Indian Journal of Poultry Science (2024) 59(1): 85–91; Research Article
https://doi.org/10.56093/IJPS.v59i1.154968

Role of fnr and narL genes in the survival of *Salmonella* Typhimurium in chicken macrophages

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(Received on September 01, 2023; accepted for publication on September 18, 2023)

ABSTRACT

Pashupathi, M., Sahoo, P.R., Sreenilav, V., Mishra, R., Priyadarsini, S., Nikhil, K.C., Saxena, M. and Kumar, A. 2024. Role of fnr and narL genes in the survival of Salmonella *Typhimurium in chicken macrophages. Indian Journal of Poultry Science, 59(1): 85-91.*

Salmonella is a gram negative, facultative anaerobic rod shaped intracellular bacteria having zoonotic importance. Salmonellosis is one of the most common foodborne zoonosis in both developed and developing countries. It has been linked to foods such as raw meat, poultry, eggs, and dairy products. Poultry and poultry products are thought to account for half of the total route of *Salmonella* transmission. Eggs are the most commonly involved food commodity in *Salmonella* outbreaks followed by chicken meat. *Salmonella* has the ability to remain in an anaerobic environment required for pathogenesis and pathogenicity in hosts. A range of sensors and several global regulatory proteins like Fnr, ArcA/ArcB dual-complex (aerobic respiratory control) and NarX/NarL systems help the bacteria to survive in the intestine for successful infection. In this study, a double knock out mutant of *fnr* and *narL* was studied for its infection, survival, and metabolic modulation in the chicken macrophages. An increased expression of ATP citrate lyase and Acetyl co-A carboxylase reported in present study indicated the diversion of Tricarboxylic acid cycle to different pathways for avoiding more ATP production and causing induction of cytokines. The other defense activities such as antigen presentation (Major histocompatibility complex class-II), DUOX oxidase, and the cytokines IL-2 and IL-4 were found increased significantly as compare to wild type. Therefore, we are concluding that the deletion of *fnr* and *narL* genes made the *Salmonella* more susceptible to macrophage internal environment and hence easier and faster clearance.

Keywords: Salmonella, Fnr, glucose uptake, ATP citrate lyase, Dual Oxidase

INTRODUCTION

Salmonellosis is one of the most common foodborne zoonosis in both developed and developing countries. It has been linked to foods such as raw meat, poultry, eggs, and dairy products (Kumawat *et al*., 2016). Poultry and poultry products are thought to account for half of the total route of *Salmonella* transmission (Pandey and Saxena, 2015; Balan and Babu, 2017; Neupane *et al.*, 2018; Rose *et al.*, 2020). *Salmonella enterica* subsp. *enterica* serovar Typhimurium (STM) is a gram negative, facultative anaerobic bacteria, which is a rod shaped intracellular pathogen of mammals and other organisms. It causes serious illness in humans and poultry. The diseases are transmitted mainly through contamination of food and water, mainly contaminated chicken meat and eggs (Babu *et al*., 2012; Pandey and Saxena, 2015). Eggs were the most commonly involved food commodity in *Salmonella* outbreaks (28% of total outbreaks), followed by chicken meat (16%), pork (9%), beef (8%), fruit (8%), and turkey (7%) (Jackson *et al*., 2013; Balan and Babu, 2017).

Infected animals, particularly layer hens, serve as reservoirs for both vertical and horizontal route of transmission (Senevirathne *et al.*, 2020; Patil *et al.*, 2022). Singh *et al.* (2019) reports that in summertime fecal shedding of *Salmonella* was higher, indicating that the pathogen's life cycle is accelerated by the environment. Immunization of layer hens has resulted in a reduction in human *Salmonella* infection in several countries, demonstrating the value of immunization in disease prevention. Novel molecular approaches that allow for precise gene deletion, strain attenuation, the identification of novel antigenic compounds, and adjuvant production have aided in the advancement of vaccine research against critical illnesses such as salmonellosis (Senevirathne *et al*., 2020; Tiwari *et al.*, 2021). The present vaccinations have limitations such as short-term immunity. As a result, there is an urgent need for the creation of an effective and long-lasting vaccination to control infection (Pandey and Saxena, 2015**).** To prevent salmonellosis, killed vaccines, subunit vaccines, and live attenuated vaccines are currently available and each has advantages and disadvantages. Live attenuated vaccines have a reputation for inducing robust humoral and cell-mediated protective immune responses in vaccinated hosts (Clark-Curtiss and Curtiss, 2018; Senevirathne *et al*., 2020).

Salmonella is a facultative anaerobe having the ability to remain in an anaerobic environment, which is required for pathogenesis and pathogenicity in hosts. *Salmonella* grown anaerobically has been demonstrated to connect to and enter host cells more successfully than *Salmonella* cultivated aerobically. According to He *et al.* (1999) a gradual decrease in oxygen partial pressure in

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the route of the stomach to rectum (Lee and Falkow, 1990; Li *et al*., 2022; Teixidó, 2010), in order for *Salmonella* Typhimurium (STM) to outlive in the lower parts of the intestine like ileum and colon, its metabolism must switch from an aerobic to anaerobic state by employing various electron acceptors.

A range of sensors and several global regulatory proteins or factors are available for *Salmonella* to detect and respond to perturbations in their surrounding environmental signals, the most important one is Fnr (fumarate and nitrate reductase regulator). The second is the ArcA/ArcB dual-complex (aerobic respiratory control), which consists of a transmembrane protein called ArcB that phosphorylates ArcA, which in turn suppresses several aerobic metabolic pathways, allowing *Salmonella* to continue anaerobic metabolism. Furthermore, it has been demonstrated that Fnr and ArcA regulate around 120 STM genes jointly (Behera *et al*., 2020; Nikhil *et al*., 2023**)**. Fnr and ArcA/B have been shown to regulate *Salmonella* anaerobic metabolism either individually or jointly (Rychlik and Barrow, 2005). Neutrophils and macrophages produce iNOS and ROS both intracellularly and extracellularly in response to the pathogen which ultimately generates peroxynitrite ($OONO^-$), through NO (nitric oxide) production. $OONO^-$ quickly isomerizes to the stable form nitrate (NO_3^-). Two more dual-regulatory complexes, NarX/NarL and NarQ/NarP, activate downstream operons and reduce NO_3^- and nitrite (NO_2^-) in anaerobic circumstances (Teixidó *et al*., 2010).

Hence, it was hypothesized that deletion of *fnr* and *narL* genes would impact the survival of bacteria inside the macrophage of the host and thus may result in faster clearance. Therefore, a double mutant of *Salmonella* Typhimurium (DM) was developed in which both *fnr* and *narL* were knocked-out. This study discusses the effect of presence or absence of these genes on bacterial survival inside the chicken macrophages via modulation of macrophage metabolism like glucose uptake, TCA cycle, innate defense and immune reactions.

MATERIALS AND METHODS

Bacterial Strains Used in This Study and Their Growth Conditions

Strains used in this study are- wild *Salmonella enterica* subspecies *enterica* serovar Typhimurium (WT) and a double mutant (DM) of it lacking *fnr* and *narL* genes, created in our laboratory by using lambda recombinase based homologus recombination method (Datsenko and Wanner, 2000). The bacterial strains were grown in Luria Bertani broth (HiMedia) and Hektoen Enteric Agar (HiMedia) at 37°C, 180rpm for overnight (16 h). For infection studies, the bacterial strains were first grown overnight at 37°C, 180 rpm and then subcultured again for up to 2 h at 37°C with constant shaking of 180 rpm. The bacterial count recorded for WT was 4.65×10^8 and for DM was 3.1×10^8 CFU/ml.

Chicken PBMC Isolation and Macrophage Differentiation

The fresh chicken blood was collected in presence of EDTA (final concentration of 5mmol/L) from adult chickens for PBMC (peripheral blood mononuclear cells) isolation. Blood was tested for absence of STM and then diluted with the equal volume of phosphate buffered saline (PBS, pH 7.4). The diluted blood is then layered over Histopaque 1077 (HiMedia) in a 15 ml tube and centrifuged at a speed of 370 ×g for 20 min. PBMCs layer was collected into a fresh 15 ml tube and washed twice with sterile PBS. Finally, the PBMC pellet was resuspended in RPMI 1640 media (HiMedia) with 10% fetal bovine serum (FBS) (Himedia) and penicillin-streptomycin (1%) and seeded into either 24 well cell culture plates or 25cc cell culture flasks after counting the number of cells. After 12hrs of incubation at 37°C in a 5% CO_2 incubator the media was changed to fresh RPMI 1640 media with 10% FBS and lipopolysaccharide (LPS) (final concentration of 0.5µg/ml) and incubated again for 48hrs for macrophage differentiation.

2-NBDG/Glucose Uptake Assay

A comparative glucose uptake by the macrophage cells with and without infections of WT and DM was measured using the fluorescent glucose analog compound, 2-NBDG (Invitrogen) (Gutiérrez *et al*., 2021). The experiment was performed in a 24 well cell culture plate. After macrophage differentiation, the cells were washed twice with sterile PBS. Infection was given with 100 multiplicity of infection (MOI) along with uninfected control in 500µl of RPMI 1640 media+10% FBS without any antibiotics. Cells were incubated for 2 h and 3 h for further analysis. At the end of each incubation period, the cells were washed with sterile PBS and added with 500µl of RPMI 1640 media without glucose but with 2-NBDG (50µM) for 20min at 37°C. Cells were washed thrice with PBS and added 500µl of lysis buffer (1%Triton X-100 and 0.01% SDS) and incubated for 10min. Finally, the cell lysate was collected, centrifuged and the supernatant was used for measuring the fluorescence in a fluorimeter with excitation and emission wavelengths of 467nm and 538nm, respectively.

RNA Isolation and cDNA synthesis

For RNA isolation, the chicken monocytes derived macrophages were grown in 25cc cell culture flasks and infected with bacterial strains -WT and DM, along with a uninfected control group. Incubation was carried out for 30min and 3hrs at 37°C. The cells were washed with sterile PBS and lysed by using 1 ml of QIAzol reagent (Qiagen) by repeated pipetting and collected for RNA isolation by standard procedure of Sambrook and Russell (2001). RNA pellet was resuspended in RNase free water and the RNA concentration was measured using

NanoDrop spectrophotometer (Thermo Scientific). cDNA synthesis was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for using it in real-time quantitative PCR. 2µg of RNA from each sample was used for preparing cDNA; the reaction mix used was: 2µl of 10X RT buffer, 0.8µl of 25X dNTPs (100mM), 2µl of random hexamers, 1µl Reverse Transcriptase enzyme, 1µl RNase inhibitor and nuclease free water (NFW) for volume make upto 20µl. The reaction conditions were: 25°C for 10min, 37°C for 120min and 85°C for 5min.

Real-Time qPCR (RT-qPCR)

The expression studies of different genes *viz.* ACLY (ATP citrate lyase), Acaca (Acetyl coA carboxylase), MHCII (Major Histocompatibility Complex class-II), DUOX1 (Dual Oxidase-1), IL2, IL4 and Calnexin were conducted by real time-quantitative PCR. The list of primers used for real time primers is given in Table 1.

The RT-qPCR reaction was set by addition of forward and reverse primers 0.5µl each, 10µl of 2× Evagreen qPCR master mix (G-Biosciences), template cDNA 0.5µl and NFW for making up the volume to 20µl. The qPCR conditions were initial denaturation of 94°C for 5min, 40 cycles of 94°C for 15sec, 56°C for 15sec and 72°C for 15sec. The samples were run in triplicates and the expression profile of different genes in different samples obtained as cycles threshold (ct) values. The final relative fold change of individual genes among the samples were calculated using the formula $2^{-\ddot{A}\ddot{A}ct}$

Statistical analysis

Both the glucose uptake assay and relative expression of various genes were analyzed by software GraphPad Prism version 8.0.1 using One-Way ANOVA and student t-test with P value <0.05.

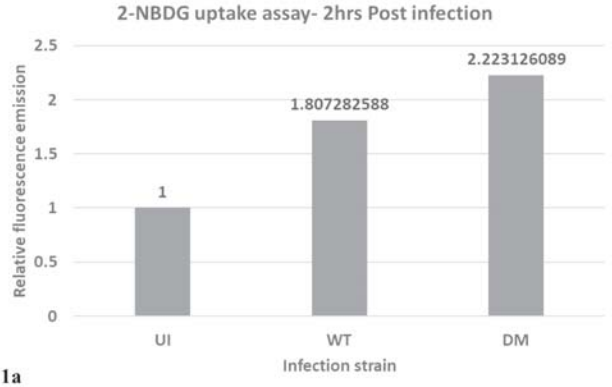
RESULTS AND DISCUSSION

The fluorescent, glucose analog compound, 2-NBDG was used to measure the relative glucose uptake by the chicken macrophages after infection with WT and DM. It was found that glucose uptake increased at 2 h post infection in both WT and DM. But between infected cells, double mutant exhibited an increase to the tune of 2.2 times compared to uninfected control whereas in WT the value was 1.8 times only. At 3 h post-infection, both the infected cells started showing a decrease in glucose uptake by 20-30%. DM was 0.71 times whereas WT was 0.8 times of the uninfected cells (Fig. 1a and 1b).

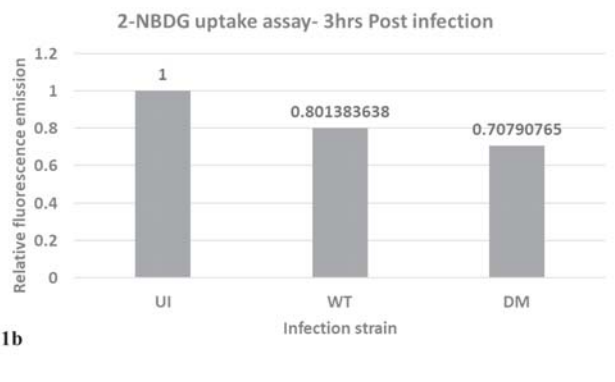
Gutie´rrez *et al.* (2021) reported that the classically activated M1 macrophages, which respond quickly to bacterial infections, get most of their energy from glycolysis. Alternatively activated macrophages (M2), which aid in tissue homeostasis, get their energy mostly from oxidative phosphorylation (OXPHOS) (Rodríguez-Prados *et al.*, 2010; Huang *et al.*, 2014).

Table 1: List of real time-qPCR primers used in this study

Gene	Primers	Product size (bp)
ATP citrate lyase (ACLY)	FP: CATGAGGAAGCACCCAGAGGRP: TGATCAGCTTGCGTGTCAGT	155
acetyl-CoA carboxylase alpha (Acaca)	FP: ATTCCCTTCCCTCCTCCTCCRP: GGTCTTCGAGTGGATTGGCA	173
MHC II	FP: GAACAGCAACGCCGAGATTCRP: CACTTCACCTCGATCTCCGG	210
DUOX1	FP: ATTCTCTGCTATCACCGCCGRP: AGGACTGTGATGAGGTTGCG	186
Interleukin 2	FP: GCAGTGTTACCTGGGAGAAGTRP: ACAGATCTTGCATTCACTTCCG	154
Interleukin 4	FP: CTCTTATGCAAAGCCTCCACAARP: TCTCCATTGAAGTAGTGTTGCC	157
Gallus gallus calnexin (CANX)	FP: GGAATAGAGTGTGGTGGGGCRP: ATGCCGGAAGATGAAGTGCA	150



1a



1b

Fig. 1a and 1b: 2-NBDG/Glucose Uptake Assay with infected (WT and DM) and uninfected samples in X-axis and relative fluorescence emission in Y-axis. Values are significant with P value <0.01.
UI-Uninfected macrophages, WT- macrophages infected with wild type *Salmonella*, DM- macrophages infected with mutant *Salmonella*.

The primary glucose transporter in macrophages, GLUT1, was dramatically enhanced early in infection, followed by a reduction by the bacteria (Gutie´rrez *et al.*, 2021; Freemerman *et al.*, 2014). *Salmonella* Typhimurium infection of macrophages and mice requires glucose and glycolysis pathway for energy (Bowden *et al.*, 2009). The initial increase in glucose was to prepare the cell defense like interleukins and other oxidase enzymes associated with pathogen elimination by the macrophages, later it starts decreasing glucose uptake and leading to starve the bacteria.

The expression profile of metabolic genes - ACLY and Acaca between the WT and DM infected cells in comparison with uninfected control has been shown in the Fig. 2a and 2b, which revealed that at 30min both ACLY and Acaca transcripts decreased almost equivalently in both WT and DM but at 3 hrs of post-infection ACLY and Acaca in DM shown an 1.8 and 1.21 fold upregulation whereas in WT the ACLY upregulated by 1.08 fold but Acaca upregulated by 0.74 times than the uninfected group.

Acetyl-CoA is required for protein acetylation, which is dependent on the activity of ATP citrate lyase (ACLY), which converts cytoplasmic citrate to acetyl-CoA and oxaloacetate. ACLY, like CIC (citrate carrier), is increased in M1 macrophages and affects the expression of several genes via histone acetylation

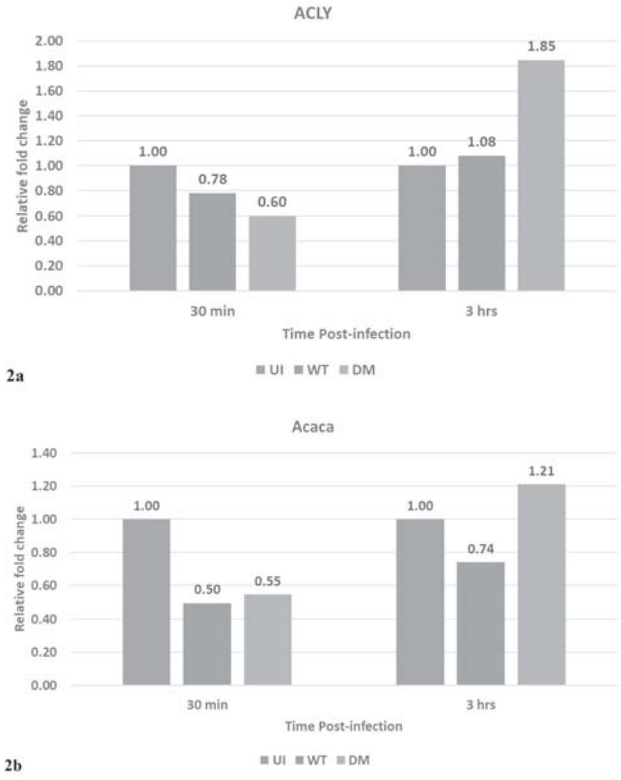


Fig. 2a and 2b: Relative fold change of ACLY and Acaca among infected and uninfected macrophages at 30min and 3h post infection by RT-qPCR. UI-Uninfected macrophages, WT- macrophages infected with wild type *Salmonella*, DM- macrophages infected with mutant *Salmonella.*

(Gutie´rrez *et al.*, 2021; Infantino *et al.*, 2013; Wellen *et al.*, 2009; Choudhary *et al.*, 2014). Many enzymes and proteins are known to be impacted by acetylation, including NF-kB, IL-6, and IL-10, in regulating epigenetic alterations by ACLY in M1 macrophages (Wang *et al.*, 2010; Greene *et al.*, 2004; Wang *et al.*, 2014).

Malate dehydrogenase can convert oxaloacetate to malate. Malate can be converted to pyruvate via the NADPH-producing malic enzyme. The acetyl-CoA will be used for acetylation purpose or for fatty acid synthesis (Iacobazzi *et al.*, 2017; Newsholme *et al.*, 1987; Pietrocola *et al.*, 2015; Viola *et al.*, 2019). Yalcin *et al.*, (2009) reported that, citrate suppresses glycolysis by directly inhibiting phosphofructokinase (PFK) 1 and 2 and indirectly inhibiting pyruvate kinase (PK); it enhances lipid synthesis by activating acetyl-CoA carboxylase (Acaca) and gluconeogenesis by activating fructose-1,6-bisphosphatase (Martin *et al.*, 1962; Gutie´rrez *et al.*, 2021). So the cells are trying to decrease the TCA cycle pathway and diverting it to NADPH production and induction of inflammatory cytokines. In addition, TCA cycle is shifted to fatty acid synthesis by the help of ACLY and Acaca. These changes were found prominent in case of DM at 3hrs of infection which were significantly higher in comparison to WT.

The defense or immune related genes like MHCII and DUOX1 expression is shown in the Fig. 3a and 3b. MHCII gene expression at 30min interval increased slightly in WT (1.17 fold) and a decrease was observed in DM (0.74 fold) whereas after 3hrs interval MHCII in WT started decreasing (1.04 fold), and, in DM it increased to 1.31 folds as compare to uninfected control group. The dual oxidase1 (DUOX1) gene revealed slight increased in both WT and DM at 30min but after 3 hours incubation the increase was 6.38 fold in WT and 56.35 folds in double mutant (DM) as compare to the uninfected control.

M1 macrophages can destroy pathogens and present their antigens to T cells, allowing adaptive responses to begin, they express CD80, MHC-II receptor, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) for the pathogen clearance (Viola et al., 2019). DUOX1 is another oxidase enzyme of nicotinamide adenine dinucleotide phosphate-oxidase family. It is reported that DUOX converts peroxide into HOCl for bacterial clearance (Dahl, *et al.*, 2015). As the DM is more susceptible to be being processed by macrophages the expression of both MHCII and DUOX1 were found increased in DM than in WT at 3hrs of incubation. Specifically, the DUOX1 expression was 56 times in DM and 6 times in WT in comparison with uninfected control group.

The expression of inflammatory cytokines such as IL-2 and IL-4 were also studied by qPCR and it was revealed that both the genes reduced at 30min interval

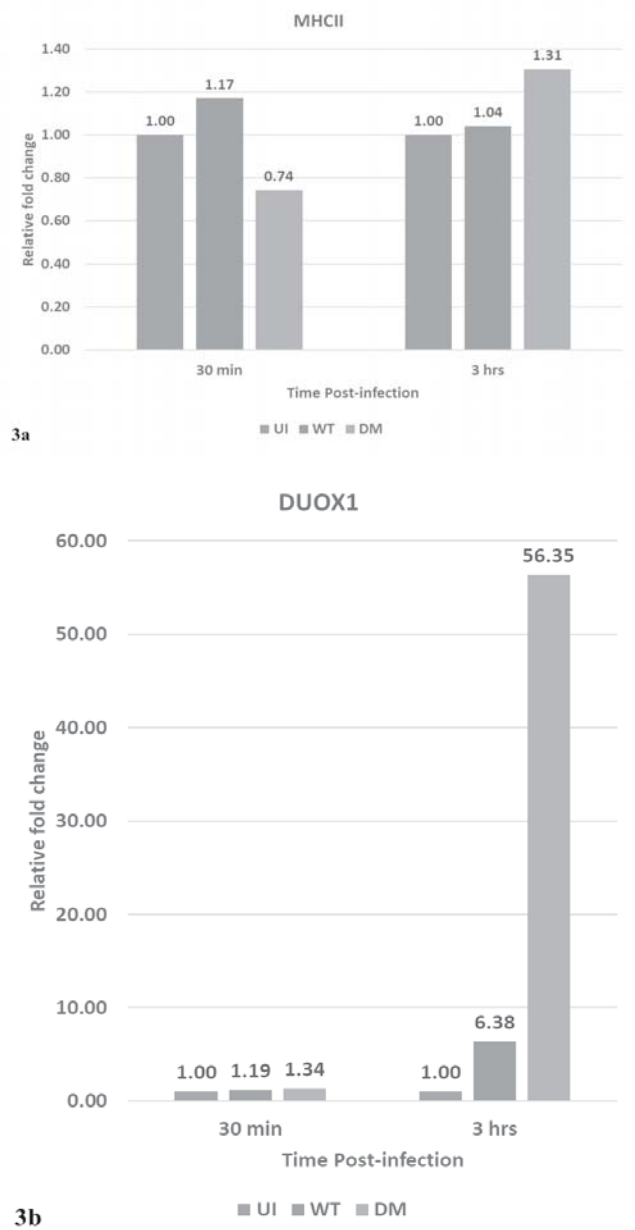


Fig. 3a and 3b: Relative fold change of MHCII and DUOX1 among infected and uninfected macrophages at 30min and 3h post infection by RT-qPCR. UI-Uninfected macrophages, WT- macrophages infected with wild type *Salmonella*, DM- macrophages infected with mutant *Salmonella.*

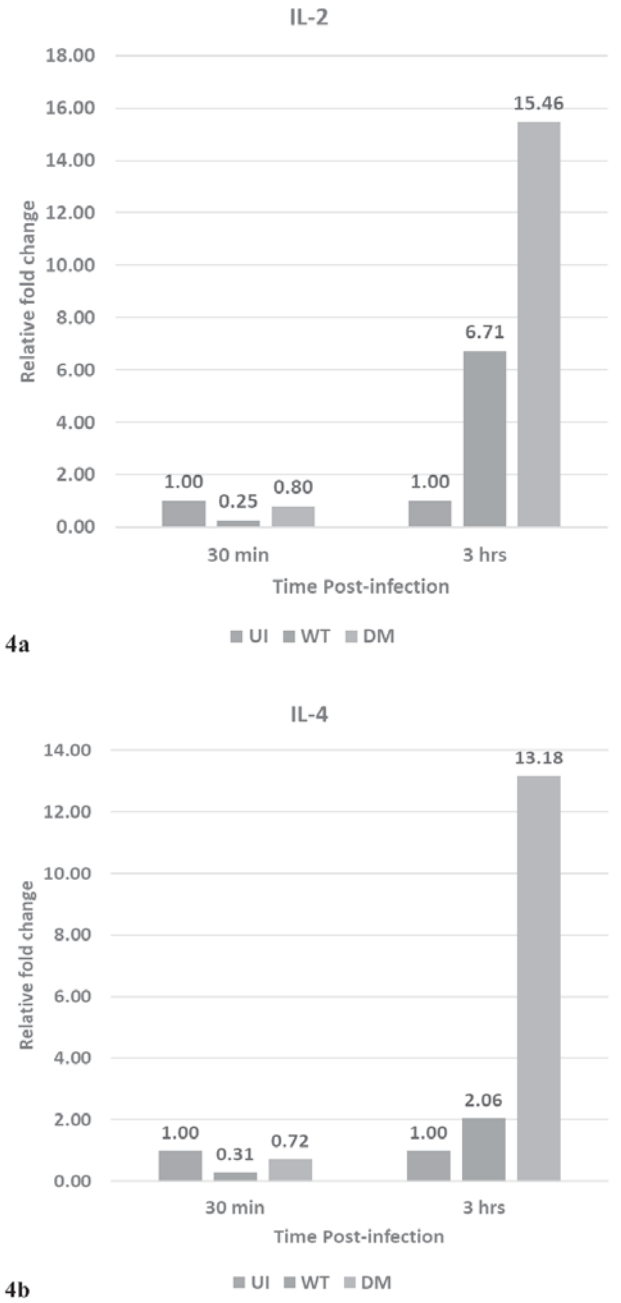


Fig. 4a and 4b: Relative fold change of IL-2 and IL-4 among infected and uninfected macrophages at 30min and 3h post infection by RT-qPCR. UI-Uninfected macrophages, WT- macrophages infected with wild type *Salmonella*, DM- macrophages infected with mutant *Salmonella.*

but significantly highly expressed after 3hrs of incubation in both, WT and DM infections (Fig. 4a and 4b). IL-2 exhibited a fold change of 6.71 in WT and 15.46 in DM than the control whereas IL-4 was upregulated by 2.06 and 13.18 folds in WT and DM infected groups, respectively. Expression of IL-2 and IL-4 genes in DM was around 2.3 and 6.5 folds upregulated than that of WT group.

Senevirathne *et al.* (2020) reported that O-antigen deficient mutant of *Salmonella* Typhimurium had a reduced cytotoxicity and had greater levels of cytokine expression, such as IL-2, IL-10, IL-4, and IFN-, than the WT strain following macrophage uptake, and the mutant did not survive in macrophages or chicken organs and cleared quickly without causing systemic infection.

Our results revealed that expression of both IL-2 and IL-4 genes were significantly higher in case of DM at 3hrs of infection. Relative fold change of IL2 and IL4 in DM were 2.3 and 6.5 than the WT infection.

CONCLUSION

The *Salmonella* after entering the macrophages, escape all the macrophage defense mechanisms for its survival and successful infection in the host system. Our

mutant *Salmonella* lacking the *fnr* and *narL* genes, was found to be more susceptible to chicken macrophage defense mechanisms when compared with the wild type *Salmonella*. The glucose uptake reduction after 3hrs of infection was to starve the bacteria and increased expression of ACLY and Acaca gene indicated the diversion of TCA cycle to different pathways to avoid more ATP production and to cause cytokine induction. The other defense activities such as antigen presentation (MHCII), DUOX oxidase, and the cytokines IL-2 and IL-4 were found increased significantly in DM compared to WT. Therefore, based on findings of present investigation, it is concluded that the deletion of *fnr* and *narL* genes made the *Salmonella* more susceptible to macrophage internal environment and hence easier and faster clearance.

ACKNOWLEDGEMENTS

This piece of work was funded by the Department of Biotechnology, India (Grant No.:BCIL/NER-BPMC/ 2017/). Pashupathi M acknowledges the fellowship support from ICAR and DBT for his PhD research work.

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