over the past ten years, as antimicrobial resistance has risen, the recommended empiric antibiotic regimen has changed (McDermott *et al*., 2018).

When susceptibilities are known, the mainstays of therapy continue to be ampicillin, ceftriaxone, and fluoroquinolones (Keintz and Marcelin, 2022). When treating antibiotic-resistant *Salmonella* infections, immunomodulatory therapies may be an effective alternative to conventional therapies (Hotchkiss *et al*., 2013). They may also help treat the immunological suppression brought on by sepsis. As a supplement to presently prescribed antibiotics, host-directed treatment (HDT) tries to improve host immune responses.

## 2.7 Antibiotic Resistance

Antimicrobial resistance in bacteria (AMR) causes a serious threat to the health of humans and animals globally (Evangelista *et al*., 2022; Murray *et al*., 2022). Four mechanisms, including decreased membrane permeability, enzymatic activation, increased efflux pumping, and downregulation of drug-binding target, have been identified as contributing factors to the resistance (Sun *et al*., 2014). Plasmids and integrons frequently contain genes that provide resistance to antibiotics like chloramphenicol, aminoglycosides, quinolones, and tetracyclines (Varela *et al*., 2021; Hao *et al*., 2016). When patients and livestock receive antibiotic therapy, inadequate antibiotic concentrations may occur. As a result, resistant bacteria may develop and become widely dispersed in the environment, including sewage water, rivers, lakes, and even drinking water (Andersson *et al*., 2014).

## 2.8 Prevention

*Salmonella* contamination has recently been linked to several food safety incidents and recalls involving ready-to-eat low-moisture products (such as milk powder, raw almonds, dry seasonings, and peanut butter) as well as other food commodities (such as meat products, eggs, and vegetables) (Hanning *et al*., 2009). Contaminations also can come from numerous sources during food production, so it is crucial to use incident investigation tools that can distinguish *Salmonella* beyond the species level (defined as *Salmonella* subtyping) (Shi *et al*., 2015).

## 2.9 Vaccination

To stop disease outbreaks, vaccination is a practical and affordable option (Bornstein *et al*., 2017). However, there are currently few licensed vaccines available to protect against *S.* Typhimurium or other NTS strains in humans, In spite of that there are some experimental vaccines for these pathogens in the preclinical phase of their development. *S.* Typhimurium can endure intracellular killing and form persisters under antibiotic stress in macrophages, culminating in persistent infection or relapse (Kurtz *et al*., 2017; Stapels *et al*., 2018). *Salmonella* infection requires both antibody- and cell-mediated immunity, particularly the T-helper type 1 (Th1) CD4+ T-cell response, for the eradication of bacteria (Benoun *et al*., 2018). Live attenuated vaccines can trigger two powerful adaptive immune response arms that can target both extracellular and intracellular bacteria and offer long-lasting protection (Tennant *et al*., 2015). These vaccines are also inexpensive to produce and easy to give to a large population orally. Therefore, it makes sense to prioritize the development of live vaccines as a means of preventing *Salmonella* infections. Due to the difficulty in balancing attenuation and protective immunity, vaccine development is quite difficult (Galen *et al*., 2016). Therefore, significant work is still required to create novel attenuated live *S.* Typhimurium vaccines that are secure, have a strong immunogenicity, and offer substantial protection. A typical method for creating live, attenuated vaccines is to target global regulator genes linked to bacterial virulence (Tennant *et al*., 2015).

In India, inactivated killed vaccine against *Salmonella* Typhimurium and *Salmonella* Enteritidis is available and given intramuscularly (manufacturer: Venky's and MSD) while live

vaccines given orally are Avipro megan Vac1 (Megan Health Inc.) and PoulVac ST (Zoetis Global).

## 2.10 Anaerobic regulators of *Salmonella* Typhimurium

### 2.10.1 *FNR* (Fumarate and Nitrate Reductase regulatory protein)

*FNR* regulator is crucial for the bacterial oxygen response. Crack and Le Brun (2018) reported that through the assembly and disassembly of iron-sulfur clusters in a facultative anaerobe, *FNR* controls several biological processes. The DNA-binding protein and a cytoplasmic oxygen sensor, *FNR* regulates more than 70 genes that are crucial for anaerobic adaptation. The regulatory domain in the N-terminal and the DNA binding domain in the C-terminal are two conserved domains of the global transcriptional regulator *FNR* (Nikhil *et al*., 2021). For the [4Fe-4S]2 or [2Fe-2S]2 iron-sulfur clusters to bind *in vivo*, the N-terminal sensory region, which contains the four cysteine residues Cys20, Cys23, Cys29, and Cys122, is necessary (Zhang *et al*., 2012). The [4Fe-4S]2 molecule that *FNR* binds without oxygen helps to promote dimerization and increases DNA binding to promoters (Mettert and Kiley., 2018). When exposed to oxygen, the active form of *FNR*, which has one [4Fe-4S]2+ cluster per protein monomer, transforms into [2Fe-2S]2+, an inactive form along with other less well-defined iron species (Crack *et al*., 2004). *FNR* can also bind to sequences of the promoter at positions -61, -71, -81, and -91. It typically binds at position -41.

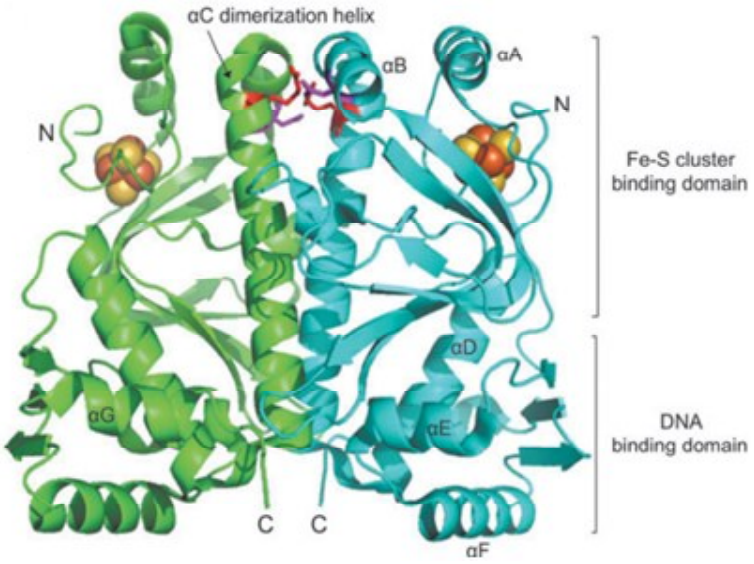



**Fig 2.2: Crystal Structure of [4Fe-4S]-FNR (Metteret and Kiley., 2018)**

*FNR* induces the expression of several genes involved in motility, anaerobic metabolism, flagellar biosynthesis, pathogenesis, and chemotaxis (Chilcott *et al*.,2000). *FNR* suppresses the transcription of the genes necessary for aerobic metabolism, energy production, and nitric oxide detoxification. *FNR* regulate several loci expression, including the SPI-1 and motility genes, which are crucial factors in the pathogenesis of *Salmonella. FNR* controls at least 311 genes either directly or indirectly in serovar Typhimurium (Fink *et al*.,2007).

To overcome colonization resistance, *S*. Typhimurium has developed several mechanisms and as a result, the host generates reactive nitrogen species, particularly nitric oxide, to prevent the pathogen from proliferation (Alam *et al*., 2002). Nitric oxide is produced by the host cells as an anti-microbial metabolite to fight against gut infection (Shelton *et al*., 2022). Nitric oxide produced by the host has the potential to interact with other substances in the intestinal lumen to produce nitrate (Rivera-Chavez and Baumler, 2015). In the intestine of the host, *Salmonella* utilizes nitrate as its alternate electron acceptor for their growth during infection (Lopez *et al*., 2012). Li *et al.* (2022) reported that there is an increased nitrate level in the spleen and liver of mice when infected by *S*.Typhimurium and it helps *Salmonella* enhance its virulence. Miller *et al.* (2022) reported that nitrate and NO are produced in the macrophages also. Its utilization is directly activated by NarX-*NarL* (nitrate sensing two-component system)

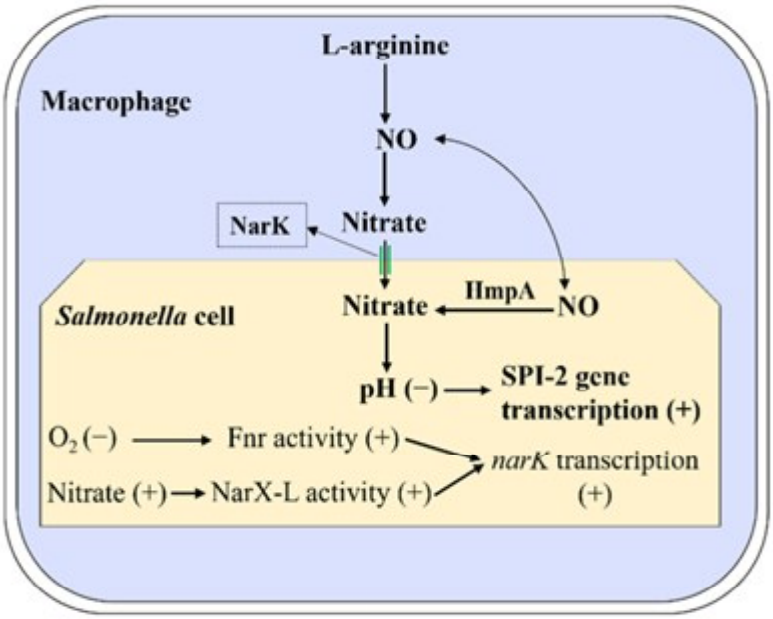



**Fig 2.3: Utilization of Nitrate by *Salmonella* Typhimurium (Li *et al.*, 2022)**

and the global regulator *FNR* under low oxygen conditions (Li *et al*., 2022). This nitrate is utilized for invasion and adhesion of epithelial cells in the gut and *Salmonella* flagellar expression (Horstmann *et al*., 2020). Transportation and utilization of nitrate leads to acidification of SCV and it causes the SPI-2 (*Salmonella* Pathogenicity Island -2) gene expression that helps in the systemic infection (Choi and Groisman, 2016).

## 2.11 Intracellular Survival Inside Macrophage

Macrophages are immune cells that are essential to the host's defense. They play a critical role in STM pathogenesis as a colonization niche (Jiang *et al*., 2021). Along with engulfing and destroying the pathogens, they also release cytokines and give T cells antigens to mount a successful adaptive immune response (Escoll and Buchrieser, 2018). The macrophages' state of activation affects their capacity to grow inside them, which is crucial to virulence. *Salmonella* growth is not permitted by M1 macrophages that are classically activated, but it is permitted by M2 macrophages that are alternatively activated (Sedivy-Haley *et al*., 2022).

*S*. Typhimurium has developed ways to avoid the unfavorable lysosome environment and cause inflammatory cell death in macrophages. Inflammatory reactions are triggered by metabolic changes that occur in macrophages due to products of *Salmonella* (Rosenberg *et al*., 2021). The host cell's intracellular way of life allows *Salmonella* to modify its metabolism during infection. The eukaryotic cell invasion, phagocyte invasion, macrophage cytotoxicity, and survival inside phagocytic cells are all made possible by the SPI-1 and SPI-2 genes. *S.*Typhimurium survival and replication in cultured cells correlate with overall survival and persistence in animal models because mutants that are unable to replicate in cultured cells are inert in animals. Therefore, *Salmonella* pathogenesis requires survival within macrophages.

## 2.12 Metabolic Reprogramming

*Salmonella* is a Gram-negative, facultative intracellular enteric bacterium with a high degree of metabolic adaptability. This trait enables it to take advantage of regional nutrient niches and survive inside skilled phagocytes (Taylor and Winter, 2020). *Salmonella* can also modify its metabolism in response to the polarization of macrophages, developing a metabolism of lipolysis in pro-inflammatory type macrophages (M1) and a glycolytic metabolism in a non-

inflammatory macrophage (M2) (Reens *et al*., 2019). Once invading bacteria are sensed by macrophages using pattern recognition receptors, the citric acid cycle is impeded, which induces the host's metabolism to switch from oxidative phosphorylation to aerobic glycolysis and causes the buildup of several metabolic intermediates (Ryan *et al*., 2020).

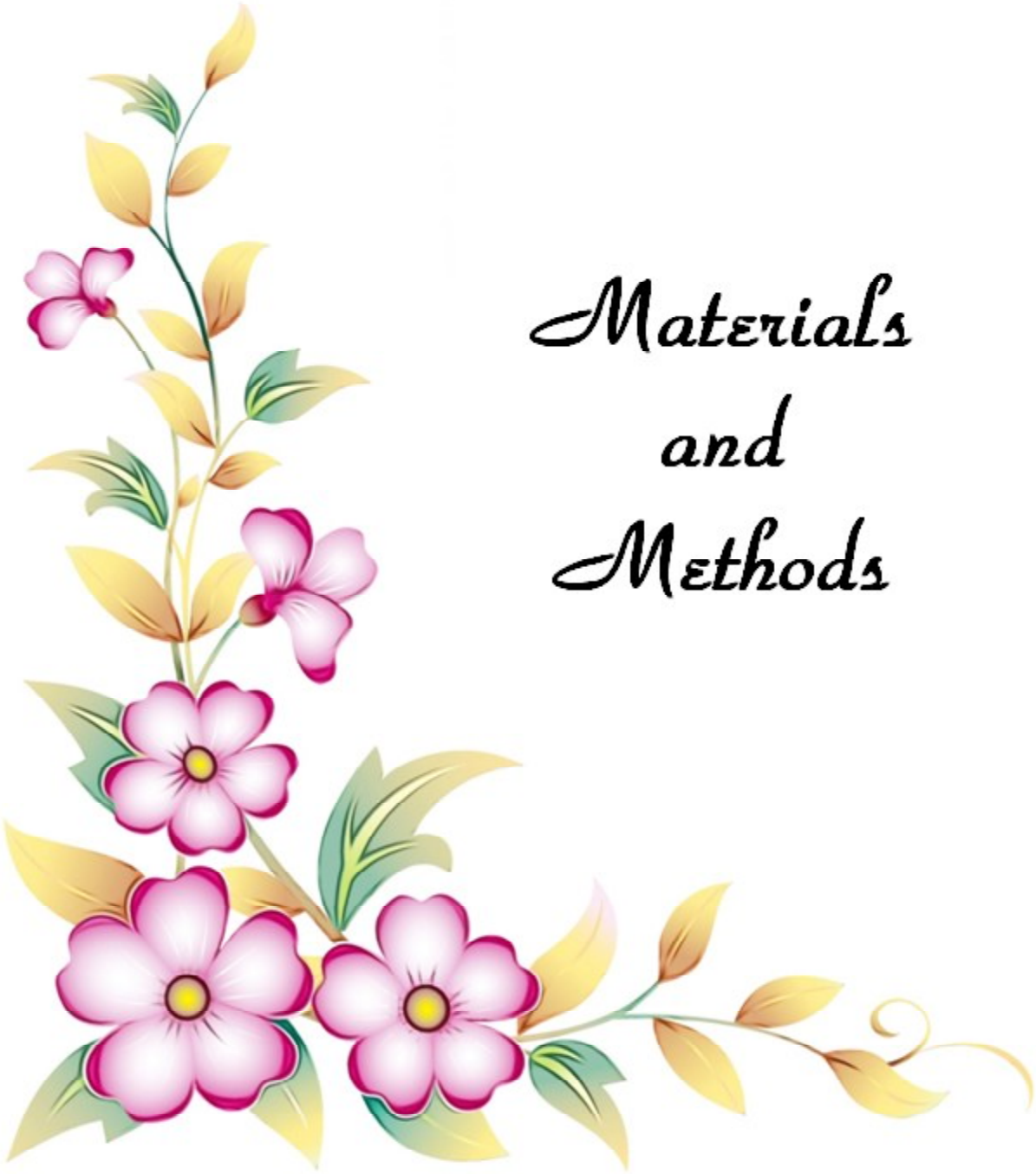
Nearly all organisms need glycolysis for respiration. As sugars are converted into pyruvate, ATP and NADH are simultaneously synthesized as part of the crucial cellular metabolic pathway. Pyruvate eventually transforms into lactic acid when anaerobic conditions prevail (Paulini *et al*., 2022). Pyruvic acid, however, enters the TCA cycle when it is present in an aerobic environment and is oxidized to produce $CO_2$ and $H_2O$. The pyruvate dehydrogenase (PDH) complex, which transforms pyruvate produced by glycolytic flux into acetyl-CoA, is inhibited by inhibitory phosphorylation caused by PDK1(Pyruvate dehydrogenase kinase1) (Tan *et al*., 2015).

In mice and macrophages, the EMP pathway is necessary for *S.* Typhimurium (STM) intracellular replication, and glucose is the main sugar that STM uses when infecting macrophages (Taylor and Winter, 2020). For accelerated intracellular replication, STM depends on the nutrient uptake from macrophages. STM must simultaneously use a variety of nutrients from macrophages, such as glucose, N-acetylglucosamine (GlcNAc), glycerol, and fatty acids, to support intracellular growth and spread infection throughout the body (Steeb *et al*., 2013; Liss *et al*., 2017). The glucose uptake rate is determined by the expression of a glucose transporter (GLUT). GLUT1 is expressed more in the inflammatory cellular environment in macrophages to take part in the flip to glycolysis (Yu *et al*., 2022).

Aerobic glycolysis replaces oxidative phosphorylation (OXPHOS) as the preferred mode of glucose metabolism when microbial ligands are recognized by macrophages. This increase in glycolysis, which triggers the production of ATP for inflammatory responses (Freemerman *et al*., 2014) and cytokine production to stop pathogen replication, is crucial for the antibacterial activities of activated macrophages (Escoll and Buchrieser, 2018). Prostaglandins, itaconate, reactive nitrogen species (RNS), and reactive oxygen species (ROS) synthesis are all produced due to glycolysis and are all involved in the antimicrobial functions of macrophages (Curi *et al*., 2017; Nonnenmacher *et al*., 2018).

In macrophages, bacterial lipopolysaccharide (LPS) increases glycolysis aerobically and is accompanied by an increase in the serine synthesis pathway via the glycolytic intermediate 3-phosphoglycerate (3PG) (Pérez-Morales., 2021). To produce the inflammatory cytokine interleukin (IL)-1 ß in activated macrophages at its highest levels, serine must be synthesized and metabolized downstream (Rodriguez *et al*., 2019). The significance of increasing serine synthesis to fight bacterial infection is that IL-1 initiates and regulates host defense (Gabay *et al*.,2010).

To start enough accumulation of carbon sources derived from macrophages to support its intracellular replication and virulence, STM can reprogram macrophage glucose metabolism. Reprogramming of metabolism in both host and STM, which takes place concurrently in the infected macrophages, leads to the buildup of those metabolic sugar intermediates (Pérez-Morales., 2021). According to Jiang *et al*. (2021) genetic or biochemical silencing of glycolysis in macrophages reduces *Salmonella* intracellular replication. Bowden *et al* (2009) reported that STM requires glycolysis to successfully infect macrophages as well as mice.



# Materials and Methods

# 3 MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Chemicals and Reagents

All chemicals, buffers, media, and reagents utilized in this study were obtained from reputable manufacturers, including Sigma (USA), Life Technologies (USA), Promega (USA), Bio-Rad Laboratories (USA), Thermo Scientific (USA), Qiagen (USA), SRL (India), Merck (India/Germany), Novagen (EMD Biosciences), Difco (BD Company, France), and Himedia (India), and were of either molecular biology or analytical grade. Detailed information about the composition of different reagents, solutions, media, and buffers employed in this study is provided in the appendix. The chemicals and kits were procured from various vendors. Specific items and their corresponding suppliers include:

- Tris-base from Himedia (India)
- Ethanol from Changsu Hongsheng Fine Chemical Co, Ltd
- 0.5 M EDTA isopropanol and chloroform from Amresco (Ohio)
- TRIzol from Qiagen (USA)
- Ethidium bromide, SDS, Triton X-100, glacial acetic acid, and glycerol from SRL (India)
- Agarose from APS labs (India)
- 50X TAE from Himedia
- Histopaque 1077 & lipopolysaccharides (L6261) from Sigma-Aldrich
- 10X Phosphate Buffered Saline from SRL (India)

- Gibco MaxSpec Fetal Bovine Serum (FBS) & Penicillin-Streptomycin (10,000 U/mL) from Thermo Fisher Scientific
- 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose) (Cat no: N13195) from Invitrogen
- Dream Taq green PCR (2X), 1 kb DNA ladder, 100 bp plus DNA ladder, 6X loading dye, XhoI FD enzyme, 10X fast digest buffer from Thermo Fisher Scientific
- Nuclease-free water from Himedia
- EvaGreen qPCR master mix from G-Biosciences

### 3.1.2 Bacteriological and Cell Culture Media

For bacterial culture, various bacteriological media including Luria Bertani (LB) agar, LB broth, Hektoen Enteric Agar (HEA), and other relevant media were obtained from Sisco Research Laboratories, India. All media were subjected to sterilization through autoclaving, except for heat-labile components such as D-Glucose and antibiotics, which were sterilized using sterile 25 mm/0.45 ìm syringe filters (Genetrix Biotech Asia Pvt Ltd, India). Autoclaving was not recommended for HEA agar, which was prepared through boiling. RPMI 1640 media (cat no-AL028) from Himedia (India) were employed for cell culture.

### 3.1.3 Plastic Glassware and Other Consumables

All plastic consumables, including Petri plates, cell culture plates/flasks, centrifuge tubes of various capacities, microcentrifuge tubes, thin-walled PCR tubes, real-time tubes & strips, and micropipette tips, were procured from reputable manufacturers such as Genaxy Scientific Pvt. Ltd (India), Nunc (Denmark), Abdos Labtech Pvt. Ltd (India), Agilent Technologies India Pvt Ltd, and Tarson (India). These plastic wares were certified as non-pyrogenic and nuclease-free, and they underwent autoclaving for proper sterilization. Glassware from Rivera (India), Borosil (India), and Schott Duran (Germany) was used, washed, and autoclaved before bacterial culture work following standard protocols. Sterile inoculating loops, Bunsen burners, biological safety cabinets, syringes, 21-G x 1½-inch needles, L-shape spreaders, and EDTA vacutainers were employed.

### 3.1.4 Equipment

The study involved a range of equipment, including:

- Air displacement micropipettes: P10, P100, P1000 (Finnpipette, Finland)
- Gel Electrophoresis apparatus: Horizontal (Bangalore, Genei)
- Gel Documentation system: Alpha Imager TM 1220, Alpha Innotech Corporation, USA
- Refrigerated microcentrifuge: Hermle, Germany
- Refrigerated centrifuge: Hermle, Germany
- Refrigerator: BPL India Ltd
- -20°C Deep freezer: Elanpro
- -80°C freezer: New Brunswick Scientific
- Spinix vortexer
- NanoDropTM: Thermo, USA
- Orbital shaker: New Brunswick
- Spectrophotometer: Varian Cary@
- Thermal cycler: Himedia & Biometra
- Circulating water bath: PMI, India
- Microwave oven: Sanyo
- Double distillation apparatus: JSGW
- Weighing balance: Sartorius, Germany
- Laminar Flow: ESCO
- MicroPulser Electroporation apparatus: Bio-Rad, USA
- $CO_2$ incubator: ESCO

### 3.1.5 Kits

The study utilized the following kits:

- GSure @ plasmid mini kit from GCC Biotech Pvt Ltd (India)
- HiPurA® Bacterial Genomic DNA Purification Kit from Himedia (India)
- ADP/ATP Ratio Assay Kit from Sigma-Aldrich (USA)
- Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific

### 3.1.6 Prokaryotic Host

For the study, *Salmonella* Typhimurium (STM)-5591 strain, Δ*fnr*: STM (Nikhil, 2019), Δ*fnr*: STM-p*fnr*, and Δ*narL*: STM (Pashupathi, 2021) strains were utilized. All bacterial strains were available in the lab (ICL Lab, Division of Biochemistry).

### 3.1.7 Blood

Blood from 4-week-old chicken broilers in CARI was collected in container containing EDTA and was used for macrophage culture.

**Table 3.1- List of primers used**

| S.no | Primers | Sequence | Purpose | Product size |
|---|---|---|---|---|
| 1 | CS-FP | TGGACAACTTCCCCACCAAC | Real-time PCR | 167 bp |
| | CS-RP | GGGCAGCTTAGCGATCAGAT | | |
| 2 | HK-FP | TGCGAGATCCGATTCGTGAG | Real-time PCR | 172 bp |
| | HK-RP | TTCAACCCTCATCCTCCGC | | |
| 3 | PTEN-FP | CAGTGGCACTGCTGTTTCAC | Real-time PCR | 180 bp |
| | PTEN-RP | ATACCGGCAAAGGTTGAGGG | | |
| 4 | GSK-FP | ATTCAGGAGAGCTTGTGGCC | Real-time PCR | 228 bp |
| | GSK-RP | GTGTCTGTTTGGCCCGACTA | | |
| 5 | PDK-FP | GCGGAGGTGTTCCTATGAGG | Real-time PCR | 156 bp |
| | PDK-RP | CGCCTTGGAAGTATTGTGCG | | |
| 6 | GLUT1- FP | ATGCCCTGGATGTCCTACCT | Real-time PCR | 156 bp |
| | GLUT1 -RP | CCAGTTAGACAGCCCAGCAA | | |
| 7 | ENO1 -FP | TATTGCCAAGGCTGTGGAGG | Real-time PCR | 182 bp |
| | ENO1 -RP | GACCAACCACCAGATCAGCA | | |
| 8 | CANX-FP | GGTGAATGGGAAGCACCTCA | Real-time PCR | 173 bp |
| | CANX-RP | GGGTTTGGGATCTTCCTGGG | | |
| 9 | TYPH-FP | TTGTTCACTTTTTACCCCTGAA | STM serotype-specific | 401bp |
| | TYPH-RP | CCCTGACAGCCGTTAGATATT | | |
| 10 | NarL out-FP | AAAACGCCGAACGCAGTAATCAC | Confirmation of | 600bp |
| | NarL out-RP | TCATTATGCCGGCCTGGGATC | Δ*narL* | |
| 11 | Fnr Outer-FP2 | GTTCCCTGCCATCGCGTCATC | Confirmation of | 500bp |
| | Fnr Outer RP2 | GGAAGCGCTTCTGCCAGATCAAT | Δ*fnr* gene | |
| 12 | SUMO del Fnr-FP | GATCAGATCTGCATGATCCCGGAAAA GCGAA | *fnr* gene cloning primers | 781bp |
| | SUMO del Fnr-RP | TTGATCGCGGCCGCTCTATTAAGCGACG TTGCGGGTATGAC | | |

### 3.1.7 Primers

Different primers used were designed using gene tool software primer 3 plus, their specificity was verified using oligo analyzer & BLAST. Primers were synthesized by GCC Biotech Pvt Ltd (India). Refer to Table 3.1 for the primers used and their corresponding sequences.

### 3.1.8 Statistical Analysis

All generated data were subjected to analysis using available software GraphPad Prism 8.0. To assess statistical significance between means of multiple groups, two-tailed unpaired t-test was conducted, with an alpha-error probability set at 0.05 ($p < 0.05$).

## 3.2 Experimental Methods

All the methods used in this work were done as per standard protocol.

### 3.2.1 Revival of the culture and Selective plating

Our laboratory's glycerol stock cultures containing WT-STM (Wild Type), Δ*fnr*: STM (*fnr* deletion mutant strain), Δ*fnr*: STM-p*fnr* (*fnr* complemented strain) and Δ*narL*: STM (*narL* deletion mutant strain) were retrieved from -80°C storage and placed at -20°C for one day. On a subsequent day, these isolates were subjected to centrifugation at 5000 x g and 25°C for 5 minutes. The resulting pellet was re-suspended in 100 µl of LB media and subsequently introduced into 5 ml of LB broth. *fnr* complemented strain was made by cloning SUMO-*fnr* into *fnr* mutant and inoculated in LB broth tubes containing kanamycin (50 µg/ml). Following this, the cultures underwent an overnight incubation within a shaker incubator, operating at 170 rpm and a temperature of 37°C. The cultures cultivated overnight were subsequently spread onto Hektoen enteric agar (HEA) plates, and these plates were left to incubate once again for an overnight period at 37°C. After this incubation, colonies that had emerged overnight were selected based on their observable traits. These chosen colonies were then subjected to subculturing in LB broth, allowing them to grow overnight at 37°C. This overnight growth was aimed at confirming the colonies' identity through colony PCR using Typhimurium serovar specific parimer and protocol are given in Table 3.2 and 3.3.

### 3.2.2 Colony PCR for confirmation of WT–STM, Δ*fnr*: STM, Δ*fnr*: STM-p*fnr* and Δ*narL*: STM

100 µl overnight culture of selected colonies was taken in a 0.5 ml centrifuge tube inside laminar airflow and centrifuged at 8000 x g for 5 mins at 25°C. The supernatant was cautiously poured off, and subsequently, 50 µl of nuclease-free water was introduced to enable the resuspension of the pellet. The upper section of the tube was securely sealed with parafilm and punctured using a needle. Following this, the tube was immersed in boiling water for 5 minutes and promptly moved to a temperature of -20°C thereafter. After undergoing a cooling process for approximately 15 minutes, the tube was withdrawn, allowing the sample to thaw at ambient room temperature. The final step encompassed another centrifugation process, this time at 8000 x g for a brief period of 1 minute at 25°C, and the resulting supernatant was employed as the template for the subsequent PCR reaction using *fnr* and *narL* outer specific primers.

**Table 3.2: PCR reaction mixture for colony PCR**

| Components | Volume (µl) |
|---|---|
| Template | 3 |
| FP (10pmol/ µl) | 1 |
| RP (10pmol/ µl) | 1 |
| 2X Dream Taq green master mix | 12.5 |
| Nuclease free water | 7.5 |
| Total | 25 µl |

**Table 3.3: PCR conditions for colony PCR**

| Steps | Temperature | Time | Condition |
|---|---|---|---|
| | 99°C | | Lid heating |
| Step I | 94°C | 5 min | Initial denaturation |
| Step II | 94°C | 20 sec | Denaturation |
| Step III | 57°C | 20 sec | Primer annealing |
| Step IV | 72°C | 75 sec | Elongation |
| | Step II to Step IV repeated 32 cycles | | |
| Step V | 72°C | 10 min | Final elongation |

### 3.2.3 Agarose gel electrophoresis

The PCR amplified products were assessed using submerged agarose gel electrophoresis on a 1% agarose gel prepared in 1X Tris Acetic acid EDTA solution (TAE), following the established methodology outlined by Sambrook and Russell (2001 To determine the molecular weights, a DNA ladder (100 bp) was employed as a reference marker Electrophoresis was conducted at a voltage of 100 volts, and subsequent visualization of the gel was achieved using UV illumination. The results were recorded using a Gel-Doc system.

### 3.2.4 Isolation of peripheral blood mononuclear cells

1. Blood was aseptically collected from 4-week-old chicken broilers using EDTA vacutainers to prevent contamination.
2. The collected blood was then diluted with an equal volume of 1X PBS (phosphate-buffered saline).
3. In a fresh 15ml centrifuge tube, an equal volume of Histopaque 1077 was added, corresponding to the volume of the undiluted blood. The diluted blood was gently overlaid onto the Histopaque 1077 to ensure separation without mixing.
4. The tube was subjected to centrifugation at 370x g for 30 minutes at a temperature of 20°C.
5. Post-centrifugation, the Peripheral Blood Mononuclear Cells (PBMCs) were carefully collected from the interface between the plasma and the Histopaque 1077 layer. The collected PBMCs were then washed three times with 1x PBS, employing the same centrifugation conditions as mentioned before.
6. In case there were Red Blood Cells (RBCs) present in the collected PBMCs, an RBC lysis buffer was utilized during the washing steps to lyse the RBCs and remove them.

### 3.2.5 Culturing of Macrophages from harvested PBMC

1. The harvested PBMC cells were suspended in RPMI 1640 media supplemented with 1% penicillin-streptomycin solution and 10% fetal bovine serum (FBS). The cell suspension was adjusted to the required concentration after counting in the hemocytometer and checked for cell viability using trypan (0.5%) blue.

2. This cell suspension was then seeded into an appropriate cell culture flask or onto plates, followed by incubation at 37°C in a $CO_2$ (5%) incubator.
3. On the subsequent day, the cells were subjected to a wash with 1x PBS to eliminate any suspended cells. Following this, RPMI 1640 media containing 10% FBS and LPS (lipopolysaccharide) at a concentration of 0.5 ng/µl was introduced to stimulate the adherent monocytes. The cells were then incubated for 48 hours under identical conditions.
4. After this 48-hour incubation period, the resulting cell population primarily composed of macrophages was deemed suitable for *Salmonella* infection studies.

### 3.2.6 Serial Dilution Plating

1. The *Salmonella* bacteria selected for infection studies were cultured by diluting them 1:100 in LB broth tubes. These tubes were then placed in a shaker incubator and allowed to incubate overnight at 37°C with agitation at 170 rpm.
2. On the following day, the bacterial cells that had grown overnight were once again sub-cultured at a 1:100 dilution in fresh LB broth tubes. These tubes were placed in the shaker incubator at 37°C and 170 rpm, and the incubation continued until the bacterial culture reached an optical density (OD) value of 0.4-0.6 at a wavelength of 600nm (OD600).
3. For the subsequent steps, 1.5 ml centrifuge tubes were prepared and labeled from 1 to 9, corresponding to each bacterial sample. Next, each tube was loaded with 90 µl of autoclaved 1x PBS.
4. To initiate the serial dilution process, 10 µl of the sub-cultured bacteria, which had reached an OD 600 value of 0.4-0.6, was added to the first centrifuge tube (Tube 1) and thoroughly mixed using a vortex mixer.
5. From Tube 1, 10 µl of the diluted bacterial solution was transferred to Tube 2 and mixed again.
6. This serial dilution process was repeated for each subsequent tube, with 10 µl from the previous tube being added to the next tube, up to Tube 9.
7. After completing the serial dilutions, a portion of each dilution was plated onto HEA (Hektoen Enteric Agar) plates.

8. The plated HEA agar plates were then incubated overnight at 37°C.
9. On the following day, the bacterial colonies that had grown on the agar plates were counted to determine the colony-forming units per milliliter (CFU/ml).

### 3.2.7 Glucose uptake assay (Gutiérrez *et al*., 2021)

1. Primary chicken macrophage cultures were grown in 24-well cell culture plates, with each well containing 1 x $10^5$ cells.
2. On the day of infection, macrophages in culture plates were washed with 1X PBS and RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), and 2g/L glucose was added. Then, the macrophages were then infected with WT-STM, Δ*fnr*: STM, Δ*fnr*:STM-p*fnr*, and Δ*narL*: STM at a multiplicity of infection (MOI) of 10:1.
3. Following the infection, the culture plates underwent washing with 1X PBS thrice to eliminate bacteria and suspended cells from the wells.
4. Subsequently, 450 µl of a solution containing 10 µM 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose), a fluorescent glucose analog and glycolysis inhibitor, was added to each well in the plates.
5. This solution was prepared in RPMI 1640 media without glucose, but still containing 10% FBS. The plates were then incubated at 37°C for 15 minutes before being washed three times with 1X PBS.
6. After the incubation and washing steps, the macrophage cells were lysed using a lysis solution (200 µl/well) and left to incubate for 5 minutes at room temperature. The lysed contents were subsequently mixed by pipetting and then centrifuged at 12,000 x g for 1 minute using 1.5 ml centrifuge tubes.
7. The resulting supernatant, containing the cellular components, was carefully transferred to a black opaque 96-well plate.
8. For further analysis, the contents of the 96-well plate were subjected to fluorimetry with excitation and emission wavelengths of 467 nm and 538 nm, respectively.

### 3.2.8 Real-time PCR

1. Primary chicken Macrophages were grown in 25 cc cell culture flasks containing 1 x $10^5$ cells.

2. The infection procedure followed the methodology outlined in the previous experiment (section 3.2.7).
3. Post-infection, the flask was washed thrice with 1X PBS, and 1 ml trypsin-EDTA was added.
4. It was kept undisturbed for 3 minutes at room temperature and then the contents were collected in the 1.5 ml centrifuge tubes.
5. Then, the total RNA was isolated by the TRIzol method, and the purity and concentration of RNA were measured using a Nanodrop spectrophotometer.
6. After that, the synthesis of cDNA was done from isolated RNA samples. The applied biosystem's high-capacity cDNA Reverse Transcription Kit was used for the quantitative conversion of up to 2 µg of total RNA to single-stranded cDNA in a single 20 µl reaction.

**Table 3.4: Reaction mixture for cDNA synthesis- Two steps**

| Components | Volume (µl) |
|---|---|
| **Step 1 – Preparation of RT master mix** | |
| 10X RT Buffer | 2 µl |
| 25X dNTP Mix (100 mM) | 0.8 µl |
| 10X RT Random Primers | 2 µl |
| MultiScribe™ Reverse Transcriptase (50 U/µl) | 1 µl |
| RNase Inhibitor | 1 µl |
| Nuclease-free water | 3.2 µl |
| Total per reaction | 10 µl |
| **Step 2 – Addition of Template** | |
| Template RNA | 2 µg |
| Nuclease free water | Make up to 20 µl |
| Total | 20 µl |

PCR tubes were briefly centrifuged to spin down the contents to eliminate any air bubbles and the cDNA synthesis reaction was carried out in a thermal cycler.

**Table 3.5: PCR condition for cDNA synthesis**

| Steps | Temperature | Time |
|---|---|---|
| Step I | 25°C | 10 mins |
| Step II | 37°C | 120 mins |
| Step III | 85°C | 5 mins |

After the synthesis of cDNA, it was checked by conventional PCR using reference gene (Calnexin)

**Table 3.6: PCR reaction mixture for checking of cDNA**

| Components | Volume (µl) |
|---|---|
| Template | 0.5 |
| Calnexin Forward primer (10 pmol/µl) | 0.5 |
| Calnexin Reverse primer (10 pmol/µl) | 0.5 |
| 2x dream Taq green master mix | 10 |
| Nuclease free water | 8.5 |
| Total | 20 µl |

**Table 3.7: PCR conditions for amplification**

| Steps | Temperature | Time | Condition |
|---|---|---|---|
| | 99°C | | Lid heating |
| Step I | 94°C | 5 min | Initial denaturation |
| Step II | 94°C | 15 sec | Denaturation |
| Step III | 56°C | 15 sec | Primer annealing |
| Step IV | 72°C | 15 sec | Elongation |
| | Step II to Step IV repeated 40 cycles | | |
| Step V | 72°C | 5 min | Final elongation |

The final PCR product was visualized in 1% agarose gel electrophoresis and gel documented with a 100 bp ladder as a reference marker.

**Table 3.8: PCR reaction mixture for Real-time PCR**

| Components | Volume (µl) |
|---|---|
| Template | 0.5 |
| Forward primer (10 pmol/il) | 0.5 |
| Reverse primer (10 pmol/il) | 0.5 |
| 2x SYBR green master mix | 10 |
| Nuclease free water | 8.5 |
| Total | 20 µl |

**Table 3.9: PCR conditions for Real-time PCR**

| Steps | Temperature | Time | Condition |
|---|---|---|---|
| Step I | 94°C | 5 min | Initial denaturation |
| Step II | 94°C | 15 sec | Denaturation |
| Step III | 56°C | 15 sec | Primer annealing |
| Step IV | 72°C | 15 sec | Elongation |
| | Step II to Step IV repeated 40 cycles | | |
| Step V | 95°C | 15 sec | Melting stage |
| Step VI | 56°C | 15 sec | |
| Step VII | 95°C | 15 sec | |

The final fold change was calculated from the ct values using formula $2^{-\Delta\Delta ct}$

### 3.2.9 Measurement of ATP levels inside macrophage (Yu *et al.*, 2020)

1. Macrophages were cultured in 96-well white opaque cell culture plates, with each well initially seeded with 10,000 cells.
2. The infection procedure followed the methodology outlined in the previous experiment (section 3.2.7).
3. Subsequent to the infection process, the culture medium was removed, and the cells underwent three washes with 1X PBS 5 min each.
4. The preparation of the working reagent was done according to the specifications provided in the table below.

**Table 3.10: Working reagent for ATP assay**

| Components | Volume (per well) |
|---|---|
| Assay buffer | 95 µl |
| Substrate | 1 µl |
| Co-substrate | 1 µl |
| ATP enzyme | 1 µl |

A volume of 90 µl working reagent was introduced into every well within the plate, after which the solution was gently mixed by tapping the plate to ensure uniform distribution.

Subsequently, the plate was subjected to an incubation period of 1 minute at room temperature. Following this incubation, the luminescence was measured in relative light units using luminometer.

# Results

# 4 RESULTS

## 4.1 Characterization of *Salmonella* Typhimurium

Isolates of *Salmonella* Typhimurium (STM)-5591 wild type strain, Δ*fnr*: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains were available in the lab (ICL Lab, Division of Biochemistry). Cultural characterization was done by streaking the strains on HEA agar plates (Fig 4.1). The colony characteristics were typical with a greenish periphery and blackish center due to the hydrogen sulfide (H2S) production. Molecular characterization of STM was done through colony PCR using Typhimurium serovar specific primers. Agarose gel electrophoresis was done using the product obtained from colony PCR, the bands obtained were indicated in Fig 4.2.

## 4.2 Culturing of Macrophages

PBMCs were isolated from blood of 4-week-old healthy chickens and cultured for 1, 2, 3, and 4 days in the presence of LPS to differentiate monocytes into macrophages. The cultures were inspected over time for changes in macrophage morphology and purity. At one day post-isolation, adherent cells were monocytes, but the floating cells were a mixed population of other cells. Non-adherent cells were removed by PBS washing. Cells became flat after two days of culture and monocyte-derived macrophages remained, as indicated by the fried eggs-like shape of the cells, which are classic mammalian M1-like macrophages (Peng *et al*. 2020) (Fig 4.3)

## 4.3 Serial dilution plating/determination of cfu/ml

The overnight-grown cultures were sub-cultured for 2hrs and serial diluted from $10^{-1}$ to $10^{-9}$. Then the diluted cultures were plated into HEA plate and incubated overnight to

calculate CFU / ml on the next day. The results are depicted in Fig. 4.4 and cfu/ml of WT-STM ($4.65 \times 10^8$), Δ*fnr*: STM ($3.6 \times 10^8$), Δ*fnr*: STM-p*fnr* ($4.2 \times 10^8$) and Δ*narL* STM ($4.4 \times 10^8$).

## 4.4 Glucose Uptake Assessment

We conducted the Glucose Uptake assay following the procedure outlined by Gutiérrez *et al*., 2021, with slight adjustments. To summarize, primary macrophages were cultivated in 24-well plates. On the fourth day, they were infected with various Salmonella strains. After infection, 2-NBDG was introduced to the infected cells, and fluorescence was analyzed. The findings are as follows:

After 30 minutes of infection, there were no notable differences in 2-NBDG uptake among the Salmonella strains. At the 3-hour mark of infection, Δ*fnr*: STM exhibited a significant increase in 2-NBDG uptake compared to the other strains. However, by the 6-hour point, glucose uptake significantly decreased in Δ*fnr*: STM compared to the 3-hour mark. Conversely, we observed a significant rise in 2-NBDG uptake in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains, as depicted in Fig. 4.5

Comparing these time points to the 30-minute post-infection point, the wild-type STM strain showed a 6.64% increase in 2-NBDG uptake at 3 hours and a substantial 53.7% increase at 6 hours. Δ*fnr*: STM displayed a 55% increase in 2-NBDG uptake at 3 hours post-infection, but this dropped to 37.8% at 6 hours compared to the 30-minute post-infection level. Δ*fnr*: STM- p*fnr* had a 44% increase at 3 hours and a 56% increase at 6 hours when compared to the 30-minute post-infection time point. In Δ*narL*: STM, there was a 30.56% increase at 3 hours and a 35.84% increase at 6 hours post-infection compared to the 30-minute point.

## 4.5 Analysis of Relative Gene Expression Changes in Infected Macrophages

### i) GLUT1 Expression

After 30 minutes of infection, there is no significant difference in the fold change in GLUT1 expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*:

STM exhibited a significant increase in GLUT1 expression compared to other strains, although this got reduced after 6 hours. Notably, at the 6-hour time point, a significant increase in GLUT1 expression was also observed in WT: STM, Δ*fnr*: STM- p*fnr*, and Δn*arL*: STM strains, as indicated in Fig. 4.6

Compared to the 30-minute post-infection time point, the wild-type STM strain displayed a 29.3% and 97% increase in GLUT1 expression at 3 and 6 hours, respectively. Δ*fnr*: STM exhibited a substantial 97.4% increase in GLUT1 expression at 3 hours post-infection, which decreased to 94% after 6 hours compared to the 30-minute post-infection time point. Δ*fnr*: STM- p*fnr* demonstrated an 82% increase at 3 hours and a 96.5% increase at 6 hours. In Δ*narL*: STM, there was a 66% increase at 3 hours and a 97% increase at 6 hours compared to the 30-minute post-infection time point.

**ii) HK-2 Expression**

After 30 minutes of infection, there is no significant difference in the fold change of HK-2 expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM displayed a significant increase in HK-2 expression compared to other strains, although this increase was reduced after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in HK-2 expression observed in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains, as indicated in Fig. 4.7

Compared to the 30-minute post-infection time point, the wild-type STM strain exhibited 22.7% increase in HK-2 expression at 3 hours and 99.5% increase at 6 hours. Δ*fnr*: STM showed a significant 98.9% increase in HK-2 expression at 3 hours post-infection, which decreased to 94.6% after 6 hours. Δ*fnr*: STM- p*fnr* displayed an 86% increase at 3 hours and 98.7% increase at 6 hours. In Δ*narL*: STM, there was an 80.8% increase at 3 hours and 99.2% increase at 6 hours compared to the 30-minute post-infection time point.

**iii) ENO1 Expression**

After 30 minutes of infection, there is no significant difference in the fold change of ENO1 expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM

displayed a significant increase in ENO1 expression compared to other strains, although this increase was reduced after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in ENO1 expression observed in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains, as indicated in Fig. 4.8

Compared to the 30-minute post-infection time point, the wild-type STM strain exhibited a 28% increase in ENO1 expression at 3 hours and 96% increase at 6 hours. Δ*fnr*: STM showed a significant 93.7% increase in ENO1 expression at 3 hours post-infection, which decreased to 92% after 6 hours. Δ*fnr*: STM- p*fnr* displayed a 70% increase at 3 hours and 93% increase at 6 hours. In Δ*narL*: STM, there was a 48% increase at 3 hours and 95.2% increase at 6 hours compared to the 30-minute post-infection time point.

**iv) PDK-1 Expression**

After 30 minutes of infection, there is no significant difference in the fold change of PDK-1 expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM displayed a significant increase in PDK-1 expression compared to other strains, although this increase was reduced after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in PDK-1 expression observed in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains, as indicated in Fig. 4.9

Compared to the 30-minute post-infection time point, the wild-type STM strain exhibited 41.5% increase in PDK-1 expression at 3 hours and 95.29% increase at 6 hours. Δ*fnr*: STM showed a significant 92.12% increase in PDK-1 expression at 3 hours post-infection, which decreased to 35% after 6 hours. Δ*fnr*: STM- p*fnr* displayed 76.4% increase at 3 hours and 85.9% increase at 6 hours. In Δ*narL*: STM, there was 63.87% increase at 3 hours and 90.6% increase at 6 hours compared to the 30-minute post-infection time point.

**v) GSK-3 Expression**

After 30 minutes of infection, there is no significant difference in the fold change of GSK-3 expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM displayed a significant increase in GSK-3 expression compared to other strains, although

this increase was reduced after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in GSK-3 expression observed in WT: STM, Δ*fnr*: STM- p *fnr*, and Δ*narL*: STM strains, as indicated in Fig. 4.10

Compared to the 30-minute post-infection time point, the wild-type STM strain exhibited 19% increase in GSK-3 expression at 3 hours and 90.8% increase at 6 hours. Δ*fnr*: STM showed a significant 91.8% increase in GSK-3 expression at 3 hours post-infection, which decreased to 75.3% after 6 hours. Δ*fnr*: STM- p*fnr* displayed 49.6% increase at 3 hours and 84.9% increase at 6 hours. In Δ*narL*: STM, there was 50.5% increase at 3 hours and 88.27% increase at 6 hours compared to the 30-minute post-infection time point.

**vi) PTEN Expression**

After 30 minutes of infection, there is no significant difference in the fold change of PTEN expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM displayed a significant increase in PTEN expression compared to other strains, although this increase was reduced after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in PTEN expression observed in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains, as indicated in Fig. 4.11

Compared to the 30-minute post-infection time point, the wild-type STM strain exhibited 2.73% increase in PTEN expression at 3 hours and 95.35% increase at 6 hours. Δ*fnr*: STM showed a significant 90.05% increase in PTEN expression at 3 hours post-infection, which decreased to 77.23% after 6 hours. Δ*fnr*: STM- p*fnr* displayed 61.96% increase at 3 hours and 90.5% increase at 6 hours. In Δ*narL*: STM, there was 31.48% increase at 3 hours and 93.9% increase at 6 hours compared to the 30-minute post-infection time point.

**vii) CS Expression**

After 30 minutes of infection, there is no significant difference in the fold change of CS expression among the Salmonella strains. However, at 3 hours of infection, Δ*fnr*: STM displayed a significant decrease in CS expression compared to other strains, although this decrease was reversed after 6 hours. Additionally, at the 6-hour time point, there was a significant increase in CS expression observed in all strains except Δ*fnr*: STM, as indicated in Fig. 4.12

In WT: STM, there was a 98.9% increase in CS expression at 3 hours post-infection, which decreased to 96.25% after 6 hours. Δ*fnr*: STM had a 95.87% increase at 3 hours and a 98.6% increase at 6 hours compared to the 30-minute post-infection time point. Δ*fnr*: STM- p*fnr* displayed a 97.5% increase at 3 hours and a 95.8% increase at 6 hours. In Δ*narL*: STM, there was a 97.94% increase at 3 hours and a 94.8% increase at 6 hours compared to the 30-minute post-infection time point.

## 4.6 ATP Measurement

Following a 30-minute infection period, there were no notable differences in ATP levels among the various Salmonella strains. However, at the 3-hour mark of infection, a significant decrease in ATP levels was observed in Δ*fnr*: STM in comparison to the other strains. Intriguingly, after 6 hours of infection, ATP levels in Δ*fnr*: STM increased. Conversely, we noted a initial increase than decrease in ATP levels in WT: STM, Δ*fnr*: STM- p*fnr*, and Δ*narL*: STM strains after 6 hours of infection, as illustrated in Fig. 4.13

Comparing these time points to the ATP levels at 30 minutes post-infection, we observed the following changes:

In WT: STM, there was a 97% increase in ATP levels at 3 hours post-infection, but this decreased to 91.29% after 6 hours compared to the 30-minute post-infection levels. Δ*fnr*: STM exhibited a 90.1% increase in ATP levels at 3 hours and a substantial 98.44% increase at 6 hours compared to the 30-minute post-infection levels. Δ*fnr*: STM- p*fnr* displayed a 97% increase in ATP levels at 3 hours, which was reduced to 91.3% after 6 hours of infection compared to the 30-minute post-infection levels. In Δ*narL*: STM, there was a 97.2% increase in ATP levels at 3 hours, but this decreased to 89.7% after 6 hours of infection compared to the 30-minute post-infection levels.

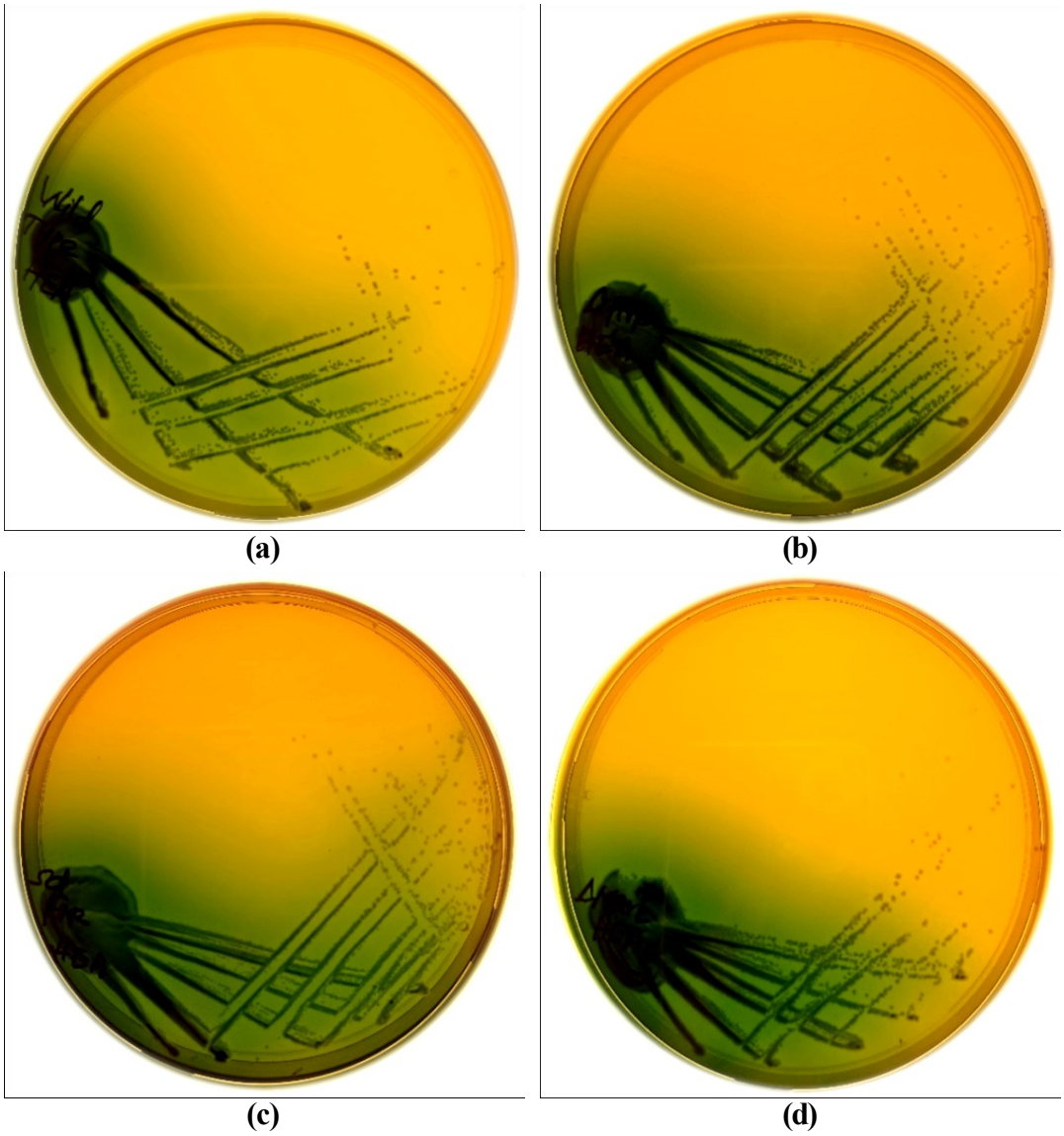


**Fig 4.1:** ***Salmonella*** **strains grown on HEA plate showing smooth colonies with black centers. (a) Wild type STM, (b) Δ*fnr*:STM, (c) Δ*fnr*:STM-*pfnr* and (d) Δ*narL*: STM**

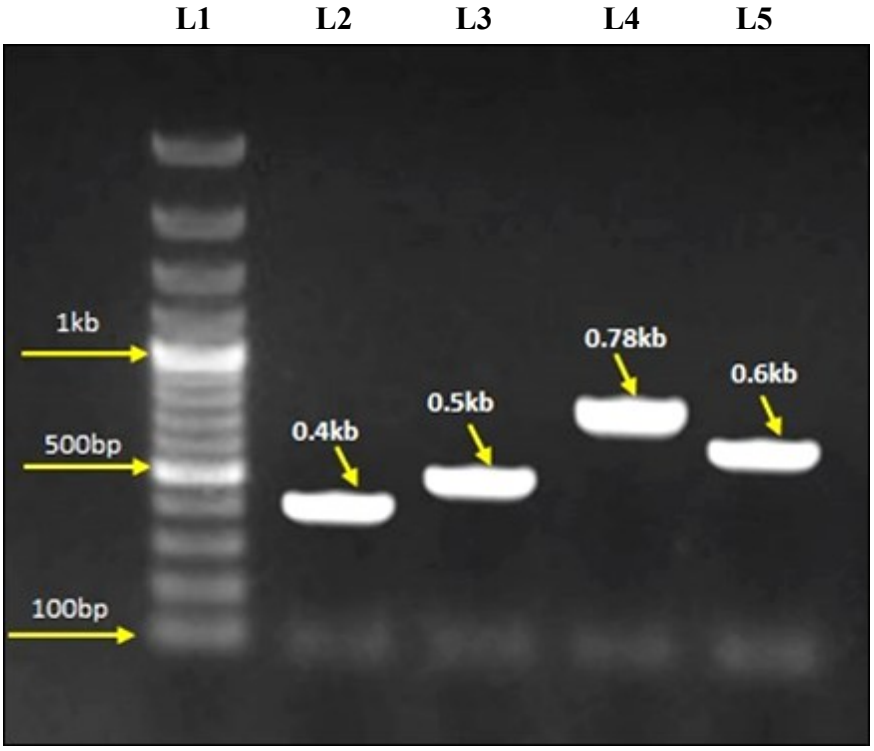
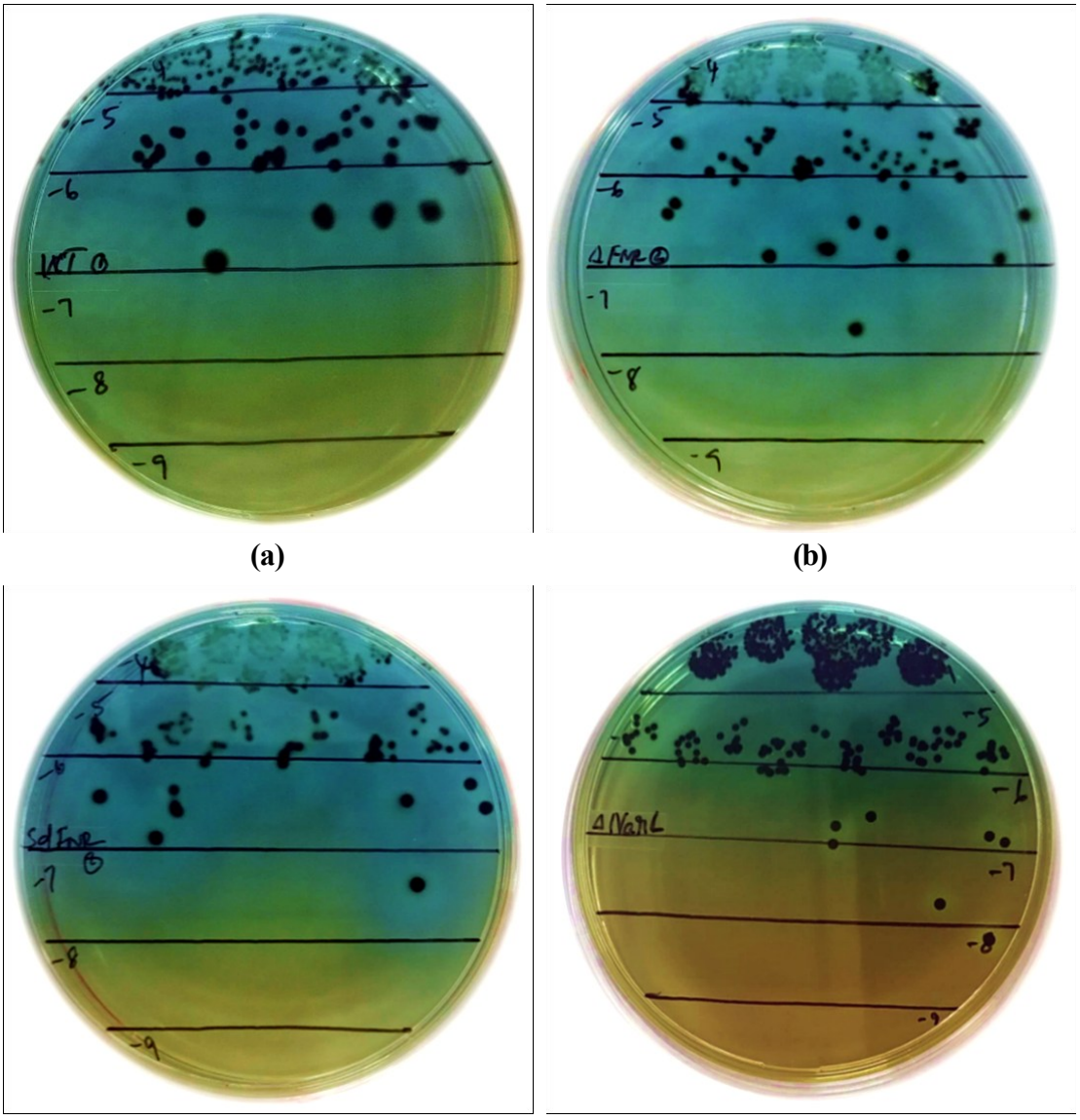



**Fig 4.2: Colony PCR for molecular confirmation**

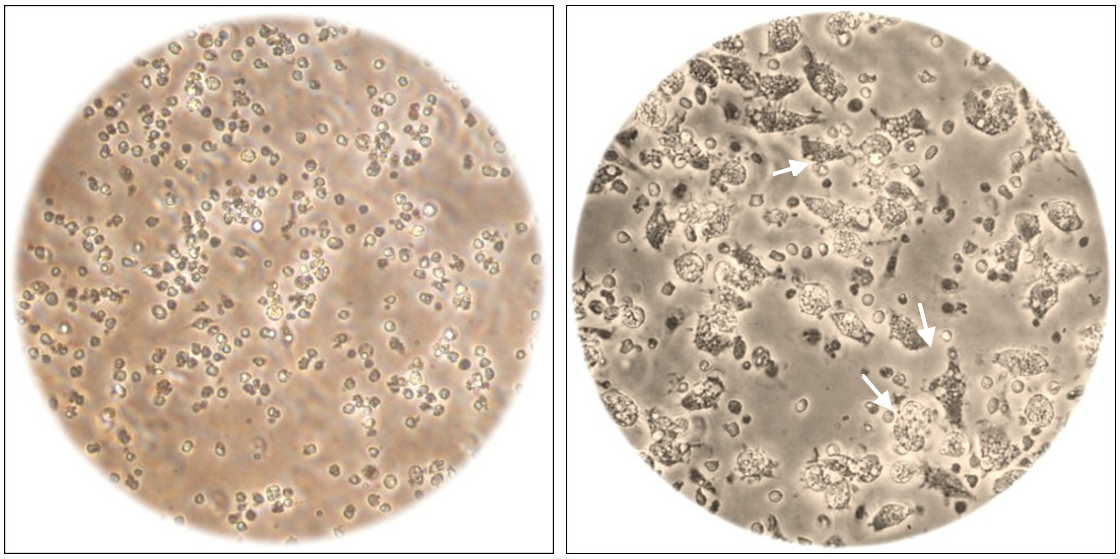
L1 : 100 bp ladder
L2 : Wild type in typh primers
L3 : *fnr* mutant in *fnr* outer primers
L4 : *fnr* complement in SUMO del primers
L5 : *narL* mutant in *narL* outer primers




**(a)** **(b)**

**(c)** **(d)**

**Fig 4.3: Serial dilution plating of STM strains in log phase** (a) Wild type STM, (b) *Δfnr*:STM, (c) *Δfnr*: STM-*pfnr* and (d) Δ*narL*: STM.



**(a)** **(b)**

**Fig 4.4: Differentiation of chicken monocytes into macrophages.** (a) 40X day 1 chicken macrophages (b) 40X day 3 chicken macrophages

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**Table 4.1: Glucose Uptake Assay**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 1.153±0.244 | 1.156±0.0596 | 2.03±0.0547 |
| Δ*fnr*: STM | 1.221±0.2557 | 2.124±0.1656 | 1.392±0.0765 |
| Δ*fnr*: STM- p*fnr* | 1.061±0.2975 | 1.565±0.1987 | 1.703±0.0255 |
| Δ*narL*: STM | 1.07±0.316 | 1.377±0.06825 | 1.811±0.0635 |

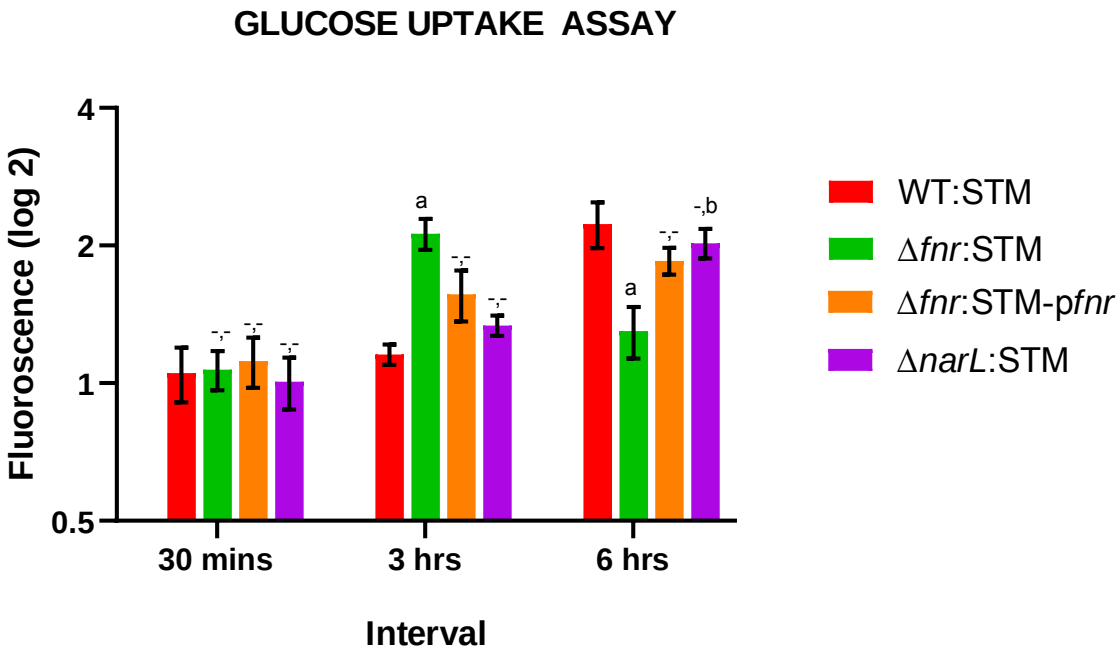



**Fig 4.5: Glucose uptake assay in *Salmonella* infected chicken monocyte-derived macrophages** (ChMoM). This graph indicates the uptake of fluorescent glucose compound (2- NBDG) by ChMoM after 30 mins, 3 hrs, and 6 hrs of infection. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '–' indicate no significant difference.

**Table 4.2: Real time PCR -GLUT 1**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.1549±0.034 | 0.2838±0.06874 | 6.74±1.368 |
| Δ*fnr*: STM | 0.2011±0.0589 | 6.57±1.628 | 2.059±0.4657 |
| Δ*fnr*: STM- p*fnr* | 0.1602±0.03594 | 0.6737±0.2136 | 4.581±1.247 |
| Δ*narL*: STM | 0.2224±0.0956 | 0.3754±0.0903 | 5.161±1.634 |

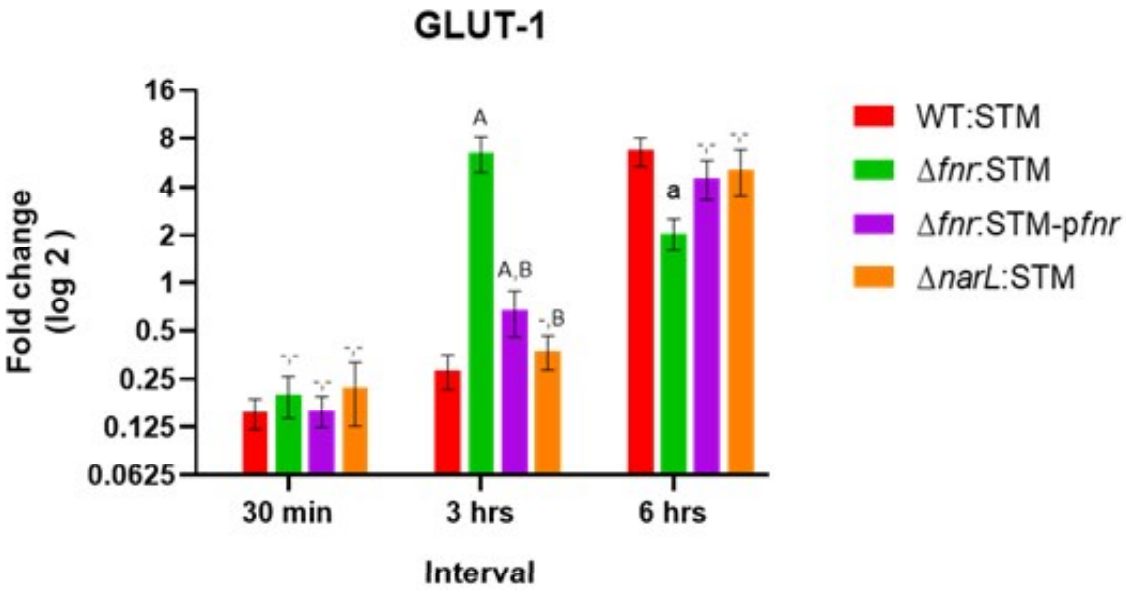



**Fig 4.6 : GLUT-1 expression in *Salmonella* infected chicken monocyte-derived macrophages.** The graph indicates GLUT-1 expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.3: Real time PCR -HK 2**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.195±0.0888 | 0.34±0.099 | 22.39±5.54 |
| Δ*fnr*: STM | 0.224±0.1053 | 14.33±2.278 | 3.042±0.4502 |
| Δ*fnr*: STM- p*fnr* | 0.2527±0.1106 | 1.455±0.3752 | 11.77±0.8964 |
| Δ*narL*: STM | 0.2189±0.0662 | 0.7625±0.2845 | 18.7±1.013 |

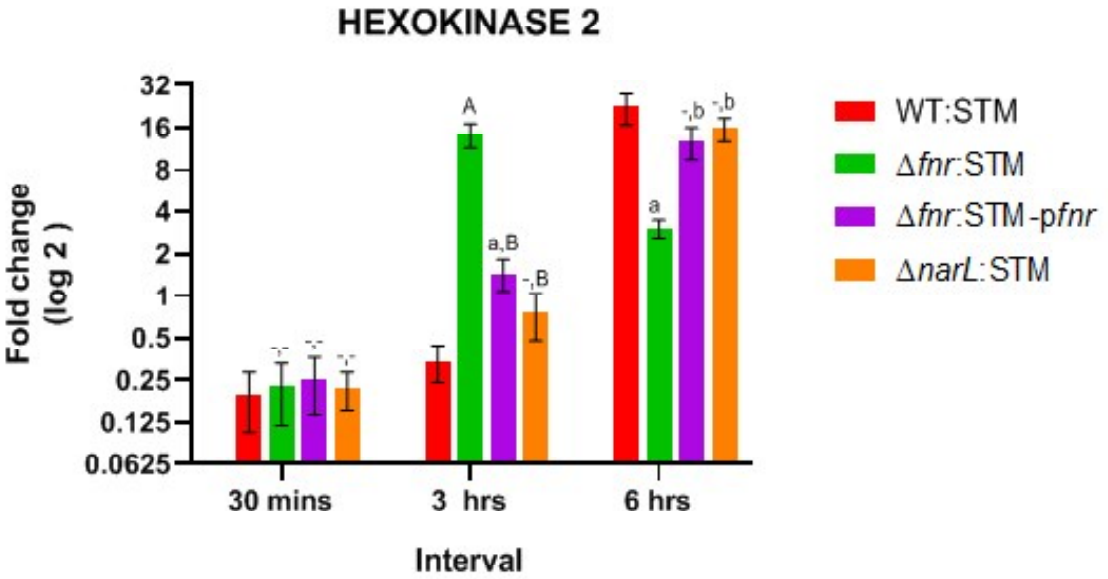



**Fig 4.7: HK -2 expression in *Salmonella* infected chicken monocyte-derived macrophages**. The graph indicates HK-2 expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote $P<0.05$, capital letters denote $P<0.01$).'– ' indicate no significant difference.

**Table 4.4: Real time PCR -ENO 1**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.3111±0.0585 | 0.3765±0.101 | 7.967±0.8767 |
| Δ*fnr*: STM | 0.3382±0.06878 | 5.086±0.7355 | 2.0170±0.3088 |
| Δ*fnr*: STM- p*fnr* | 0.3195±0.0848 | 1.526±0.2621 | 5.271±0.8272 |
| Δ*narL*: STM | 0.2683±0.03277 | 0.7615±0.2011 | 5.3±0.6522 |

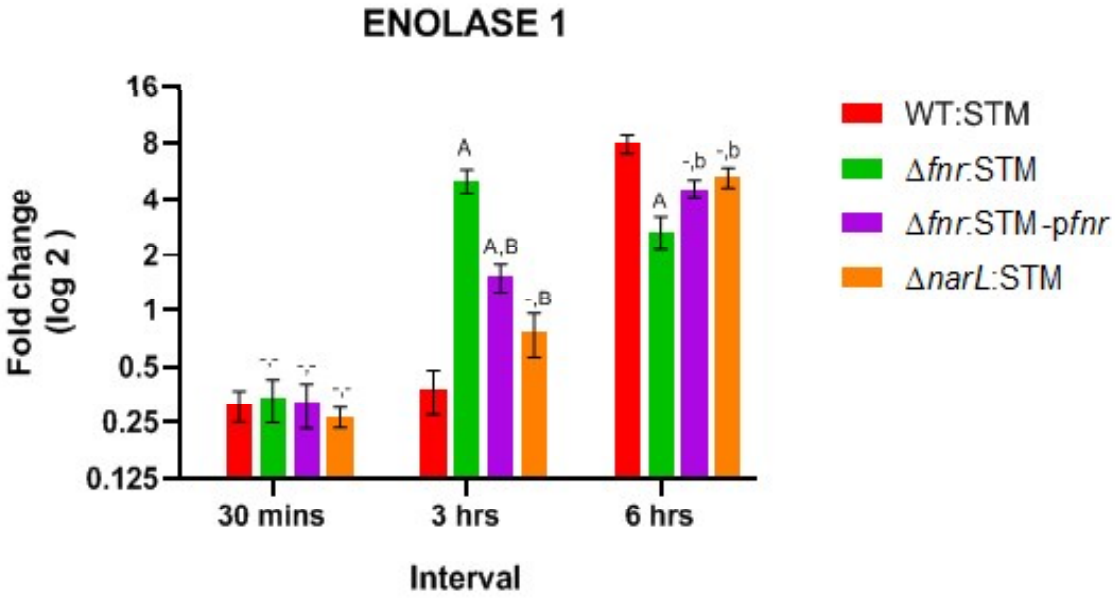



**Fig 4.8 : ENO -1 expression in *Salmonella* infected chicken monocyte-derived macrophages.** The graph indicates ENO-1 expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.5: Real time PCR -PDK 1**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.184±0.0424 | 0.3476±0.0519 | 4.569±0.7387 |
| Δ*fnr*: STM | 0.1847±0.0323 | 2.678±0.4286 | 1.286±0.0248 |
| Δ*fnr*: STM- p*fnr* | 0.2005±0.02005 | 0.8827±0.1047 | 2.762±0.381 |
| Δ*narL*: STM | 0.1739±0.02964 | 0.5757±0.0863 | 4.183±0.8977 |

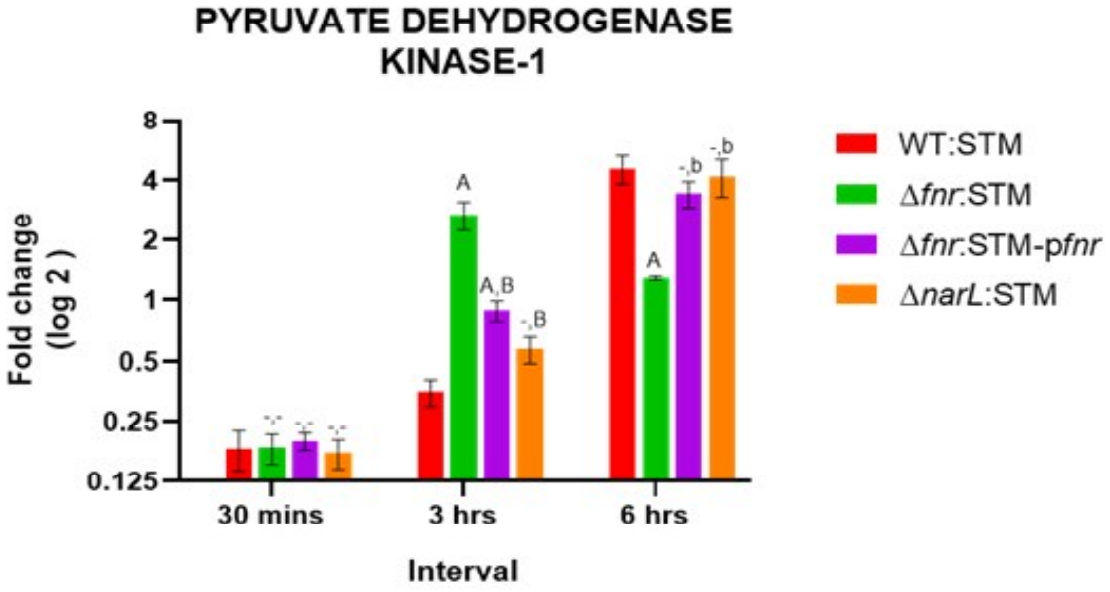



**Fig 4.9 : PDK -1 expression in *Salmonella* infected chicken monocyte-derived macrophages**. The graph indicates PDK-1 expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.6: Real time PCR -PTEN**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.2528±0.0284 | 0.4266±0.1076 | 4.441±0.68 |
| Δ*fnr*: STM | 0.2619±0.0377 | 2.966±0.3884 | 1.15±0.2928 |
| Δ*fnr*: STM- p*fnr* | 0.2837±0.0449 | 0.9996±0.1545 | 3.66±0.3551 |
| Δ*narL*: STM | 0.2332±0.0442 | 0.4147±0.1122 | 4.195±0.6171 |

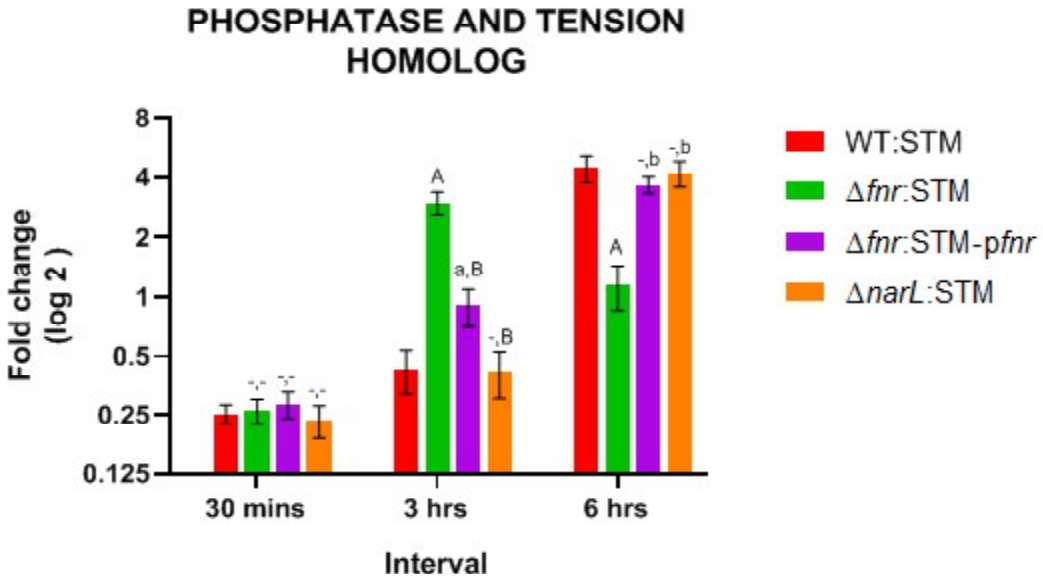



**Fig 4.10: PTEN expression in *Salmonella* infected chicken monocyte-derived macrophages**. The graph indicates PTEN expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference,a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.7: Real time PCR -GSK 3**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.2671±0.4344 | 0.4799±0.081 | 6.279±0.5059 |
| Δ*fnr*: STM | 0.2425±0.06524 | 4.041±0.302 | 1.560±0.4074 |
| Δ*fnr*: STM- p*fnr* | 0.2517±0.071 | 1.479±0.2904 | 3.823±0.5712 |
| Δ*narL*: STM | 0.2701±0.028 | 0.7755±0.1559 | 4.418±0.4931 |

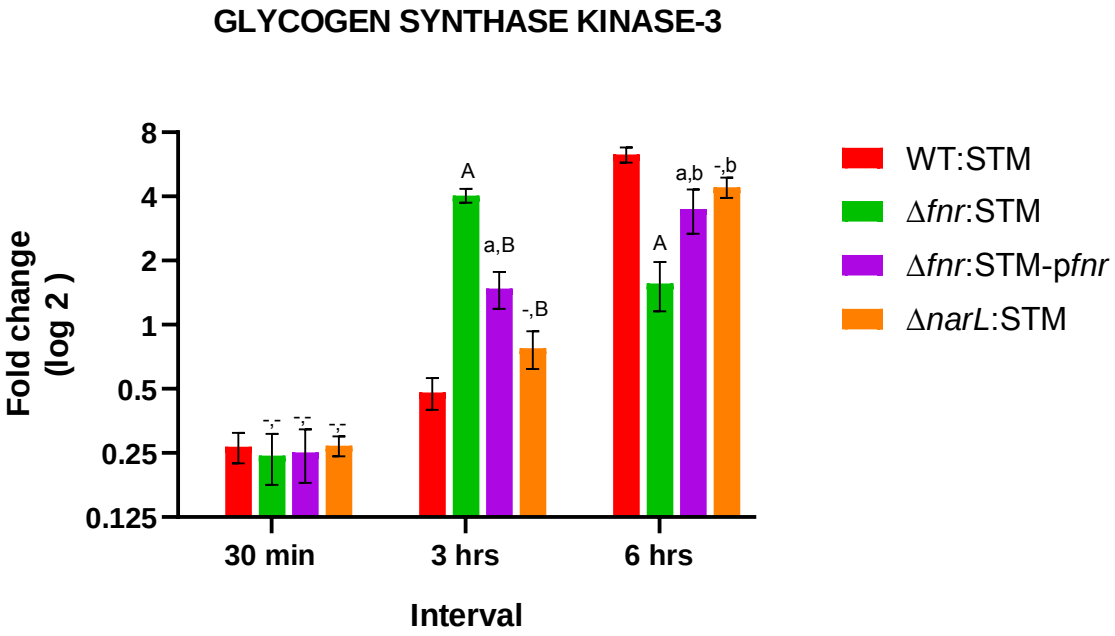



**Fig 4.11: GSK -3 expression in *Salmonella* infected chicken monocyte-derived macrophages.** The graph indicates GSK-3 expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.8: Real time PCR -CS**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 0.6486±0.1565 | 3.957±0.6005 | 1.494±0.2339 |
| Δ*fnr*: STM | 0.7151±0.1512 | 1.109±0.1773 | 4.303±0.5898 |
| Δ*fnr*: STM- p*fnr* | 0.6851±0.2835 | 1.499±0.4237 | 1.462±0.3279 |
| Δ*narL*: STM | 0.6646±0.0794 | 1.924±0.243 | 1.271±0.2042 |

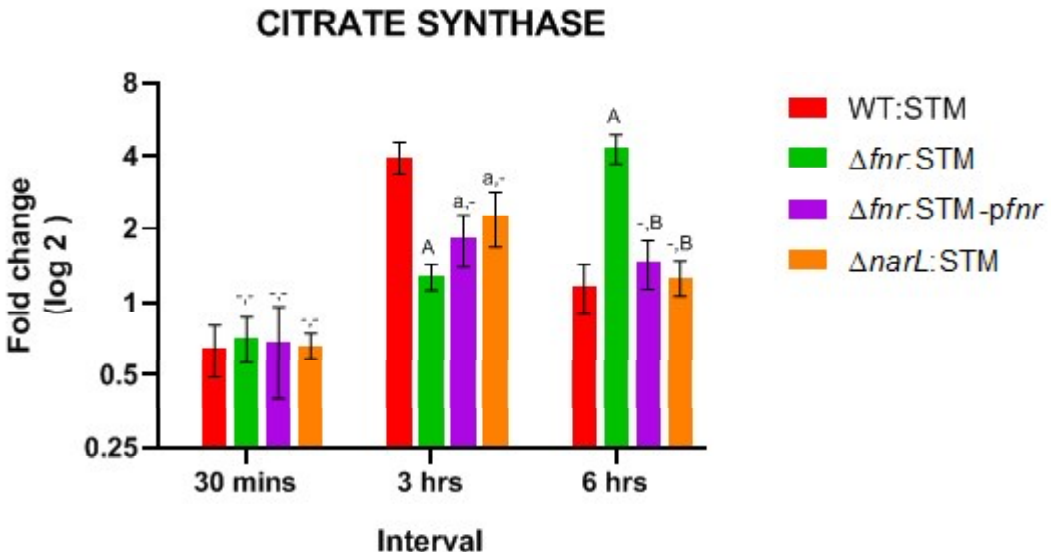



**Fig 4.12: CS expression in *Salmonella* infected chicken monocyte-derived macrophages**. The graph indicates CS expression in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference,a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

**Table 4.9: ATP Assay**

| Strains | 30 min | 3 hrs | 6 hrs |
|---|---|---|---|
| Wild type | 76±16 | 1652±205.5 | 358.3±1.25 |
| Δ*fnr*: STM | 85.5±17.5 | 308.8±8.750 | 1220±191 |
| Δ*fnr*: STM- p*fnr* | 69±21 | 923±55 | 449.5±145.5 |
| Δ*narL*: STM | 74.5±22.5 | 1167±68 | 380.8±66.25 |

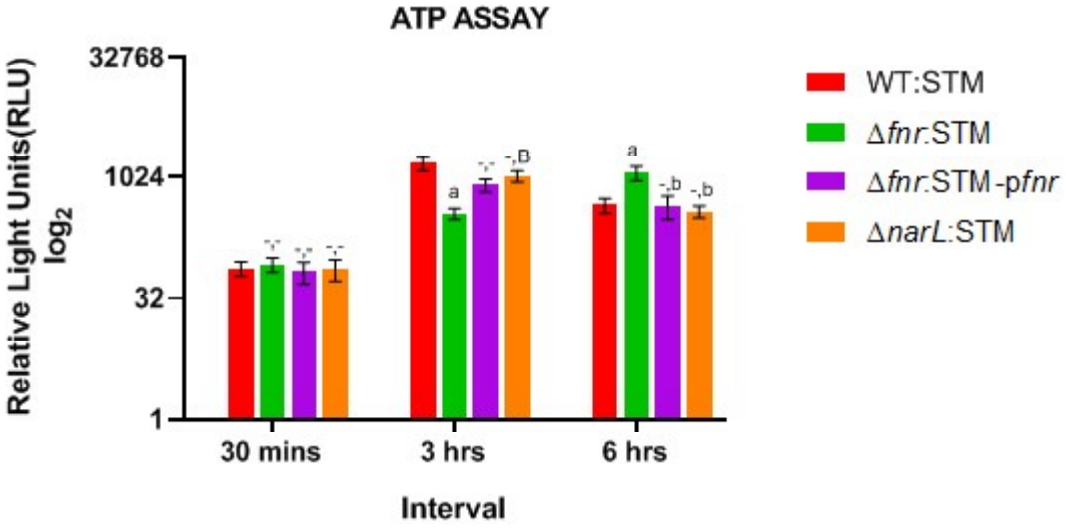



Fig 4.13: **ATP assay in *Salmonella* infected chicken monocyte-derived macrophages**. The graph indicates ATP level in ChMoM after 30min, 3hrs, and 6hrs of infection with different STM strains. Data is representative of the mean± SEM (n=3). Letters on histograms denote statistically significant difference, a/A: Difference between each individual mutant as compared to WT analyzed by unpaired t- test, b/B: Difference between three mutants analyzed by one-way ANOVA (small letters denote P<0.05, capital letters denote P<0.01). '– ' indicate no significant difference.

# Discussion

# 5 DISCUSSION

*Salmonella* Typhimurium is a prevalent serotype of *Salmonella* that poses a significant threat to both domestic animals, humans and poultry. This gram-negative bacterium belongs to the *Enterobacteriaceae* family and is responsible for causing a range of diseases in various host species. Within the *Salmonella* genus, over 2,500 different serotypes or serovars have been identified, with *Salmonella* Typhimurium and *Salmonella* Enteritidis being the two most prevalent serotypes in human infections. These serotypes are often transmitted from animals to humans through contaminated food, including eggs, meat, poultry, and milk. Additionally, contact with infected animals, including pets, and consuming infected animal's meat can also lead to human infections, even if the animals do not show signs of disease. *Salmonella* Typhimurium infections in humans typically result in acute onset of symptoms, including fever, abdominal pain, diarrhoea, nausea, and sometimes vomiting.

Controlling *Salmonella* Typhimurium in domestic animals and poultry requires a multi-faceted approach that encompasses various stages of the food chain. Prevention measures include implementing strict biosecurity measures in animal production facilities, such as proper hygiene practices, all-in-all-out production systems, and thorough cleaning and disinfection between batches. Heat-treating animal feed to eliminate *Salmonella* bacteria is also crucial in preventing contamination.

*Salmonella* is a common bacterium that causes gastroenteritis and systemic disease in mammals, including humans. During infection, *Salmonella* can invade macrophages and replicate within them. The ability of *Salmonella* to survive and replicate within macrophages is crucial

for its pathogenicity. *Salmonella* for *in vivo* proliferation requires synthesis of biomass components such as proteins, carbohydrates, lipids, and nucleic acids from small molecule precursors. *Salmonella* can obtain these precursors from internal storage such as glycogen or lipids (for a limited number of replication) or directly from the host micro environment (e.g., amino acids) and/or synthesize them from a few basic host carbons (such as acetate, glycerol, and glucose), nitrogen (e.g., ammonium), sulfur (e.g., sulfate), and phosphorus (e.g., phosphate) sources (Bumann and Schothorst., 2017).

Glycolysis plays a crucial role in the growth and survival of *Salmonella* within macrophages. Studies have shown that *Salmonella* mutants cannot catabolize glucose due to the deletion of genes involved in glucose transport and metabolism and also offer reduced replication within macrophages. These findings indicate that glucose and its utilization through glycolysis are essential for *Salmonella* replication within macrophages.

In infected macrophages, there is an increase in glycolysis, leading to the accumulation of lactate. The shift towards glycolysis and lactate production provides a source of energy for *Salmonella* and may contribute to the survival and replication of the bacteria within macrophages. In return, macrophages produce immune effector molecules, such as reactive oxygen species and cytokines, which play important role in preventing bacterial infections. The role of glycolysis in *Salmonella* infection of macrophages is crucial for the pathogenesis of the bacteria. Glycolysis provides a source of energy for *Salmonella* and is required for its replication and survival within macrophages. *Salmonella* drives metabolic changes in macrophages to create an environment favorable for its replication. The bacterium activates host pathways, such as the mTOR pathway, to enhance glycolysis and lipid metabolism. Overall, the study of glycolysis in *Salmonella* infection highlights the complexity of host-pathogen interactions and the importance of metabolic reprogramming in infectious diseases.

During pathogenesis, *Salmonella* Typhimurium encounters less oxigen tension in the intestine and switches over from aerobic to anaerobic metabolism which is mediated by various anaerobic regulators and their operons (Sahoo et al.,2023). Nitrate anaerobic regulator L (NarL), Fumarate nitrate reductase regulator (Fnr), nitrate reductase (NarG), and secretion

system regulator (ssrB) are important regulators that control the pathogenesis of *Salmonella* in anaerobic conditions (Godfrey *et al.*, 2017). NarL is one important component of NarX/ NarL dual regulatory complexes that regulate the nitrate/nitrite redox reaction and modulate the anaerobic respiration (Teixido *et al.,* 2010). *fnr* is a well-known global anaerobic regulator and contributes to the virulence of many bacterial pathogens that encounter changes in $O_2$ availability. *fnr* is also required for *S.* Typhimurium systemic infection, as the *fnr* mutant was completely attenuated in both orally and i.p.-infected mice and the lack of *fnr* resulted in a dramatic reduction in the ability of *S.* Typhimurium to replicate in poultry macrophages (Jiang *et al.,* 2023).

2-NBDG, a fluorescent analog of glucose is a substrate for glucose transporters. 2-NBDG uptake in bacteria is mediated by a mannose or a glucose/mannose transporter system (Sinclair *et al.,* 2020). Here in this study, we found that 2-NBDG uptake was upregulated by macrophages in *fnr* knockout strain at 3 hrs post-infection (P.I.) and then it was downregulated after 6 hrs P.I. Wild type, *narL* knockout, and *fnr* complemented strains were up-regulated at 6 hrs P.I. Gutierrez *et al*. (2021) reported that uptake of fluorescent glucose analog 2-NBDG immediately upon infection (0.5-2h) was increased followed by a steady decline (4 h) during *S.* Typhimurium SL1344 infection in BMDMs. This is in contrast to our findings that increased 2-NBDG uptake was seen in chicken primary macrophages at 0.5 hrs during STM 5591 infection. This may be due to different salmonella strains and macrophages.

Freemerman *et al.* (2014) reported that GLUT1 is the primary glucose transporter in murine BMDM and RAW264.7 macrophages and that proinflammatory activation of macrophages either through M1 polarization of BMDM or LPS stimulation of RAW increased GLUT1 expression. In this study, GLUT -1 expression was upregulated after 3 hrs of infection compared to 30 mins. *fnr* knockout strain, it was more upregulated at 3 hrs P.I and then downregulated after 6 hrs P.I. WT: STM, *Δfnr*: STM- *pfnr,* and Δ*narL*: STM strains are upregulated at 6 hrs P.I. Gutierrez *et al.* (2021) reported that the expression of GLUT1, the main glucose transporter in macrophages, was significantly increased early upon infection (0.5-2h) followed by a decline during the later phase of infection (4h).

Jiang *et al.* (2021) reported that the mRNA levels of key glycolytic enzymes, including hexokinase (Hk), 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 3 (Pfkfb3), and dominant glucose transporter Slc2a1 were significantly upregulated in infected peritoneal macrophages (PMs) relative to untreated PMs, which are consistent with the observed increase in the levels of glycolysis and glucose uptake of STM-infected PMs. Na *et al* (2016) reported that GM-CSF increases macrophage glycolytic capacity in response to LPS activation at 12 hpi in primary BMDMs.

We found that HK 2 expression was upregulated in the *fnr* knockout strain at 3 hpi and expression was reduced after 6hpi opposite to that of other strains used in this study. Wang *et al.* (2021) reported that HK3 was up-regulated in WT-infected samples at 5 hpi in mice models. Gutierrez et al. (2021) reported that the expression of HK2 was significantly increased early upon infection (0.5-2h) followed by a decline during the later phase of infection (4h).

We found that ENO1 expression was upregulated in the *fnr* knockout strain at 3 hpi and expression was reduced after 6hpi opposite to that of other strains used in this study. Gutierrez et al. (2021) reported that the expression of enolase was significantly increased early upon infection (0.5-2h) followed by a decline during the later phase of infection (4h). Enolase is generally known as the glycolytic pathway enzyme present in the cytoplasm of eukaryotic cells and some microorganisms (Serek *et al.,* 2020).

We found that PTEN expression was upregulated in the *fnr* knockout strain at 3 hpi and expression was reduced after 6hpi opposite to that of other strains used in this study. Gutierrez et al. (2021) reported that the expression of PTEN was significantly increased early upon infection (0.5-2h) followed by a decline during the later phase of infection (4h). Glover *et al.* (2022) reported that PTEN can promote phagocytosis of bacteria by macrophages during listeriosis.

We found that PDK-1 expression was upregulated in the *fnr* knockout strain at 3 hpi and expression was reduced after 6hpi opposite to that of other strains used in this study. PDK1 phosphorylates components of the PDH complex in the mitochondria. This inhibitory

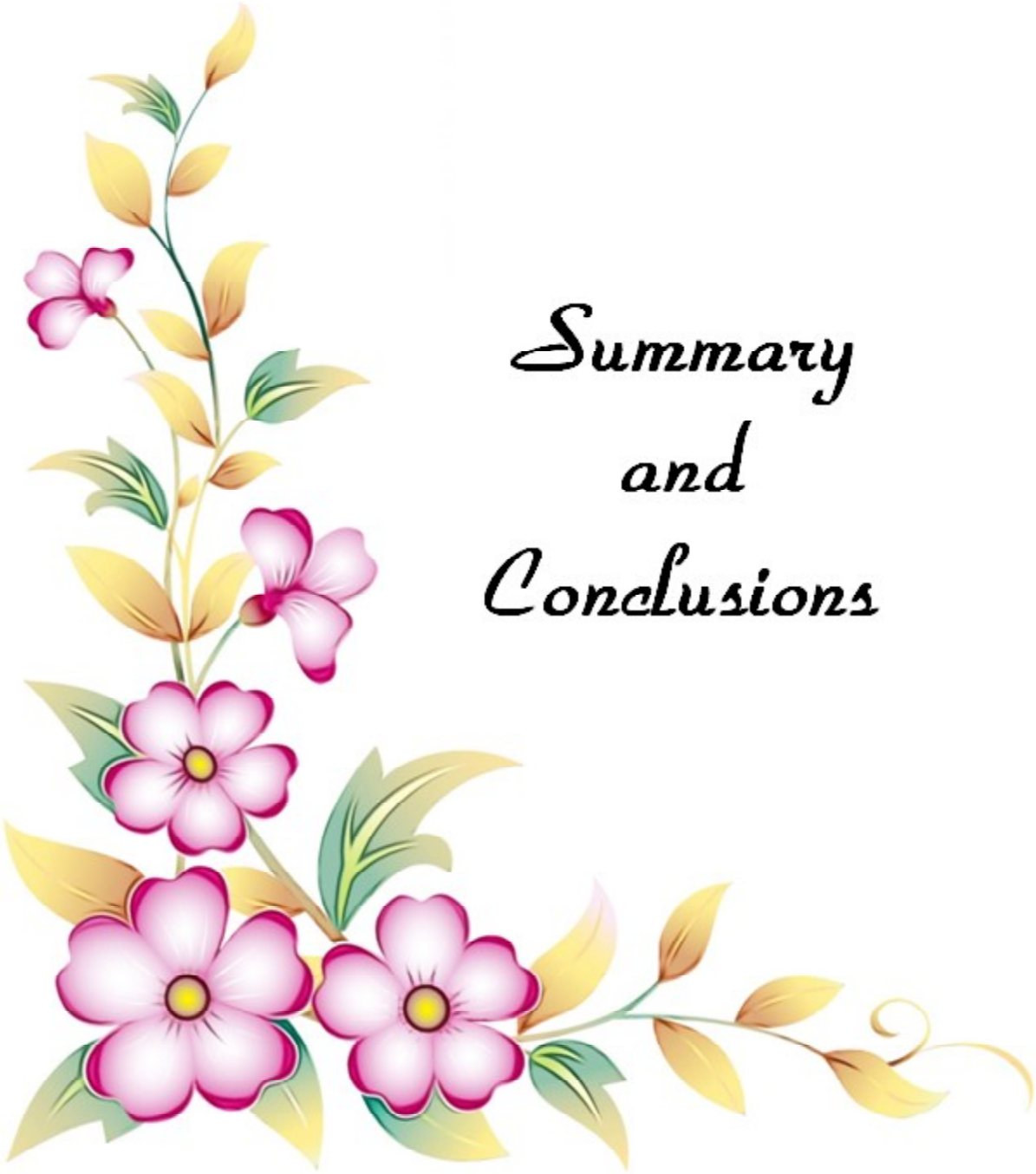
phosphorylation suppresses pyruvate conversion to acetyl-CoA, thereby augmenting lactate production (Tan *et al.,* 2015). He also reported that PDK1 is required for enhanced glycolysis in LPS-treated macrophages, thereby promoting the full activation of M1 macrophages.

We found that GSK-3 expression was upregulated in the *fnr* knockout strain at 3 hpi and expression was reduced after 6hpi opposite to that of other strains used in this study. Gutierrez *et al*. (2021) reported that the expression of phosphorylated GSK-3 was significantly reduced early upon STM infection. Kogut *et al.* (2014) reported a significant increase in GSK 3β transcription within 30 min that was maintained at 60 min in *S.* Enteritidis-infected heterophils. Glycogen synthase kinase 3β (GSK3β) plays a fundamental role during the inflammatory response induced by bacteria. Depending on the pathogen and its virulence factors, the type of cell, and probably the context in which the interaction between host cells and bacteria takes place, GSK3β may promote or inhibit inflammation (Cortés-Vieyra *et al.,* 2012). GSK-3 is a multifunctional serine/threonine kinase that has been recently shown to regulate elements of both innate and acquired immune responses (Kogut *et al.,* 2014). Inhibition of GSK-3β activity switched the response after LPS stimulation from proinflammatory, IL-12 releasing to anti-inflammatory, IL-10 producing. This anti-inflammatory response to TLR stimulation in monocytes demonstrated that GSK-3β could regulate the inflammatory responses (Martin *et al.,* 2005). Duan *et al.* (2007) revealed that glycogen synthase kinase 3β (GSK-3β) kinase activity was increased in response to WT *Salmonella* in mouse epithelial cells.

We found that CS expression was downregulated in the *fnr* knockout strain at 3 hpi and expression was upregulated after 6hpi opposite to that of other strains used in this study. In macrophages, citrate accumulates in the cytosol as a result of LPS-induced expression of the mitochondrial citrate carrier SLC25A1 which is responsible for citrate efflux from mitochondria (Infantino *et al.,* 2011). citrate is redirected to the cytosol where it is metabolized into acetyl-CoA and oxaloacetate, providing a source of nicotinamide adenine dinucleotide phosphate (NADPH) for ROS and NO production (Ramond *et al.,* 2019).

We found that ATP level was less in the *fnr* knockout strain at 3 hpi and it was increased after 6hpi opposite to that of other strains used in this study. Although glycolysis produces ATP less efficiently than oxidative phosphorylation, the rate of ATP production is faster. Cells may

have lower oxygen levels in an infectious/ inflammatory environment, so increased glycolytic metabolism may be a self-protective mechanism for cells to maintain cellular ATP levels and cell viability during a robust immune response (Wang *et al.*, 2021). Yu *et al*. (2020) reported that glycolysis was lower in the WT and sseK3-complemented groups than in the ΔsseK3 mutant group. Moreover, the ATP levels of macrophages in the mock group were significantly higher than those in the infection's groups at 4 h, 6 h, and 8h, which suggested that aerobic metabolism was predominant in the mock group since less ATP is produced by glycolysis than by aerobic oxidation. Marzan *et al.* (2011) reported that *fnr* gene knockout may have caused the cra gene to be downregulated and thus activated the glycolysis genes, and eventually caused upregulation of the specific glucose uptake rate under oxygen limitation in *E. coli* infection. He also reported that *fnr* mutant caused less of a growth rate, which caused less biomass concentration which in turn caused glucose concentration to be increased. In this study, we found that *fnr* knockout has increased glycolysis earlier in macrophages compared to other strains used.



# Summary and Conclusions

# 6 SUMMARY AND CONCLUSIONS

Many studies have documented how various bacteria and viruses manipulate cellular metabolism to facilitate their infection (Shi et al., 2015). They actively modify the host cell's metabolic pathways to redirect glycolysis and mitochondrial TCA cycle intermediates towards producing the essential components such as lipid droplets, fatty acids, amino acids, and nucleotides required for the pathogen's survival and nutritional needs (Richardson *et al.,* 2015). Meanwhile, the host cell's metabolic responses and pathways play a crucial role in regulating the extent and duration of both innate and adaptive immune responses, ultimately influencing the progression of infection (Ganesan and Chawla, 2014). In many cases, this leads to a decrease in the TCA cycle and an increase in aerobic glycolysis, resulting in the conversion of pyruvate to lactate in the cytoplasm, producing two ATP molecules from each glucose molecule (Tiku *et al.*, 2020).

In the case of M. tuberculosis-infected macrophages, there is a noticeable reduction in the TCA cycle and a shift towards aerobic glycolysis. Inhibiting this metabolic shift leads to reduced cytokine production and promotes the growth of intracellular bacteria (Gleeson *et al.,* 2016). Studies have also shown that disrupting the TCA cycle enhances the survival of *Salmonella* Typhimurium within murine macrophages, both in their resting and activated states (Bowden *et al.,* 2010).

*Salmonella enterica* comprises facultative gram-negative bacteria. Recent research suggests that *Salmonella* can manipulate host cell metabolism to increase energy resources and metabolites available for intracellular replication. He *et al.* (2023) discovered that S.

Enteritidis infection caused significant phosphorylation changes in key glycolytic pathway proteins in chicken macrophage HD-11 cells, indicating altered glycolysis due to *Salmonella* infection. The infection reduced glycolysis and enhanced oxidative phosphorylation (OXPHOS) in chicken macrophages. Different *Salmonella* strains had varying effects on macrophage polarization and glycolysis. Among the tested strains, *S.* Enteritidis was the most effective at downregulating glycolysis and promoting M2 polarization, while *S.* Senftenberg did not significantly alter glycolysis and may promote M1 polarization (He *et al.,* 2023). Remarkably, *Salmonella* strains could survive within macrophages despite these cells' ability to produce bactericidal substances like reactive oxygen species (ROS), nitric oxide (NO), lysozyme, and proteolytic enzymes when exposed to *Salmonella* (Braukmann *et al.,* 2015). The strain-specific impact on host cell glycolysis likely contributes to Salmonella's virulence and fitness for intracellular survival within macrophages (He *et al.*, 2023).

Fnr is a global anaerobic regulator that governs over three hundred genes, including those related to *Salmonella* Typhimurium's metabolism and virulence. It upregulates genes associated with anaerobic metabolism while repressing genes encoding enzymes involved in aerobic pathways such as electron transport, oxidative phosphorylation, and certain tricarboxylic acid cycle enzymes (Fink *et al.,* 2007). Fnr also controls ethanolamine and propanediol utilization and activates pyruvate metabolism, glycerol metabolism, and glycerophospholipid metabolism, indicating adaptation to anaerobic environments (Behera *et al.,* 2020). Furthermore, Fnr influences the DNA binding protein Fis, a global regulator of *Salmonella* Typhimurium's virulence. Nikhil *et al.* (2022) reported that deletion mutants of the fnr gene were outcompeted by wild strains in nutrient-deprived media. These mutants exhibited reduced motility, biofilm formation, defective intracellular survival, and replication within macrophages, along with attenuation in vivo. They also showed increased adhesion and invasion of epithelial cells both in vivo and in vitro. Recoding the fnr gene in STM resulted in significantly reduced bacterial pathogenicity. Swagathika *et al.* (2019) constructed a mutated fnr promoter that exhibited a 70-80% reduction in strength compared to the wild fnr promoter. This mutated promoter could potentially replace the wild fnr promoter in the *Salmonella* Typhimurium genome, reducing FNR protein expression and thereby attenuating the organism's pathogenicity.

In our current study, we investigated the role of *fnr* in regulating glycolysis in *Salmonella* Typhimurium-infected macrophages. In a glucose uptake assay, we observed that the fnr mutant strain outcompeted wild, *fnr*-complemented, and *narL* mutant strains after 3 hours, and vice versa after 6 hours. Real-time analysis revealed upregulation of GLUT1, HK2, ENO1, PDK-1, GSK3, and PTEN in the *fnr* mutant strain at 3 hours, followed by downregulation at 6 hours compared to the wild, *fnr*-complemented, and *narL* mutant strains. Additionally, ATP levels were lower in the fnr mutant strain at 3 hours but higher at 6 hours compared to the other strains.

**CONCLUSION**

- The fnr mutant strain exhibited increased glycolysis in chicken macrophages during the early stage of infection compared to the wild, *fnr*-complemented, and *narL* mutant strains.
- During the later stage of infection, the *fnr* mutant strain displayed decreased glycolysis compared to the other strains.
- The wild-type strain showed an increase in glycolysis during the later stage of infection.
- The *narL* mutant strain had a glycolytic impact similar to the wild-type strain.


Mini Abstract

# 7 MINI ABSTRACT

*Salmonella* Typhimurium, a prominent serotype of the *Salmonella genus*, poses a substantial threat to both domestic animals and poultry, with significant implications for human health. This discussion explores the intricate interplay between *Salmonella* Typhimurium and macrophages, shedding light on the critical role of glycolysis in *Salmonella* pathogenesis within these immune cells. The study begins by emphasizing the importance of controlling *Salmonella Typhimurium* in domestic animals and poultry through stringent biosecurity measures and proper hygiene practices. Heat treatment of animal feed is also crucial to prevent contamination at the source. Within macrophages, Salmonella's ability to adapt and thrive is examined, with a focus on glycolysis as a vital component of its survival strategy. Glycolysis is shown to be essential for *Salmonella* replication within macrophages, with alterations in macrophage metabolism impacting the production of immune effector molecules. Furthermore, the discussion delves into the metabolic shift that *Salmonella* undergoes upon encountering the intestine, transitioning from aerobic to anaerobic metabolism. Key anaerobic regulators, such as NarL, fnr, and others, are highlighted for their roles in modulating pathogenesis under anaerobic conditions. The study also presents findings on glucose uptake and GLUT1 in infected macrophages, shedding light on their dynamic regulation during *Salmonella* infection. The research provides valuable insights into the expression of glycolytic enzymes, including hexokinase (HK2), enolase (ENO1), PTEN, PDK-1, and GSK-3, in the context of *Salmonella infection*. In our study, we investigated the influence of Fnr on glycolysis in *Salmonella Typhimurium*-infected macrophages. Glucose uptake assays showed that the fnr mutant strain displayed differential competition dynamics compared to wild-type and complemented strains. Real-time analysis revealed distinct expression patterns of glycolytic genes and related factors, suggesting fnr's role in modulating glycolysis during infection. ATP levels also varied with Fnr status, further highlighting its impact on metabolic regulation. Overall, this discussion underscores the complexity of host-pathogen interactions, emphasizing the pivotal role of metabolic reprogramming in *Salmonella Typhimurium* pathogenesis within macrophages. The findings contribute to a deeper understanding of bacterial survival strategies and open new avenues for potential therapeutic interventions in combating *Salmonella* infections.

# लघु सारांश

# 8 लघु सारांश

साल्मोनेल्ला टाइफिमुरियम, साल्मोनेल्ला जीनस का एक प्रमुख सीरोटाइप, महत्वपूर्ण प्रभाव के साथ, घरेलू पशुओं और मुर्गीपालन दोनों एवं मानव स्वास्थ्य के लिए एक बड़ा खतरा पैदा करता है। यह कार्य साल्मोनेल्ला टाइफिम्यूरियम और मैक्रोफेज के बीच जटिल परस्पर क्रिया की पड़ताल करती है, जो इन प्रतिरक्षा कोशिकाओं के भीतर साल्मोनेल्ला रोगजनन में ग्लाइकोलाइसिस की महत्वपूर्ण भूमिका पर प्रकाश डालती है। अध्ययन की शुरूआत कड़े जैव सुरक्षा उपायों और उचित स्वच्छता प्रथाओं के माध्यम से घरेलू पशुओं और पोल्ट्री में साल्मोनेल्ला टाइफिम्यूरियम को नियंत्रित करने के महत्व पर जोर देकर की जाती है। स्रोत को दूषित होने से रोकने के लिए पशु चारे का ताप उपचार भी महत्वपूर्ण है। मैक्रोफेज के भीतर, साल्मोनेल्ला की अनुकूलन और पनपने की क्षमता की जांच की जाती है, जिसमें इसकी जीवित रहने की रणनीति के एक महत्वपूर्ण घटक के रूप में ग्लाइकोलाइसिस पर ध्यान केंद्रित किया जाता है। मैक्रोफेज के भीतर साल्मोनेल्ला प्रतिकृति के लिए ग्लाइकोलाइसिस को आवश्यक दिखाया गया है, मैक्रोफेज चयापचय में परिवर्तन से प्रतिरक्षा प्रभावक अणुओं के उत्पादन पर असर पड़ता है। इसके अलावा, यह कार्य उस चयापचय बदलाव पर भी चर्चा करती है जो साल्मोनेल्ला आंत से मिलने पर गुजरता है, एरोबिक से एनारोबिक चयापचय में परिवर्तित होता है। प्रमुख अवायवीय नियामक, जैसे कि एनएआरएल, एफएनआर और अन्य को अवायवीय स्थितियों के तहत रोगजनन को संशोधित करने में उनकी भूमिकाओं के लिए उजागर किया गया है। अध्ययन संक्रमित मैक्रोफेज में ग्लूकोज अवशोष्ज्ञण और GLUT1 पर निष्कर्ष भी प्रस्तुत करता है, जो साल्मोनेल्ला संक्रमण के दौरान उनके गतिशील विनियमन पर प्रकाश डालता है। यह शोध साल्मोनेल्ला संक्रमण के संदर्भ में हेक्सोकाइनेज (HK), एनोलेज (ENO1), PTEN, PDK-1 और GSK-3 सहित ग्लाइकोलाइटिक एंजाइमों की अभिव्यक्ति में मूल्यवान अंतदृष्टि प्रदान करता है। हमारे अध्ययन में, हमने साल्मोनेल्ला टाइफिम्यूरियम–संक्रमित मैक्रोफेज में ग्लाइकोलाइसिस पर एफएनआर के प्रभाव की जांच की। ग्लूकोज ग्रहण परीक्षण से पता चला कि एफएनआर उत्परिवर्ती तनाव ने जंगली–प्रकार और पूरक उपभेदों की तुलना में अंतर प्रतिस्पर्धा गतिशीलता प्रदर्शित की। वास्तविक समय के विश्लेषण से ग्लाइकोलाइटिक जीन और संबंधित कारकों के विशिष्ट अभिव्यक्ति पैटर्न का पता चला, जो संक्रमण के दौरान ग्लाइकोलाइसिस को नियंत्रित करने में एफएनआर की भूमिका का सुझाव देता है। एटीपी स्तर भी एफएनआर स्थिति के साथ भिन्न होता है, जो चयापचय विनियमन पर इसके प्रभाव को और अधिक उजागर करता है। कुल मिलाकर, यह कार्य मेजबान–रोगजनक इंटरैक्शन की जटिलता रेखांकित करती है, मैक्रोफेज के भीतर साल्मानेल्ला टाइफिम्यूरियम रोगजनन में चयापचय रिप्रोग्रामिंग की महत्वपूर्ण भूमिका पर जोर देती है। निष्कर्ष जीवाणु अस्तित्व रणनीतियों की गहरी समझ में योगदान करते हैं और साल्मोनेल्ला संक्रमण से निपटने में संभावित चिकित्सीय हस्तक्षेप के लिए नए रास्ते खोलते हैं।

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# Appendix

# APPENDIX

## I Solutions for agarose gel electrophoresis

**0.5 M EDTA (pH 8.0)**

| | |
|---|---|
| EDTA (Disodium dihydrate salt) | 37.4 g |
| Double distilled water | 200 ml |

The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 200 ml.

**TAE buffer (50 X)**

| | |
|---|---|
| Tris base | 60.50 g |
| Glacial acetic acid | 14.25 ml |
| 0.5 M EDTA solution (pH 8.0) | 25.0 ml |

The final volume was made up to 250 ml with double distilled water

**Ethidium bromide stock (10 mg/ml)**

| | |
|---|---|
| Ethidium bromide | 10 mg |
| Double distilled water | 1 ml |

The ethidium bromide was dissolved in water and stored at 4°C in amber-colored tubes.

**Gel Loading Dye (6X)**

| | |
|---|---|
| Tris-HCI (pH 7.6) | 10 mM |
| Bromophenol blue | 0.03% |
| Xylene cyanol FF | 0.03% |
| Glycerol | 60% |
| EDTA | 60 mM |

## II Antibiotic stocks

**Kanamycin stock (50 mg / ml)**

| | |
|---|---|
| Kanamycin | 50 mg |
| Autoclaved distilled water | 1 ml |

Finally, the solution was sterilized by a 0.22 μM filter and stored at -20 °C.

## III Media for bacteriological culture

**Luria-Bertani broth**

Add 25 grams of powder to distilled or deionized water.

Bring volume to 1 L and mix thoroughly.

Gently heat and bring to boiling.

Autoclave at 15 psi pressure at 121°C for 15 minutes.

**Luria-Bertani agar**

Add 40 grams of powder to distilled or deionized water.
Bring volume to 1 L and mix thoroughly.
Gently heat and bring to boiling.
Autoclave at 15 psi pressure at 121°C for 15 minutes.

**Hektoen Enteric Agar**

Suspend 76.67 grams in 1L distilled water.
Heat to boiling to dissolve the medium completely.
Do not autoclave.

## IV Media for macrophage culture

**RPMI 1640 cell culture media (Media 1)**

| | |
|---|---|
| RPMI 1640 w/ Sodium bicarbonate w/o L-Glutamine | |
| Fetal bovine serum | 10 % |
| Penstrep solution (100 X) | 1% |

The above ingredients were mixed and filtered using a 0.45 µm filter. Stored at 4°C

**RPMI 1640 cell culture media (Media 2)**

| | |
|---|---|
| RPMI 1640 w/ Sodium bicarbonate w/o L-Glutamine | |
| Fetal bovine serum | 10 % |
| LPS (1 mg/ml) | 0.5 ng/ml |

The above ingredients were mixed and filtered using a 0.45 µm filter. Stored at 4°C

**RPMI 1640 cell culture media (Media 3)**

| | |
|---|---|
| RPMI 1640 w/ Sodium bicarbonate w/o L-Glutamine | |
| Fetal bovine serum | 10 % |

The above ingredients were mixed and filtered using a 0. 45 µm filter. Stored at 4°C

**RPMI 1640 cell culture media (For 2-NBDG)**

| | |
|---|---|
| RPMI 1640 w/ Sodium bicarbonate w/o L-Glutamine w/o glucose | |
| Fetal bovine serum | 10 % |

The above ingredients were mixed and filtered using a 0. 45 µm filter. Stored at 4°C

## V Buffers

**1X PBS solution**

| | |
|---|---|
| 10 X PBS cell culture grade | 10 ml |
| Distilled water/Deionized water | 90 ml |

Autoclave at 15 psi pressure at 121°C for 15 minutes.

**Lysis buffer**

| | |
|---|---|
| Triton X-100 | 0.1% |
| SDS | 0.01% |

# VITAE

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**Awards / Fellowship**

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- ICAR-AIEEA(PG)- 2021: All India rank-2 (JRF)
- ICAR NET 2023-JRF