Chapter 3

Studying the systemic dissemination of E. cloacae SBP-8 during infection in mammalian (murine) model host.

3.1 Introduction

Enterobacter cloacae (*E. cloacae*), a natural inhabitant of human gut, can cause various life-threatening infections like neonatal meningitis, urinary tract and respiratory infections in immunocompromised hosts [1,193-195]. In addition, it has been the reason for multiple nosocomial infections and outbreaks [1]. Despite the fact that this opportunistic pathogen is involved in a broad array of infections, there are very few studies reporting its pathogenic potential and ability to produce legitimate virulence-associated factors. It produces siderophores, toxins like hemolysin, and thiol activated cytotoxins, and has genes encoding different secretion systems [6, 34, 161]. Nevertheless, the pathogenic mechanism of *E. cloacae* is poorly understood due to a lack of an established model system for validating and studying the systemic infection process.

Different non-mammalian model organisms like *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* have been pivotal in understanding the pathogenesis of multiple pathogens [152, 164, 196]. As stated in the previous chapter, we established *C. elegans* as a model host to study the pathogenicity of *E. cloacae* SBP-8 and the corresponding host innate response [197]. To gain further insights into the systemic infection process of *E. cloacae* SBP-8, we used mouse as a mammalian model host. The murine model provides an advantage over other model hosts like flies and worms owing to its anatomical, physiological, and genetic similarity with humans. The model offers a suitable means to study the complex host-pathogen interactions at various stages of infection. The murine model is a preferential model and has been successfully used for studying the pathogenesis of several enterobacterial pathogens like *Salmonella, Shigella, Yersinia,* and Enterotoxigenic *Escherichia coli* [198]. The ease of performing intragastric infection in the mice makes it an attractive model to study foodborne pathogens. *E. cloacae* SBP-8 is a soil isolate and possesses the ability to cause infection, as established through the *C. elegans* infection model. Thus, it has a fair potential to cause food-borne infections and spread *via* the feco-oral route. A murine model for infection studies could shed light on the ability of *E. cloacae* SBP-8 to infect mammals (including humans) while providing insights into the mode of infection and systemic dissemination of the pathogen.

The aim of the present study is to evaluate the infectivity of *E. cloacae* in murine model (mammalian model host) and its ability to cause systemic infection. When injected through intraperitoneal (i.p) route, the bacterium encounters the phagocytic cells present in the peritoneum. If the bacteria possesses pathogenic ability, it can survive the phagocytic attack and spread systemically. Swiss albino mice were infected with *E. cloacae* SBP-8 using the i.p route. After confirming the ability of *E. cloacae* SBP-8 to cause infection in mice *via* i.p route,

we used the intragastric (i.g) infection route to study the feco-oral transmission of this bacterium. The host immune response was assessed at the molecular, histopathological and immunological level. *In-vitro* cell culture models, intestinal epithelial and macrophage cell lines, were used to evaluate the invasion, proliferation, and intracellular localization of *E. cloacae* SBP-8 considering them as a major entry points for systemic dissemination.

3.2 Materials and Methods

3.2.1 Bacterial culture, animals and cell lines

E. cloacae SBP-8 used in the present study is a rhizospheric bacteria isolated from the *S*orghum *bicolar* plant [147]. Bacteria were grown in Luria Bertani broth (Himedia, India) at 37°C. Bacterial culture was grown and maintained as discussed in previous chapters. For infection studies, ten weeks old Swiss albino mice weight at least 20 g reared in Central Animal Facility, BITS-Pilani, Pilani were used. Institutional Animal Ethics Committee approved all experiments pertaining to mice with protocol number IAEC/RES/22/07. Macrophage cell line derived from BALB/c (colorectal adenocarcinoma cell line with epithelial morphology (HT-29) was a generous gift from Prof. Rajdeep Chowdhury, Dept. of Biological Sciences, BITS Pilani, India.

3.2.2 *In-vivo* infection assay

All procedures were carried out as per institutionally approved protocols. Swiss albino mice were infected intra-peritoneally and intra-gastrically with 10^7 CFU (colony forming unit) of *E. cloacae* SBP-8 per mouse. After 6 h and 24 h of infection, mice were anesthetized with an intraperitoneal injection of 2 mg ketamine and 0.15 mg of xylazine. Organs like lungs, spleen, kidney, and liver were aseptically isolated, weighed, and homogenized in 0.5 ml of sterile PBS (Phosphate-buffered saline) using Qiagen bead homogenizer, Germany. Serial dilutions of homogenate were plated onto LB agar containing ampicillin to count number of colonized *E. cloacae* SBP-8. Results were recorded as CFU per gram for liver, spleen, lungs, and kidney.

3.2.3 *ERIC*-PCR based DNA fingerprinting

To confirm the identity of colonized *E. cloacae* SBP-8, Enterobacterial Repetitive Intergenic Consensus (*ERIC*) PCR was performed as described in previous chapter [11]. The banding pattern of the amplicons was captured with a gel documentation system (Biorad, USA) and compared with that obtained with pure culture of *E. cloacae* SBP-8.

3.2.4 Histopathology of infected organs

For histological analysis, lungs, kidney, liver, and spleen were removed aseptically as mentioned in section 2.2. The organs were washed with sterile PBS and fixed in 10% formalin before embedding in paraffin. 5 μm tick tissue sections were obtained using a Zeiss microtome, Germany and then stained with hematoxylin and eosin for examination under light microscope, BX43, Olympus, India.

3.2.5 Cytokine analysis

Three groups each having six mice were labelled as uninfected, intraperitoneally infected, and intragastrically infected as mentioned in section 2.2. The blood from experimental mice was collected by disrupting the retrobulbar venous sinus using a capillary tube (a retro-orbital bleeding method). The blood was allowed to clot to obtain serum, and serum from six mice within each group was pooled. 50 μL of serum was used for estimation of cytokines (IL-1 α, IL- β, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and GM-CSF) using Multi-Analyte ELISA Array Kit (Qiagen, India) as per manufacturer's instructions. The readings were recorded using Thermo scientific Multiscan plate reader (Thermo Scientific, India) at 450 nm. Standard errors (SE) of the means were calculated from two technical repeats of independent experiments performed.

3.2.6 Haematological and biochemical analysis of blood serum

In order to assess host-immune response against *E. cloacae* SBP-8 infection haematological and biochemical analysis of blood serum was conducted. After 6 h and 24 h of infection, blood samples (500 μl) were collected from the retrobulbar venous sinus of the animals. Various haematological parameters including haematocrit, haemoglobin content, red blood cells count, total leukocytes count (TLC), differential leucocytes count (DLC), total platelets count (TPC), packed cell volume (PCV), mean corpuscular haemoglobin concentration (MCHC) and blood sugar (random) were assessed using Hematology Analyser-3, labtronics, India as per the manufacturer's instructions. For biochemical evaluation, total protein, albumin, serum creatinine, serum alkaline phosphate, bilirubin, urea, cholesterol, triglyceride were determined by commercial reagent kit (Gold site diagnostic, India) and biochemistry analyzer, labtronics, India to test the liver and kidney functions.

3.2.7 Bacterial invasion assay

The test of bacterial phagocytosis by macrophage and bacterial invasion were performed using RAW 264.7 and HT-29 cell lines respectively. For the given tests, gentamicin protection assay was performed as described previously [199]. Briefly, the cells were seeded in DMEM

(Dulbecco's Modified Eagle Medium) with 10% FBS (Fetal bovine serum). The plates were incubated at 37°C with 5% CO_2 for 24 h in DMEM media. Cells were infected with *E. cloacae* SBP-8 at a multiplicity of infection (MOI) of 50 for 30 min, centrifuged it for 30 min, washed the pellets three times with sterile PBS and treated with 100 µg/ml gentamicin for 1 h to kill extracellular bacteria. The infected cells were washed three times with PBS and then incubated with 25 µg/ml of gentamicin till the time of lysis. 2 and 16 hours post-infection (hpi) (time starting after 30 minutes infection period), the cells were washed three times with PBS and lysed with 0.5 ml of 0.1% TritonX-100. The serial dilutions of the lysates were plated onto LB agar supplemented with ampicillin (100 µg/ml) to determine the CFU. The percent invasion/phagocytosis was calculates as,

$$\frac{\textit{E. cloacae}\text{ SBP-8 recovered after gentamicin treatment}}{\textit{E. cloacae}\text{ inocula used for infection}} \times 100$$

3.2.8 Visualization of bacterial colonization by imunofluorescence confocal microscopy

HT-29 and RAW 264.7 cells (1×10^4 cells) seeded on coverslips were infected with green fluorescent protein-tagged *E. cloacae S*BP-8 as described in section 2.7. At 2 and 16 hpi, the cells were washed with PBS and stained with Lyso-Tracker dnd-99. The coverslips were then processed for confocal microscopy, and images were obtained with the 488 nm(green) and 594 nm (red) wavelength filter using confocal laser scanning microscope-880, Zeiss Germany. Co-localization of *E. cloacae* SBP-8 with Lyso-Tracker was analyzed with Image J [200].

3.2.9 Statistical analysis

Bacterial colonization assay in mice was performed with 6 mice per group, while intracellular survival assay in cell lines were performed in triplicate with three biological replicates. Data were analyzed using Graph pad prism 8 and expressed as mean ± standard deviation. Student's *t*-test or Mann Whitney U test was performed to determine the statistical significance of the data.

3.3. Results

3.3.1 Infiltration of *E. cloacae* SBP-8 in different systemic organs

The progression of urinary tract infection and respiratory disease caused by opportunistic pathogen, *E. cloacae* is very rapid, and symptoms may develop within initial hours of infection [201]. Our previous study reported that *E. cloacae* SBP-8 colonized the intestine of worm and eventually killed it. To study the systemic dissemination in mice, *E. cloacae* SBP-8 at a dose of 10^7 CFU per mice were injected *via* i.p route for direct and rapid dissemination to different organs. The infected mice were monitored for up to 24 h for the occurrence of disease

symptoms. At 6 h post-infection, most of the mice looked healthy with no signs of disease symptoms. By 24 h, 4 out of 6 mice were listless, huddled, hunched up, and breathing rapidly. The examination of bacterial burden in these mice at 6 and 24 h revealed colonization of *E. cloacae* SBP-8 in reticuloendothelial organs with CFU of 10^4-10^5/gm of lungs, liver, spleen and kidney (Fig.3.1 A and B). The bacterial burden in different organs increase from 6 h to 24 h indicating survival of the pathogen and probably some proliferation. The above results confirmed that *E. cloacae* SBP-8 systemically disseminate to different organs while making the mice sick with observable symptoms.

E. cloacae is a part of normal gut microbiota, and almost all reported infections are of endogenous origin. Additionally, *E. cloacae* SBP-8 is a soil isolate having good probability of causing oral infection. Therefore, gastro-oral infections seem to be the most natural infection route for this pathogen. We tested the ability of *E. cloacae* SBP-8 to successfully infect mice *via* i.g route by monitoring the organ infiltration by this pathogen. Similar to i.p infection, mice infected intragastrically showed similar symptoms like, they were listlessness, huddled behaviour and had breathing difficulty, with significant bacterial load in each of these organs [Fig.3.1 C].

ERIC PCR profiling was performed to confirm the bacteria isolated from different organs are indeed *E. cloacae* SBP-8. Identical DNA banding pattern to that of the pure culture of *E. cloacae* SBP-8 were observed in all the screened colonies indicating the presence of *E. cloacae* SBP-8 in different organs of mice [Fig.3.2].

3.3.2 Serum cytokine profile in mice infected with *E. cloacae* SBP-8.

We further characterized the *E. cloacae* SBP-8 induced inflammatory immune response and its potential implication in the pathogenesis. Serum levels of a panel of 10 pro-inflammatory and anti-inflammatory cytokines such as IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GM-CSF were determined at an early (6 h) and late time point (24 h) of infection. All the cytokines showed significant increase in the blood/serum during infection. Some of them namely IL-4, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GM-CSF increased (atleast till 24 h) with the progress in infection. The increased levels of pro-inflammatory cytokines, IL-1 β, IL-12, IL-17A, IFN-γ, TNF-α and GM-CSF, could be attributed to activation of the cell-mediated immunity by the bacterial antigens while high levels of anti-inflammatory cytokines, IL-4, IL-10 and IL-6 indicated a host response to control the inflammation and facilitate antibody production.

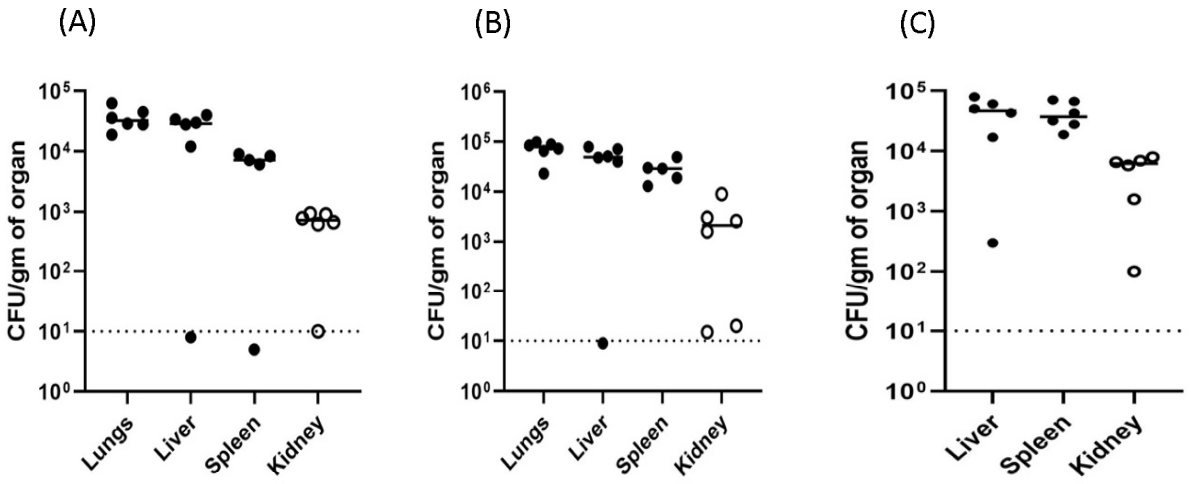

Fig.3.1 Kinetics of *E. cloacae* SBP-8 infection in mice. A group of six Swiss albino mice were infected intraperitoneally with *E. cloacae* SBP-8 (1×10^7) and CFU per gram of organ (lungs, spleen, liver and kidney) was determined at 6 h (A) and 24 h (B) post-infection. Similarly, six Swiss albino mice were infected intragastrically and CFU monitored at 24 h post-infections. A dashed line indicates the limit of detection. Each point indicates CFU recovered from a single animal. Symbols below the limit of detection represent no detectable numbers of bacteria in the given organ. A solid line indicates the median of CFU recovered.

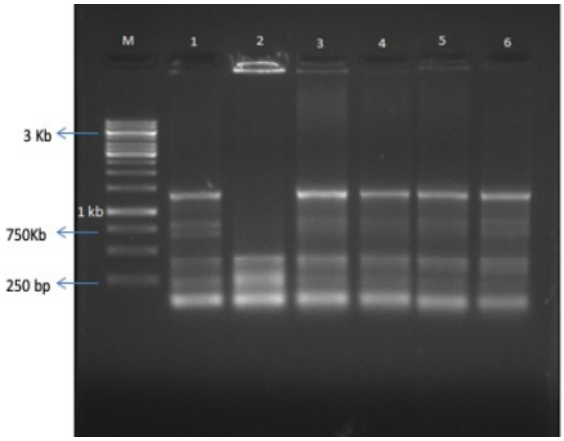

Fig.3.2 Image showing the amplicons obtained *via ERIC* PCR of bacterial colonies obtained in CFU analysis of different organs of infected mice. Lane 1 (pure *E. cloacae* SBP-8) and lanes 3 to 6 (colonies from CFU analysis) show a similar banding pattern, confirming the identity of the bacteria from the organs as *E. cloacae* SBP-8. Lane 2: *E. coli* DH5α was used as a negative control.

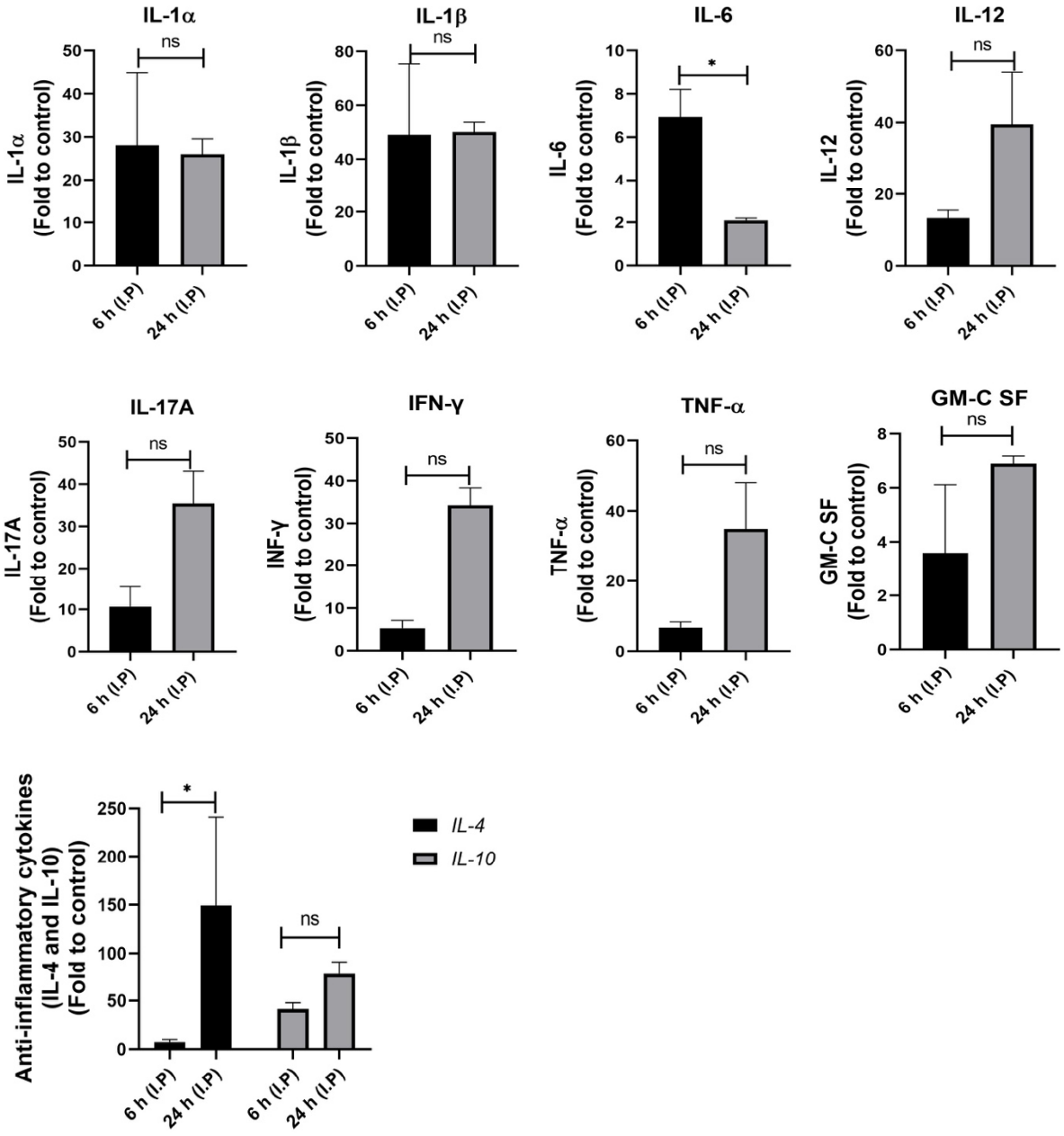

Fig.3.3 Analysis of cytokine levels in the sera of Swiss albino mice infected with *E. cloacae* SBP-8. Two groups of each 6 Swiss albino were infected intraperitoneally with 1×10^7 CFU/mice of *E. cloacae* SBP-8. The sera was collected from infected mice at 6 and 24 hpi. Proinflammatory cytokines, IL-1α, IL-1β , IL-6, IL-12, IL-17A, IFN-γ, TNF-α, GM-CSF and anti-inflammatory cytokines, IL-4 and IL-10 were determined using Qiagen Multi-Analyte ElISA Kit (MEM-004A).Results were represented as mean± SD pooled sera samples (six animals per pool) for each infected and control groups

3.3.3 Histopathological changes in the mice organs due to *E. cloacae* SBP-8 infection

Histopathological analysis was performed to examine the changes in the pathology of reticuloendothelial organs of Swiss albino mice infected with *E. cloacae* SBP-8. Histopathological analysis of lung sections of mice infected with *E. cloacae* SBP-8 *via* i.p and i.g route showed similar signs of inflammation/damage i.e., mild edema (congestion) and interalveolar septa thickening with mild fibrosis [Fig.3.4]. Infiltration of inflammatory cells increased with infection time [Fig3.4]. Maximum damage in the alveolar cells was observed by 24 hpi in both i.p, and i.g infected mice. The histological section of the control (PBS-injected/fed) mice retained normal architecture of alveolar spaces without any signs of toxicity/damage.

Histopathological assessment of kidney sections revealed that in all the treatment groups, the glomerulus, proximal convoluted tubules (PCT), and distal convoluted tubules (DCT) were intact without any signs of degeneration [Fig 3.4]. However, mild infiltration of inflammatory cells was observed in the infected mice. Histopathological analysis of the liver sections displayed normal intact hepatocytes in all the treatment groups with no infiltration of inflammatory cells from the central vein [Fig.3.4]. Similarly, histopathological analysis of the spleen sections revealed intact red pulp (pink in colour) and white pulp (blue in colour) and absence of splenocytes in all the groups [Fig.3.4].

3.3.4 Assessment of immune response and organ damage due to *E. cloacae* SBP-8 colonization based on hematological and biochemical indicators

To assess host immune response and evaluate the functionality of reticuloendothelial organs of *E. cloacae* SBP-8 infected mice, total blood profiling and various biochemical assays were performed. Overall, no significant differences were observed in the haematological parameters after *E. cloacae* SBP-8 infection in mice. All the parameters were within the normal range except total red blood cell count (TRBC) and total leucocyte count (TLC) at 24 h of infection in i.g route with respect to the control group. No significant difference in TRBS and TLC was observed at both the time points on i.p infection. In all the groups infected with *E. cloacae* SBP-8, the serum levels of major inflammatory markers *viz.*, AST/SGOT, and ALT/SGPT were high in infected mice with respect to control, indicating liver injury. The results revealed that *E. cloacae* SBP-8 is pathogenic to mice inflicting damage to the host [Table 1].

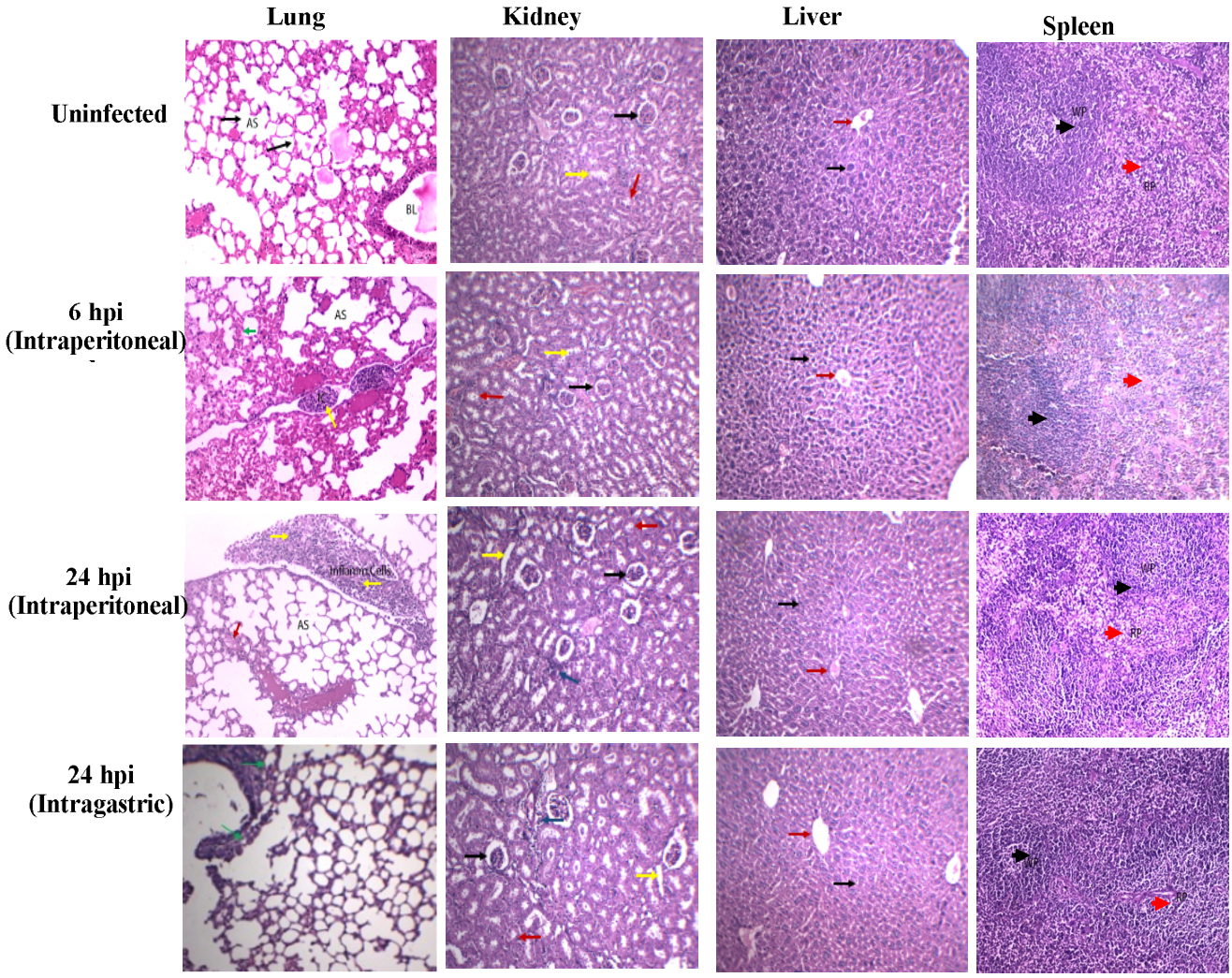

Fig.3.4 Histopathological evaluation (H&E staining, 40X) of various organs of Swiss albino mice infected with *E. cloacae* SBP-8 at 6 h and 24 h of infection for i.p route while 24 h for i.g route of infection. Control mice were injected with sterile PBS. For lungs, black arrows indicate the normal architecture of alveolar spaces with thin alveolar septa; red arrow shows mild edema or congestion; green arrow refers to interalveolar septa thickening, and ; yellow arrow points to infiltration of inflammatory cells. Similarly, for kidney, black arrows indicate glomerulus; red arrow refers to proximal convoluted tubules (PCT); yellow arrow shows distal convoluted tubules (DCT), and blue arrow points to infiltration of inflammatory cells. For liver, the different arrows indicate as follows: black arrow: hepatocytes, and; red arrow: central vein (CV). For spleen, black arrow: indicates white pulp (lymphatic tissue) whereas red arrow shows red pulp (venous sinuses).

Table 3.1. Haematological (Total blood profiling) and biochemical examination of serum (Liver/ kidney function tests) from mice infected with *E. cloacae* SBP-8, after 6 h (i.p) and 24 h (i.p and i.g) post infection.

Parameters	Control (PBS) Injected	6 hpi via i.p route	24 hpi via i.p route	24 hpi via i.g route
Animal Body weight (gm)	21	20	20.5	21
Haemoglobin (Hb gm %)	12.30	12.35	11.80	12.10
Total leukocytes count (TLC)/cumm	12743	13654	13821	**14500**
Differential Leucocytes Count (DLC)				
Neutrophil (%)	62.9	70.69	78.28	79.70
Lymphocytes (%)	24.9	25.4	24.5	20.3
Eosinophils (%)	4.56	4.33	5.12	4.77
Total Red Blood Cell Count (TRBC) (million/cu.mm)	7.54	7.67	6.10	**9.41**
Total Platelets Count (TPC) (lacks/cu.mm)	5.16	4.87	5.89	5.69
Packed cell volume (PCV) (%)	37	38	27.30	41.57
Mean corpuscular haemoglobin Concentration(MCHC) gm/dl	31.5	32.50	33.70	31.67
Total Eosinophils Count (TEC) cells/cumm	543	511	498	556
Blood sugar Random (mg%)	137	126	142	139
AST/SGOT (U/L)	22.33	**55.60**	**72.67**	**68.33**
ALT/SGPT (U/L)	21.63	**72.67**	**101.22**	**106.67**
Serum Bilirubin (mg%)				
Total	0.50	0.77	0.87	0.83
Direct	0.28	**0.50**	**0.63**	**0.61**
Indirect	0.22	0.27	0.24	0.23
Serum alkaline phosphatase (U/L)	124	153	**167**	**182**
Total protein (gm %)	6.2	6.38	5.87	6.50
Albumin (gm/dl)	3.67	2.84	3.19	3.23
Serum creatinine (mg%)	0.37	0.27	0.32	0.34
Globulin (gm/dl)	2.50	3.4	3.70	4.17
Blood urea (gm/dl)	13.67	13.0	14.33	13.67
Serum cholesterol (mg%)	112.3	132.6	128	124

- Above values are from pooled blood and serum of 5 mice in each group infected with *E. cloacae* SBP-8 by i.p and i.g route. PBS injected mice were used as control.

3.3.5 Intracellular survival of *E. cloacae* SBP-8 in mammalian cell lines.

In order to cause systemic infection, foodborne pathogen are required to cross the intestinal barrier to reach different organs. As *E. cloacae* SBP-8 colonized systemically in mice infected intragastrically, we tested its ability to infect intestinal epithelial cells. We used HT-29, a colon carcinoma intestinal epithelial cell line, to evaluate the invasion and proliferation of the *E. cloacae* SBP-8 using gentamicin protection assay. *E. cloacae* SBP-8 was able to invade the HT-29 cells, showing 0.2197 ± 0.02114 percent invasion [Fig.3.5 A]. It was able to survive and proliferate into these cells exhibiting 2.5-fold increase from 2 to 16 h [Fig.3.5 B].

For most of the intracellular pathogens, macrophages act as a protective shield disseminating them to different systemic organs [202]. Ability of *E. cloacae* SBP-8 to survive and proliferate in intestinal epithelial cells suggests it may cross the intestinal barrier thereby reach GALT Gut-associated lymphoid tissue). The pathogen could then utilize the macrophages within GALT for systemic dissemination. In addition, the dissemination of *E. cloacae* SBP-8, when injected intrapertoneally, to systemic organs is very likely through macrophages in peritoneum. To examine the survival of *E. cloacae* SBP-8 in macrophages, we used RAW 264.7 murine macrophage cell lines for the intracellular survival assay. We observed that macrophages are able to phagocytose the *E. cloacae* SBP-8 engulfing 0.5589 ± .0901 percent of the bacteria provided (Fig.3.5 A). The *E. cloacae* SBP-8 did not proliferate in these cells but was able to sustain upto 16 hpi [Fig.3.5 B]. We could detect intracellular bacteria *via* CFU analysis at 16 hpi within the macrophages. This indicates *E. cloacae* SBP-8 could probably use macrophages for disseminating to different organs.

The primary defence mechanism that macrophages use is lysosomal degradation of the invading pathogen. Most intracellular pathogens bypass this fusion thereby preventing lysosomal degradation. To test if *E. cloacae* SBP-8 were killed by lysosomal degradation we performed colocalization analysis using confocal microscopy for tracking its lysosomal fusion using Lyso-tracker red dye. RAW264.7 macrophages infected with GFP-tagged *E. cloacae* SBP-8 were stained with Lyso-tracker red dye at 2 and 16 hpi [Fig.3.6 A]. Around 50% of the bacterial cells colocalized with the lysosomes at 2 hpi while the percent (~20%) colocalization decreased at 16 hpi [Fig 3.6 C]. This suggests that most of the intra-macrophage bacteria could have been cleared through lysosomal degradation during the initial hours of infection. However, it remains to be identified if at the later time-point (16 hpi), *E. cloacae* SBP-8 escapes the lysosomal fusion by surviving in the vacuolar compartment or escaping into the cytoplasm.

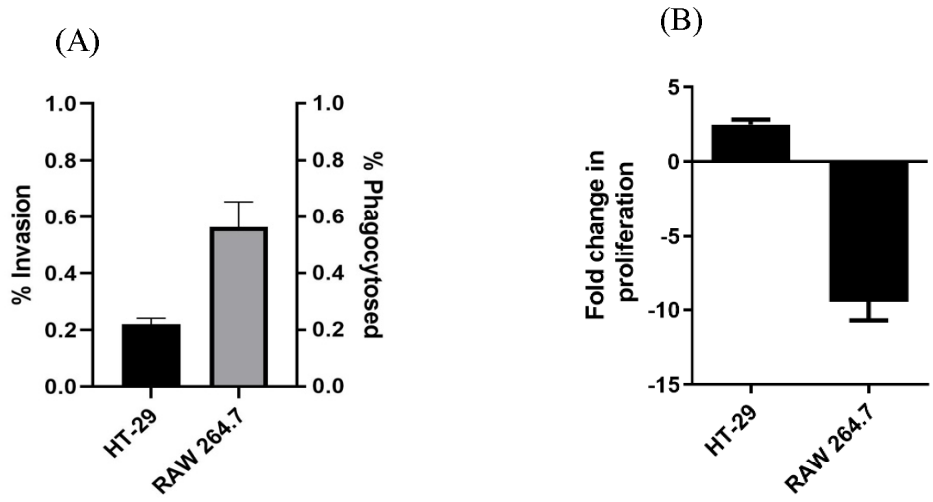

Fig.3.5 Bacterial invision and proliferation assay. (A) Percentage of *E. cloacae* SBP-8 invading and phagocytosed in HT-29 cells and RAW 264.7 murine macrophages cell lines, respectively. (B) Fold change (from 2 h to 16 h) in CFU recovered from *E. cloacae* SBP-8 infected HT-29 and RAW 264.7 cell lines.

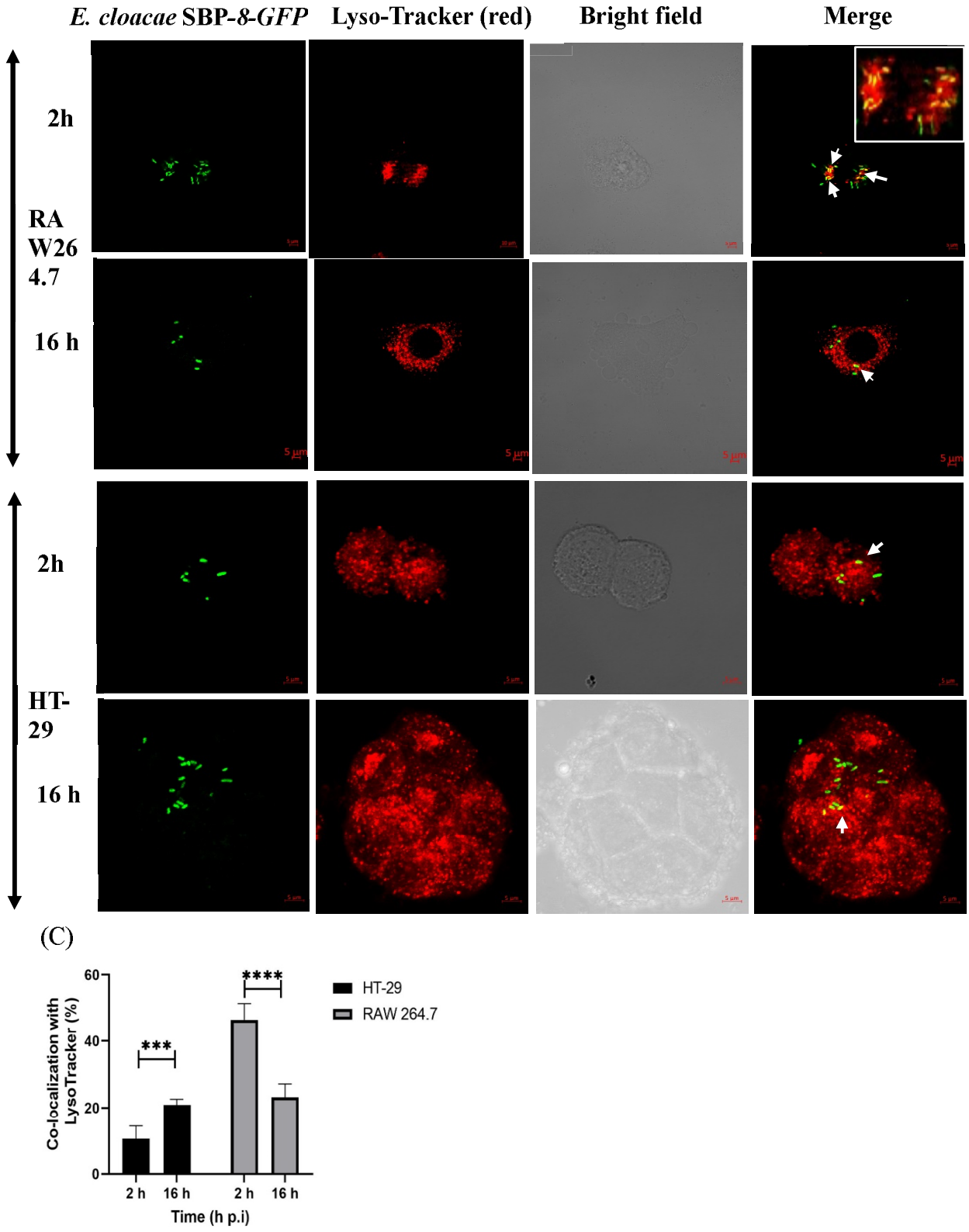

Fig.3.6 Representative fluorescent microscopic images depicting lysosomal fusion of *E. cloacae* SBP-8-GFP in RAW 264.7 (A) and HT-29 (B) cell lines at 2 and 16 hpi. Both the cells were infected with *E. cloacae* SBP-8-GFP at an MOI of 50 followed by gentamicin treatment to get rid of extracellular bacteria. The infected cells on the coverslips were collected, fixed, and then stained with lysotracker-dnd-99 (red). White Arrows indicate the co-localization of *E. cloacae* SBP-8 with the Lyso-tracker. (C) Percentage co-localization of *E. cloacae* SBP-8-GFP with Lyso-tracker at 2 and 16 hpi in HT-29 and RAW 264.7 cell lines.

The lysosomal fusion of *E. cloacae* SBP-8 was also tested in HT-29 at 2 and 16 hpi [Fig.3.6 B]. On contrary to the colocalization results with macrophages, the percent colocalization of *E. cloacae* SBP-8 with lysosomes increased from 2 hpi (9%) to 16 hpi (20%) in this cell line [Fig.3.6 C]. Qualitative analysis of the bacterial number within the images confirms the decreased bacterial number in RAW264.7 macrophages and an increased bacterial number in HT-29 epithelial cells. The results of lysosomal fusion and intracellular proliferation assay appears to be counterintuitive and needs further investigation.

3.4 Discussion

It is arduous to treat infections caused by *E. cloacae* complex due to its antibiotic resistance and poorly studied pathogenic mechanisms [17]. In chapter 2, we demonstrated that *E. cloacae* SBP-8 is pathogenic gradually colonizing the intestine of *C. elegans* and eventually killing it. Understanding how *E. cloacae* establishes infection and spreads throughout the body may allow us to discover effective ways to diagnose and treat *E. cloacae* infections. The mammalian model (like mice) is often used as a preferred model to investigate and analyse different stages of bacterial infection as well as understand the pathogen invasion and proliferation mechanisms [39, 203]. Thus, Swiss albino mice were used as model host to assess the pathogenesis of *E. cloacae* SBP-8. Based on the visual observations of the infected mice, the disease symptoms including lethargy and difficulty in breathing appeared at a late time point (24 hpi) of infection. Further, the systemic dissemination of the test pathogen was examined by recovery of the bacterial colonies from different organs of the infected mice followed by the confirmation of bacterial colonies as *E. cloacae* SBP-8 using ERIC-PCR. The infected mice exhibited significant bacterial infiltration in reticuloendothelial organs like liver, lungs, spleen, and even kidneys at 6 hpi with the increase in bacterial load at later time point (24 hpi) indicating infection severity and systemic spread. Appearance of disease symptoms at late time point could be due to high bacterial load activating the immune response. The lungs of infected mice showed pathological changes including excessive infiltration of inflammatory cells, intraseptular ticking, and mild edema. Similarly, mild infiltration of inflammatory cells was also observed in the kidneys of infected mice. This is corroborated with the haematological parameters of infected mice sera, where total leukocyte count was significantly higher compared to that of uninfected mice. The influx of these cells into the alveolar spaces could have been triggered by the surge in pro-inflammatory cytokines like TNF-α and IL-1β in such as IL-6, IL-10, IL-17A, and IFN-γ indicates activation of host immune response against *E. cloacae* SBP-8 like those observed in mice infected with pathogens like *Listeria monocytogenes* [205], *S. aureus* [206], and *V. cholerae* [207]. The increased levels of pro-inflammatory cytokines at 6 hpi correlates well with the cellular infiltration in the lungs and kidneys [208]. These pro-inflammatory cytokines enhance leukocyte transmigration into the site of infection by facilitating the expression of cell adhesion molecules while triggering the production of eicosanoids and prostaglandins which in turn perpetuate the inflammatory [209-211]. *E. cloacae* SBP-8 infection significantly increased AST and

consequently AST/ALT ratio to over 2:1, which is an indication of cirrhosis [212]. This observation is supported by the study of Fei & Zhao who reported *E. cloacae* B29 induced liver damage [213]. Elevated levels of serum ALT and AST in *E. cloacae* SBP-8 infected mice indicates infection induced liver injury. However, no morphological changes were observed in the hepatocyte cells stained with haematoxylin and eosin stain. The *in-vivo* results indicate *E. cloacae* SBP-8 is an invasive pathogen causing systemic dissemination in reticuloendothelial organs while hampering function of some organs like liver. The pathogenicity of this strain can be attributed to the presence of several virulence factors like hemolysins, leukotoxins, thiol-activated pore-forming cytotoxins and secretion systems in the genome [147]. Nevertheless, further investigation is required to identify the role of these factors in *E. cloacae* SBP-8 pathogenicity.

Although *E. cloacae* is a part of normal gut microbiota and generally viewed as non-invasive opportunistic pathogen, some recent studies have reported *E. cloacae* to be invasive [161]. Results of our study also suggest the invasive nature of this species but the source is of exogenous origin. Most pathogens encounter two major cell types during infection, namely epithelial cells and macrophages cells that are the important in also supporting the bacterial survival *in vivo* [214]. It has been reported that many gastrointestinal pathogens deceive the gut defence mechanisms and breach the intestinal epithelial barrier to disseminate to systemic sites. Additionally, when mice are infected *via* i.p route, major cells arriving at the sites are macrophages. Thus, macrophages could act as the disseminator of the pathogen to various systemic sites when infected intragastrically or intraperitoneally. Gentamicin protection assay in intestinal epithelial and macrophage cell-lines suggested that that *E. cloacae* SBP-8 survives inside the macrophages (for upto at least 16 h), invades and multiplies (2.5-fold) within the epithelial cells. This indicates *E. cloacae* SBP-8 to be an intracellular pathogen probably disseminating by surviving within the phagocytic cells. Similar to our results, Bustos *et al.* demonstrated that *E. cloacae* adhere and proliferate inside epithelial cells and macrophages [35]. After crossing the intestinal barrier, they manipulate antigen-presenting cells in the blood to house and disseminate itself to systemic organs [215, 216]. Pathogens have evolved mechanisms to evade the defences of the host and survive in the macrophages. When tested, *E. cloacae* SBP-8 were killed by the RAW264.7 macrophages but the pathogen survived within them for at least upto 16 h giving sufficient time for dissemination to different organs. One of the defence mechanisms of macrophages is lysosomal degradation of the invading pathogen. Intracellular pathogens survive this lysosomal degradation by inhibiting phagosome maturation

or escaping in the cytoplasm. Thus, we analysed if *E. cloacae* SBP-8 could inhibit or divert the normal process of phagosome maturation and withstand within the hostile environment of the phagosome. Around 50% of *E. cloacae* SBP-8 fused with the lysosomes at an early time point of infection (2 h), thereby being degraded by the lysosomal enzymes. This could possibly account to the reduction in its number, as estimated at 16 hpi, in RAW 264.7 cells. Whereas, in HT-29, most of the *E. cloacae* SBP-8 inhibited lysosomal fusion, which could be the reason for gradual increase in the bacterial number during infection. However, it remains to be investigated how *E. cloacae* SBP-8 escapes lysosomal fusion in epithelial cells but not in macrophages and what leads to the reduction in its number within macrophages.

To summarize, we established that mice (Swiss albino) could be used as a mammalian model host to study the pathogenicity of *E. cloacae* SBP-8. We demonstrated its systemic dissemination to reticuloendothelial organs of the mice. *E. cloacae* SBP-8 infection induced the recruitment of the major immune response cells at the sites of infection. It also elevated host innate immune response leading to the systemic production of proinflammatory cytokines. Further, we hypothesize that the most likely route of *E. cloacae* SBP-8 infection is feco-oral whereby it crosses the intestinal epithelium and disseminates *via* phagocytes, at least macrophages. Thus, studying various pathogenesis aspects using murine infection model may provide an useful tool to gain insights into the pathogenesis of *E. cloacae*.