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***Original Article***

# Pathological, Immunological, and Hematological Parameters Associated with Experimental Infection of *Citrobacter Freundii* in Rabbits

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**Abstract**

*Citrobacter freundii* is one of the most important nosocomial opportunistic pathogens, which causes sepsis, as well as different gross and histopathological lesions in various internal organs in humans and animals, especially dogs and fish. This study aimed to investigate the hematological parameters, immunological responses, and pathological effects of the infection induced by the virulent strain of *C. freundii* on rabbits. A total of 42 rabbits (local breed; male and female), with a mean weight of 1.5-2 kg, were housed under controlled environmental conditions (20±2˚C, 14:10 h light: dark cycle) and allowed *ad libitum* access to food and water. After two weeks of adaption, the rabbits were divided randomly into three groups of 14 animals per group. Group one (G1) received $3\times10^8$ CFU/ml of the virulent isolate (intraperitoneally [IP]) of *C. freundii*. Group two (G2) was injected subcutaneously (SC) with $3\times10^8$ CFU/ml of the virulent strain of *C. freundii*, while group three was IP injected with phosphate buffer saline and considered a negative control group. Results showed the variable gross pathological effects which included hemorrhage, edema, and congestion of visceral organs. Furthermore, the microscopic lesions showed pneumonia due to inflammatory cells infiltration, mainly neutrophils, macrophages, plasmacytes, and lymphocytes, severe interstitial and intra-alveolar edema, extensive pulmonary hemorrhage, emphysema, and atelectasis. The recorded data from the liver samples revealed hepatitis which was characterized by perivascular and periportal leukocyte cuffing, marked centrilobular with periportal necrosis, extensive hepatic edema, and periportal edema in addition to extensive fibrosis in interlobular septa and periportal fibrosis with severe interstitial hemorrhage. In the kidneys, there were severe renal edema, mixed inflammatory exudation, mainly neutrophils, macrophages, plasmacytes, lymphocytes, fibroblast infiltration in renal parenchyma and renal cortex, extensive renal hemorrhage, edema, as well as fibrosis and severe renal tubular necrosis. In addition, enteritis appeared in the intestine with mucosal edema, especially in lamina propria; moreover, necrosis of entire villi, epithelial necrosis, mucosal and submucosal hemorrhage, and fibrosis were observed. The present study revealed a significant increase in total leukocytes count and the concentration of TNF-α in the infected groups. To the best of the authors' knowledge, this study is considered the first attempt aimed to detect the pathological effects of *C. freundii* on visceral organs in rabbits. It is concluded that this bacterium could induce a significant pathological, hematological, and immunological changes in the infected animals.

**Keywords:** *Citrobacter freundii*, Enteritis, Granulomatous pneumonia, Hepatitis, Iraq

## 1. Introduction

*Citrobacter freundii*, known as an opportunistic nosocomial and ubiquitous bacterium, is an anaerobic facultative and Gram-negative bacteria, which belongs to the family *Enterobacteriaceae* (1). It has long rod-shaped bacilli with a typical length of 1-5 µm.

Generally, the cells of *C. freundii* have several flagella used for motility (2). *C. freundii* is revealed in MacConkey agar around small with dark pink cambered colonies (3). Moreover, it is considered as oil organism, most commonly found in food, water, soil, natural environment, sewage, and intestinal tracts of humans and animals (1, 4).

In humans, *C. freundii* can cause diarrhea and food poisoning and plays an important role in public health as an opportunistic pathogen in causing food-borne diseases (3, 4). It is also an important infectious bacteria in veterinary and medical sciences, as itis responsible for different pathological lesions, such as systemic hemorrhages with gastroenteritis in animals and human patients (5). Some strains of this bacterium have been associated with nosocomial opportunistic infections in different tissues, such as blood stream, biliary tract, liver, urinary tract, intestines, soft tissue, peritoneum, meninges, endocardium, bone, respiratory tract, and wounds, especially in immunocompromised patients (6, 7).

In humans, this pathogenic bacterium is able to infect people who exhibit low immunity resulting in several diseases, such as neonatal meningitis, pneumonia, sepsis, urinary tract infection, septicemia, bacteremia, and abscessation of the brain (8, 9). In veterinary medicine, *C. freundii* is capable to infect fish, especially trout, cyprinids, and tilapia, thereby causing many diseases, such as severe enteritis, hemorrhagic septicemia, serious lesions in gills and kidney of catfish, systemic infection in common carp, septicemia, tail necrosis, as well as bleeding and reddening of the body, especially in Mozambique tilapia (10). In addition, high mortality rates in Nile tilapia, cutaneous bleeding in zebrafish, gastroenteritis with a progressive high-mortality rate in rainbow trout have been observed so far (11). Therefore, the current study aimed to isolate, identify, and investigate the pathological, immunological, and hematological effects of *C. freundii* on rabbits.

## 2. Materials and Methods

### 2.1. Animals and Experimental design

A total of 42 rabbits (local breed; male and female), with a mean weight of 1.5-2kg, were housed under controlled environmental conditions (20±2˚C, 14:10h light:dark cycle) and allowed *ad libitum* access to food and water. After two weeks of adaption, rabbits were divided randomly into three groups of 14 animals per group. Group one (G1) received $3\times10^8$ CFU/ml of the virulent isolate (intraperitoneally [IP]) of *C. freundii*. Group two (G2) was injected subcutaneously (SC) with$3\times10^8$ CFU/ml of the virulent strain of *C. freundii*, while group three (G3) received IP phosphate buffer saline and was considered a negative control group.

### 2.2. Bacterial Isolation

Swabs were taken from the ulcers of the gills, skin, and fans of the infected fish, especially *Cyprinus carpio L*., which were obtained from fish cultivated in floating cages in the Al-Hay district. They were then transferred to the laboratory and cultured in MacConkey agar. Following that, the bacterial isolates were confirmed and identified by an API 20 NE system.

### 2.3. Inoculation

In total, seven rabbits from each group were sacrificed on the 3rd and 7th days post-inoculation after anesthetization by the IP injection of ketamine/xylazine (12). All relevant internal organs were taken aseptically for gross and histopathology evaluations. For microscopic investigation, specimens, such as the lung, liver, intestine, and kidney were collected and preserved directly in 10% neutral buffered formalin as described by Snedecor and Cochran (13). Blood was aspirated from the rabbit's marginal ear vein with Venoject (BD Life Sciences, Cockeysville, Md, USA) without any anticoagulants. The required amount of blood (15 ml)was collected, and serum was separated for hematological evaluation. White blood count was measured on the 3rdand 7thdays post-inoculation.Following the manufacturer's instruction of the sandwich ELISA kit (SunLong Biotech/China), the concentration of the tumor necrosis factor-alpha (TNF-

α) was estimated on the7thday post-injection.

### 2.4. Statistical Analysis

The obtained data were analyzed using a two-way ANOVA and the least significant difference to make comparisons among the means of the groups. A p-value less than 0.05 was considered statistically significant (14).

## 3. Results

### 3.1. Isolation and Identification of Bacteria

Identification of the *C. freundii* isolates was conducted using an API®20E analytical system (a confirmation test) for confirming and approving the diagnosis of *C. Freundii* isolates in addition to studying the biochemical tests. This test was performed according to the manufacturer's protocol (BioMarieux/France) (Figure 1).

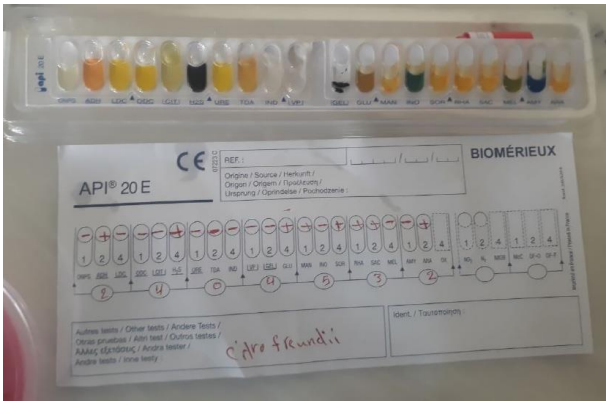


**Figure 1.** Results of the API 20 E system of the *Citrobacter freundii*

### 3.2. Pathological Findings

Various pathological findings in the vital organs of rabbits, such as the lungs, intestine, liver, and kidneys, included all-organ congestion, hemorrhage and edema, consolidation of lungs, severe gross and microscopic lesions in different organs of the experimental animals in G1 and G2 in descending order, compared to G3.

Microscopically, the lungs (Figure 2) appeared with interstitial thickening, as well as thickening of the alveolar wall [blue arrows (B), black arrows (D)] due to mixed inflammatory cells infiltration, mainly neutrophils with few lymphocytes, plasmacytes, and macrophages [black arrows (A), blue arrows (B)], congestion of alveolar capillaries with proliferation of pneumocyte type II and peri-bronchial lymphoid tissue hyperplasia. Ina certain section of the lungs, there was severe interstitial and intra-alveolar edema [blue arrows (A), black arrows (B)] after three days post-inoculation with the virulent strain of *C. freundii.*

After seven days post-infection, the microscopic lesions in the lungs showed multifocal granulomatous pneumonia characterized by caseous necrosis[black arrows (F)] in the center with a colony of bacteria[arrows head(F)]surrounded by chronic mononuclear cell infiltration, mainly lymphocytes, macrophages, plasmacytes, and fibroblasts[blue arrows (F), black arrows (E)].

Additionally, extensive interstitial hemorrhage of the lungs [blue arrows (E)] with pulmonary emphysema and formation of emphysematous knobs [blue arrows (D)] with areas of pulmonary atelectasis [blue arrows (C)] and thickening of blood vessel walls of the lungs [black arrows (C)] with thrombosis [(arrows head (C)] was observed in this study.

After three days post-inoculation with the virulent strain of *C. freundii*, the livers (Figure 3) of the infected group revealed hepatitis (Figure 3) characterized by perivascular and periportal leukocyte cuffing[blue arrows (C), black arrows (D)], especially mononuclear cells, such as macrophages, lymphocytes, and plasmacytes, with few neutrophils and focal aggregation in the interstitium [black arrows (A)], marked congestion of central vein, centrilobular necrosis [black arrows (B)], particularly in hepatocytes around the central vein with periportal necrosis [black dual arrows (C)]. In this case, the nucleus of the necrotic hepatocyte was lost, and the cytoplasm was homogenous and more eosinophilic. Furthermore, severe hepatic edema [blue arrows (A), black dual arrows, black arrows (C and B), black arrows (E)] was observed in the interstitium, as well as periportal edema. In addition, there was a dilation of hepatic sinusoids [blue arrows (A)]. On the 7th day of the

experiment, granulomatous hepatitis was observed due to multifocal infiltration of mononuclear cells, mainly macrophages, lymphocytes, plasmacytes, and fibroblast in interstitial tissue of the liver which was disrupted of the liver parenchyma with extensive fibrosis in interlobular septa [black dual arrows (D)] and periportal fibrosis [black dual arrows (E), blue arrows (F)] with severe interstitial hemorrhage [black arrows (F)].

Furthermore, centrilobular and periportal necrosis [blue arrows (E)] were obvious in some sections of the liver; additionally, extensive dilation of sinusoids [arrows head (F)] and congestion of portal vein [blue arrows (D)], with bile duct hyperplasia [arrows head (C)], was observed in this study.

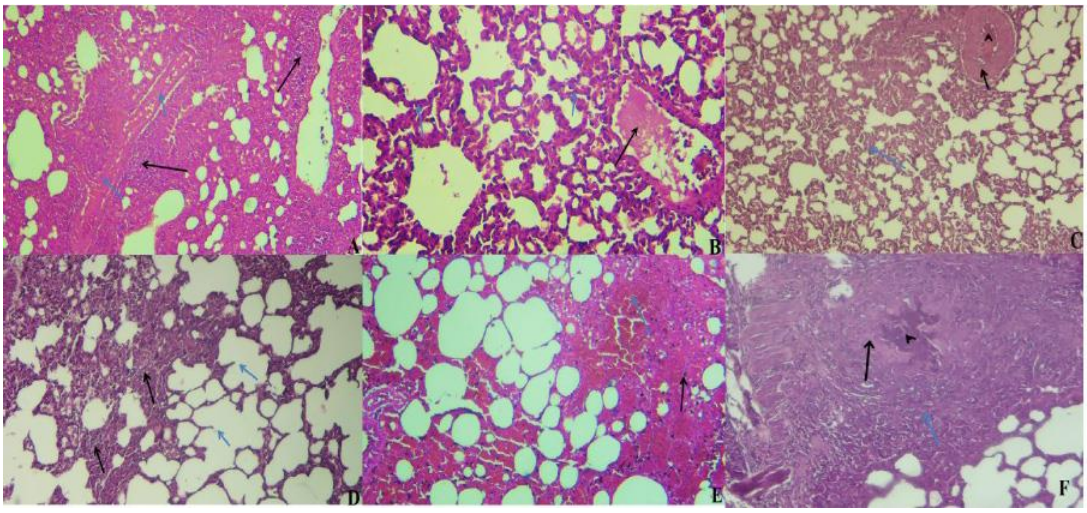


**Figure 2.** Hematoxylin and eosin-stained representative images of the histopathological changes of the lungs in the infected rabbits

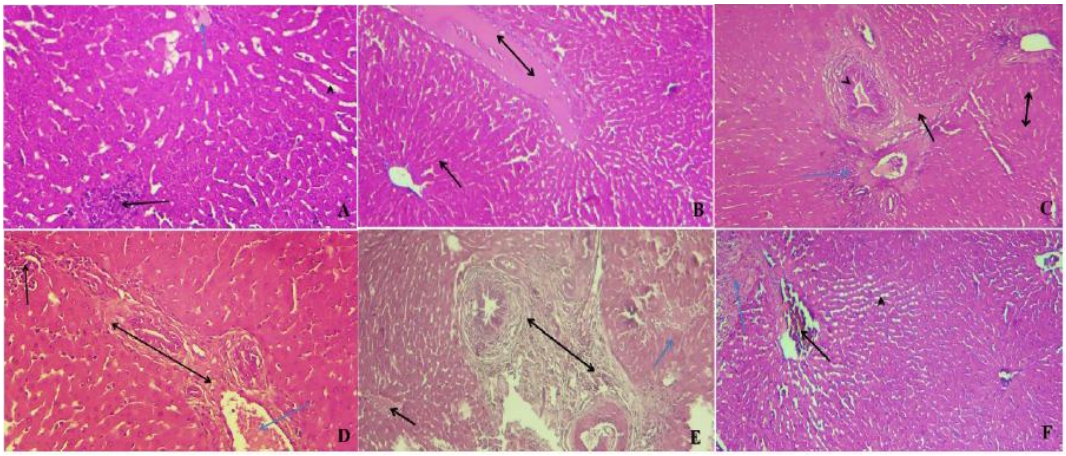


**Figure 3.** Hematoxylin and eosin-stained representative images of the histopathological changes of the liver in the infected rabbits

Histopathological examination for the kidneys of the infected groups (Figure 4) showed severe renal edema [black arrows (B)], mixed inflammatory exudation, such as neutrophils, predominately with few macrophages, lymphocytes, and plasmacytes [black arrows (A)] in the interstitial of the kidney and renal cortex, especially proximal and distal convoluted tubules, followed by the congestion of renal blood vessels. These changes were observed after three days post-inoculation with the virulent strain of *C. freundii*.

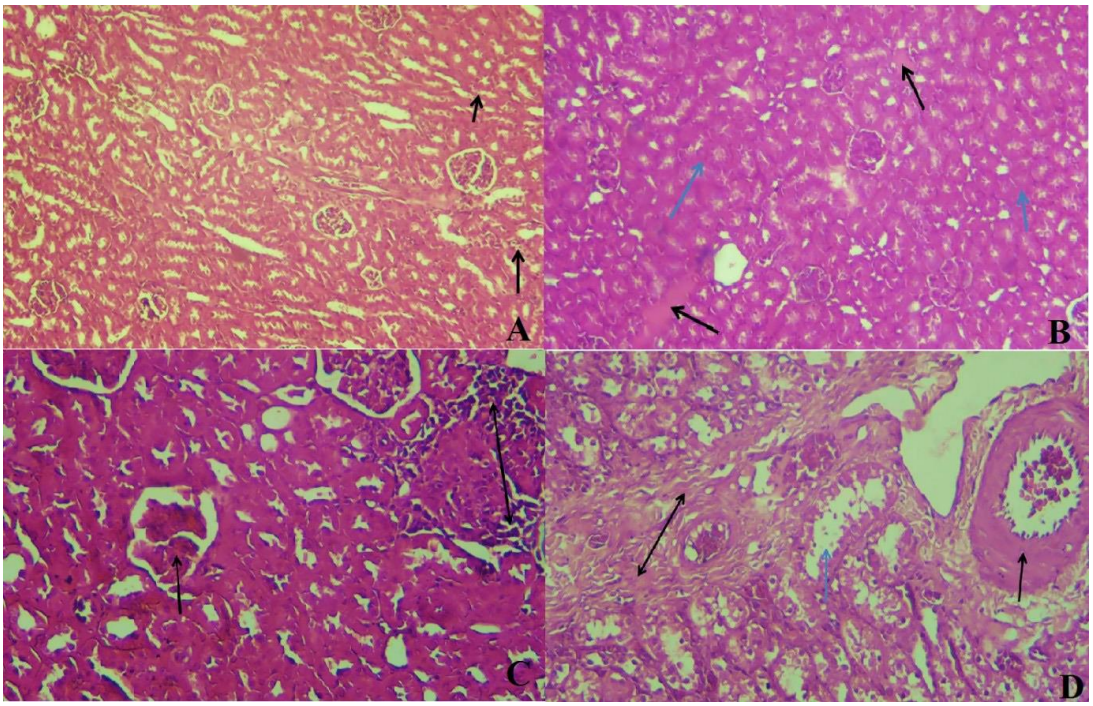


**Figure 4.** Hematoxylin and eosin-stained representative images of the histopathological changes of the kidneys in the infected rabbits

On the7thday of the experiment, there was chronic granulomatous nephritis of the kidneys due to focal infiltration of mononuclear cells [black dual arrows (C)], mainly macrophages, lymphocytes, plasmacytes, and fibroblastin the interstitial tissue of the kidney, and atrophy of glomerular tuft with inter glomerular hemorrhage [black dual arrows (C)]. Moreover, extensive renal hemorrhage and edema were observed in some sections of the kidney. Furthermore, extensive renal fibrosis [black dual arrows (D)] was observed, especially in interstitial due to fibrous connective tissue proliferation with hyperplasia of fibroblast. In addition, severe renal tubular necrosis [blue arrows (B)] was noted, and in this case, the cells were swelling and sloughing into the lumen of renal tubules with the epithelial necrosis of the proximal and distal renal convoluted tubules, followed by the congestion and thickening in the walls of the renal blood vessels [black arrows (D)].

On the 3rd day of the experiment, enteritis in the intestine (Figure 5) were noted in the infected rabbits (Figure 5) due to severe neutrophilic infiltration with few macrophages, plasmacytes, and lymphocytes aggregation in mucosa and submucosa [black arrows(A)] along with mucosal edema, especially in lamina propria, followed by moderate vascular congestion found in the mucosa and submucosa of the intestine, the necrosis of entire villi with epithelial necrosis [blue arrows (A)], and desquamation in the epithelial lining of the intestinal mucosa. On the 7th day of the experiment, severe microscopic findings were noticed in the intestine with severe granulomatous enteritis characterized by focal mononuclear cell aggregation [black dual arrows (B), black dual arrows (C), blue arrows (D)], mainly macrophages, lymphocytes, plasma cells, and fibroblast in mucosa and submucosa of the intestine in the certain sections of the intestine, as well as mucosal and submucosal hemorrhage [blue arrows (C)] and fibrosis [black arrows (C), black dual arrows (D)] due to fibroplasia. In addition, the severe necrosis of villi with epithelial necrosis of intestinal mucosa and goblet cell hyperplasia secreted mucin were noted in this period [black arrows (D)].

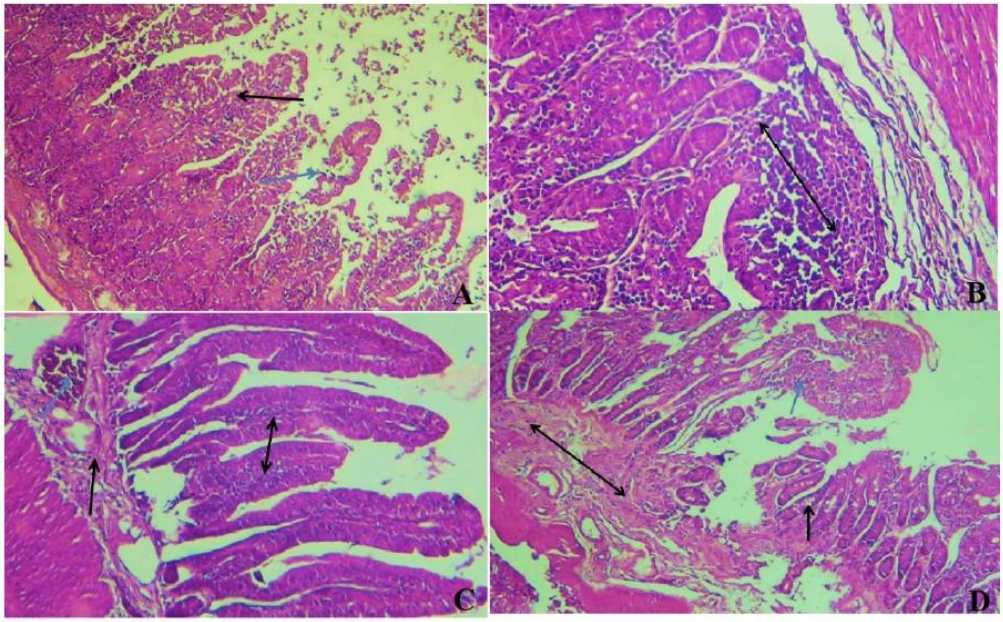


**Figure 5.** Hematoxylin and eosin-stained representative images of the histopathological changes of the intestine in the infected rabbits

### 3.3. Hematological Findings

The findings revealed a significant difference between the infected groups (G1 and G2 in descending order) and the G3 regarding an increase in the mean values in total leukocyte count on the 3rd and 7th days of the experiment ($P$<0.05; Figure 6).

### 3.4. Immunological Findings

In our study, a significant difference was observed between the infected groups and the G3 regarding an increase in the concentration of TNF-α. There was also a highly significant difference between the IP and SC groups in descending order in terms of TNF-α concentration ($P$<0.05), compared to the G3 (Figure 7).

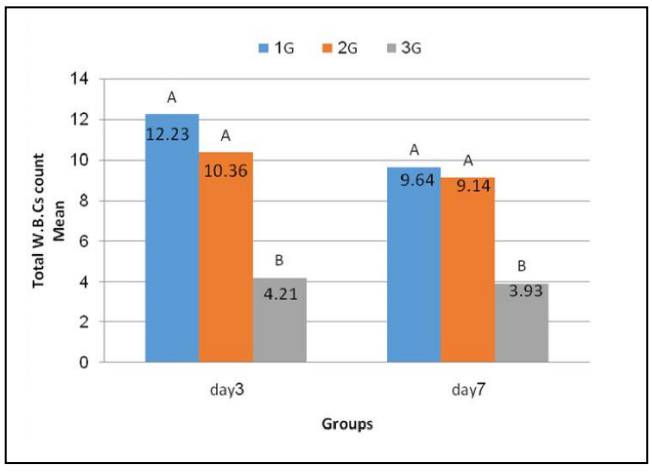


**Figure 6.** Total leukocyte count (×10$^3$) in the rabbits of the infected and control groups

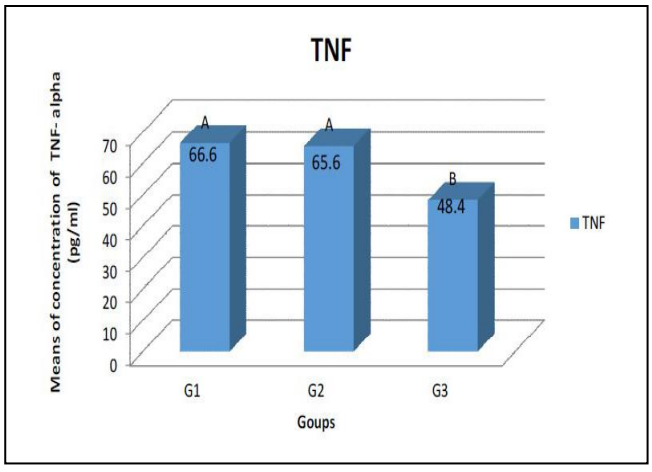


**Figure 7.** Concentration of TNF-α in the rabbits of the infected and control groups after seven days post-inoculation

## 4. Discussion

The isolate of *C. freundii* was obtained from the ulcers of the gills, skin, and fans of the infected fish and cultured in Mac Conkey agar. It was then confirmed and identified by an API 20 NE system. These findings were consistent with the results obtained from a study conducted by Al-Haider, Al-Niaeem (14). They isolated the bacterial isolates from the skin, intestine, and gills of fish cultivated in floating cages in the Al-Hilla river of Babylon province and cultured on MacConkey agar medium and identified by the Vitek II system.

Our data revealed various pathological lesions in all vital organs demonstrating grossly congestion, hemorrhage, and edema, while the different microscopic observations in visceral organs for the lung noticed pneumonia with the thickening of the alveolar wall due to neutrophils with lymphocytes, macrophages, plasmacytes, and fibroblasts aggregation, congestion of alveolar capillaries, and severe interstitial and intra-alveolar edema, followed by extensive pulmonary hemorrhage and pulmonary emphysema with areas of pulmonary atelectasis. These findings are in agreement with the results of previously conducted studies (8, 15), in which researchers showed that *C. freundii* was responsible for many nosocomial infections, pneumonia, septicemia, wounds infections and bloodstream, urinary tract infection, gastroenteritis, endocarditis, brain abscesses, and meningitis with high morbidity and mortality (5).

Our microscopic findings of the liver revealed hepatitis characterized by perivascular and periportal leukocyte cuffing, especially mononuclear cells, such as macrophages, lymphocytes, plasmacytes, and fibroblast with few neutrophils, as well as centrilobular and periportal necrosis. Furthermore, severe interstitial hepatic edema, as well as periportal edema, extensive fibrosis in interlobular septa, and periportal fibrosis with severe interstitial hemorrhage were observed in this study. However, the kidneys showed renal edema and mixed inflammatory exudation, mainly macrophages, lymphocytes, plasmacytes, fibroblast, and neutrophils in the interstitial of the renal parenchyma and renal cortex, atrophy of glomerular tuft with inter glomerular hemorrhage, followed by extensive renal hemorrhage and edema with extensive renal fibrosis, especially in interstitial tissue of the kidney, and severe renal tubular necrosis with the renal epithelial necrosis.

These histopathological observations in the liver and kidneys in the present study were in agreement with the findings of another previously conducted study by Galarneau, Fortin (16). They inoculated *C. freundii* IP with $1.0\times10^8$ CFU/mL in fish for15 days, and after the necropsy of fish, the microscopic changes in the internal organs revealed severe hemorrhages interstitial tissue of the kidney, tubular degeneration, and necrosis associated with acidophilic materials in the tubular lumen, followed by fibrin deposition with coagulative necrosis. The liver appeared with severe hydropic degeneration and centrilobular necrosis, and the gills showed hydropic degeneration with focal areas of coagulate necrosis, interstitial edema, mononuclear infiltration, especially macrophages and lymphocytes, and ulceration with cellular desquamation and telangiectasia (17).

Our histopathological observations noticed enteritis with mucosal and submucosal hemorrhage, edema, and fibrosis in the intestine. These results were consistent with the findings of a study by Kordekag (17) who investigated old Collie and old Maltese puppies that were infected by *C. freundii* and suffered from septicemia. The small intestine of the dogs showed hemorrhagic lesions, and the foci of congestion were often observed in the mucosa of the colon and small intestine.

The results of the current study showed different mean values of total leukocyte count in the infected and control groups of rabbits. Moreover, a significant difference ($P<0.05$) was observed between the infected groups (G1 and G2) in descending order and G3 regarding an increase in the mean values in total

leukocytes on the 3rd and 7th days of the experiment. These data of the present study were in accordance with the results of another previous study by (18). The results revealed a strongly significant increase in the C-reactive protein and procalciton in concentrations, as well as a highly significant increase in the leukocytes and the I:T neutrophil ratio due to an infection by nosocomial Gram-negative bacteria that was higher than that of the Gram-positive bacteria.

A significant difference was also observed in the infected groups, compared to the control negative group regarding an increase in the concentration of TNF-α. These data were compatible with the results of another previous study that observed a significant increase in the TNF-αamong the in pediatric patients infected by Gram-negative bacteria which led to the elevated levels of TNF-α (19).

Additionally, they demonstrated a significant increase in the mean levels of TNF-αin Gram-negative bacteria, which was in accordance with the results obtained from the present study (19).

## Authors' Contribution

Study concept and design: S. R. S. A.
Acquisition of data: G. J. K. A.
Analysis and interpretation of data: S. R. S. A.
Drafting of the manuscript: G. J. K. A.
Critical revision of the manuscript for important intellectual content: S. R. S. A.
Statistical analysis: G. J. K. A.
Administrative, technical, and material support: S. R. S. A.

## Ethics

The study protocol was approved by the veterinary medicine college council in Wasit University, Wasit, Iraq.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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