# THE ROLE OF *IFN*- γ AND *TNF*- α IN EXPERIMENTAL MASTITIS

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**ABSTRACT**

**This study was design to characterize the immune response in experimentally *Pseudomonas aeruginosa* mastitis mice treated probiotic bifidocin and cazacin of *Bifidobacterium spp*. and *Lactobacillus casei*. We quantified the level of the IFN-γ and TNF-α cytokines in blood by ELISA technique. IFN-γ level was significantly higher in infected group compared to control (340.21 ± 41.61, 8.45 ± 0.83 pg/ml, respectively). While the level of IFN-γ was significantly higher in mastitis mice than bifidocin and cazacin treated mice. Also, TNF-α level showed a significant increase in mastitis mice compared to controls (320.11±40.33, 8.45±0.83pg/ml, respectively). Among mastitis and bifidocin (9 and 18 mg/ml), cazacin (11 and 22 mg/ml) treated mice a high level of TNF-α was observed in these groups without variant significant differences. These suggest that *Pseudomonas aeruginosa* caused mice's mastitis developed a cell-mediated response. In addition, the extracts of bifidocin and cazacin have a possibility to use in treatment and prevention of mastitis infections and able to modulate the levels of cytokines in lactating mice.**

**Key words: *Pseudomonas aeruginosa*, immune response, ELISA, bifidocin and cazacin.**



## دور IFN- γ و TNF- α في الفئران المصابة تجريبيا بالتهاب الضرع

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**المستخلص**

**من أجل وصف الاستجابة المناعية في الفئران المصابة تجريبيا ببكتيريا *Pseudomonas aeruginosa* المسببة لالتهاب الضرع والتي تم معالجتها بمستخلصات المعززات الحيوية bifidocin وcazacin لبكتريا.*Bifidobacterium* spp و*Lactobacillus casei*. تم تقييم مستوى انترفيرون- كاما وعامل التنخّر الورمي-ألفا في الدم باستخدام تقنية الفحص المناعي المرتبط بالإنزيم. كان مستوى انترفيرون-كاما مرتفع معنويا في مجموعة الفئران المصابة مقارنة بمجموعة السيطرة (340.21 ± 41.61 ,8.45 ± 0.83 الغرام/ مل،على التوالي). كذلك كان مستوى الانترفيرون- كاما مرتفع معنويا في مجموعة فئران التهاب الضرع مقارنة بمجموعة الفئران المعالجة بمستخلصات bifidocin وcazacin. أظهرت نتائج مستوى عامل التنخر الورمي-ألفا أيضا زيادة معنوية في مجموعة الفئران المصابة مقارنة بمجموعة السيطرة (320.11 ± 40.33 ,8.45 ± 0.83 الغرام/مل ،على التوالي). بين مجموعة الفئران المصابة ومجموعة الفئران المعالجة بمستخلص bifidocin (9 و18ملغم/ مل)، ومستخلص cazacin (11و22ملغم/مل)، لوحظ ارتفاع عامل التنخر الورمي-ألفا في هذه المجاميع باختلافات إحصائية غير متباينة. اشارت النتائج الى ان مجموعة الفئران المصابة ببكتيريا *Pseudomonas aeruginosa* طورت الاستجابة الخلوية، بالإضافة الى أن مستخلصات bifidocin وcazacin لها قدرة في معالجة او الوقاية من التهاب الضرع و تعديل مستويات السايتوكينات في الفئران المرضعة.**

**الكلمات المفتاحية: *Pseudomonas aeruginosa*،الاستجابة المناعية، تقنية الفحص المناعي المرتبط بالانزيم، bifidocin وcazacin.**

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## INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen associated with the mastitis in dairy animals (14). The infections caused by *P. aeruginosa* are sophisticated to cure and oftentimes required combination therapies. In addition, the antibiotic resistance caused by *P. aeruginosa* is an increasing problem (27). In severe cases, the affected animal gland might be necrotic or even gangrenous and the milk bloody (6). These complications due to the contaminated environment with the causing disease's microorganisms when the host defenses are decreased by stresses, concomitant diseases, or the nutritional imbalances (16). Often, the environmental sources of these pathogenic bacteria are the soil and water-related (such as hoses and muddy pastures) (7). As mentioned previously, *P. aeruginosa* considers the most common bacterial pathogens that widespread distribution in the health care settings (32). Also, bacteria especially that *Bifidobacterium and Lactobacillusrelated* genera are the most known probiotic organisms (22). Pathogens are microorganisms that often-caused diseases in their hosts. So, the potential mechanisms to inhibit the pathogen invading include the production of anti-microbial substances such as bacteriocins, enhancing the epithelial barrier through attachment, competition for pathogenic-binding sites, and modulation the immune system (4). The *Lactobacillus casei* bacteria are of interest as a probiotic through its role for health promotion in treatment or prevention of a number of diseases and disorders (8). Also, it found to produce many bioactive metabolites that confer host benefits when it consumed (9). Natural-killer cells (NK), T-helper lymphocytes class one cells (Th1) and antigenic presenting cells (APCs) secreted IFN-γ cytokines. Else, IFN-γ plays a role in resistance and immune response to parasites, viruses, intracellular and extracellular bacteria (30,33). The production of IFN-γ is affected by the stimulation of Interleukin 12 (IL-12) secretion via neutrophils, macrophages, dendritic cells after the stimulation with lipopolysaccharide (LPS), other microbial products (12), affected and promotes of IFN-γ secretion by naive $CD_4^+$ T cells and NK cells (18). Else, TNF-α has been indicated as an important mediator of neutrophil recruitment to the inflammation sites. Some previous studies have shown that the level of TNF-α is increased in the milk from udders infected with *P. aeruginosa* (13). The aim of the research is to treat experimental mastitis infection in mice as an alternative laboratory model to cows by the extracts of bifidocin and cazacin from probiotic bacteria and evaluate their probiotic role in immunity**.**

## MATERIALS AND METHODS

### Samples collections for *Pseudomonas aeruginosa* isolates

Fifty milk samples were collected from cow's mastitis aseptically in 10 ml sterile plastic vials from each animal in different areas of Baghdad to isolate Pseudomonas aeruginosa. Samples of cow's milk were streaked on MacConkey and blood agar and incubated at 37 ºC for 24 hr., the suspected colonies were sub-cultured by using a selective media (Chromogenic and Cetrimide media) to obtain pure isolates, biochemical tests were used to identify Pseudomonas aeruginosa isolates such as citrate utilization, catalase, oxidase, motility, Indole production, methyl red, Voges-Proskauer, urea hydrolysis and TSI (20).

### Samples collections for *Bifidobacterium spp.* and *Lactobacillus casei.* Isolates

Fifty samples of healthy bovine milk were collected to isolate *Bifidobacterium spp. and Lactobacillus casei* from the different area around Baghdad city, then 10 ml of each 50 samples of milk were centrifuged at 1250 rpm for 10 minutes, then the supernatant was discarded. While the sediment was cultured on Man Rogosa Sharpe medium (MRS) with tween 80, then incubated anaerobically for 48 hrs.at 37 ºC, then detection by Sugar fermentation test was used to identify the isolated colonies (2).

### A Confirmation Diagnosis Test (Remelrapid$^{tm}$ And Ii System) For *Bifidobacterium spp.* and *Lactobacillus casei* detection

The principle of RapID$^{TM}$ ANA II System that used is based on microbial degradation of the specific substrates revealed by various indicator systems. The reactions used are a combination of traditional exams and single-substrate chromogenic exams.

**Detection of bifidocin and Cazacin Extracts Produced By *bifidobacterium spp*. and *Lactobacillus casei***

Bifidocin and Cazacin extracts were purified from the 50 milk samples as described by (11,19). The protein concentration was determined by Bradford method, then purified by Gel filtration chromatography technique (31). The molecular weight of extract was determined by gel filtration chromatography method by sepharose-6B based on the standard curve made by standard protein, the *lactobacillus casei* was 4.3 (KD) while the *Bifidobacterium spp.* was 17.6 (KD)**.**

**Expermintal design**

Mastitis induced in mice by infected experimentally with *Pseudomonas aeruginosa*, then infected lactating mice treated by IP injected bifidocin with two concentrations 18 and 9 mg/ ml, and cazacin extracts (22 and 11 mg/ ml) for a week. From total 50 mice, blood samples were collected from 5 mastitis infected mice and from 10 of each bifidocin (9 and 18 mg\ml) and cazacin (11, 22 mg/ ml) mastitis treated mice in addition to 5 blood samples from healthy control mice group to determine the serum IFN-γ and TNF-α levels by sandwich ELISA technique (peproTech,USA), after centrifuged the clotted blood in 3000 R.P.M. for near 10 min, the serum were stored at −80 °C until use. The statistics with Least significant difference – LSD test Analysis of the Variation ANOVA used to compare between means in this research (28).

**RESULTS AND DISCUSSION**

**Assessment of Ifn- Γ In Mastitis Group**

The results of IFN-γ serum level showed a significantly increased P≤0.01 level of IFN-γ serum level in infected mice group compared to the healthy control group (340.21 ± 41.62, 8.45 ± 0.8 pg/ ml, respectively), as shown in (Table1). When a comparison between mastitis infected mice and mastitis treated mice with bifidocin extracts with doses 9 and 18 mg/ ml and cazacin extracts with doses 11 and 22 mg/ ml at the first week, The mean of IFN-γ serum level significantly increased P≤0.01in infected mice group compared to the two bifidocin extract doses (9 and 18 mg/ ml) of the treated mice group (340.22± 41.6,51.86 ±18.35and 162.43 ± 36.55, respectively). Also, a significant increase in IFN-γ serum level in mastitis treated mice with 18 mg/ml bifidocin than 9 mg/ml (162.43 ±36.55,51.86±18.35, respectively), (Table2). Such, variants result appearedP≤0.01 when a comparison of the infected mice group and the mastitis treated mice with cazacin extract doses group (11 and 22 mg/ ml) in the first week (340.22 ± 41.6, 104.04 ± 30.55and 78.75± 4.08, respectively), (Table 3). The results of mastitis infected mice and mastitis treated mice with bifidocin extracts with doses 9 and 18 mg/ ml and cazacin extracts with doses 11 and 22 mg/ ml for the second week appeared a significantly increased P≤0.01 level of IFN-γ in infected mice compared to mastitis treated mice in both doses of bifidocin 9 and 18 mg/ml (340.22 ± 41.61, 51.74 ± 17.07 and 143.09 ± 35.57, respectively) (Table4), Differ appeared P≤0.01 when comparisons among the infected mice group (340.22 ± 41.61pg/ml )and mastitis treated mice with cazacin extract doses 11 and 22 mg/ ml (107.07 ± 33.99, 77.50 ± 7.69) (Table 5).

**Assessment of TNF-α in Mastitis group**

Blood TNF-α was significantly higher in the infected group of mice (320.11 ± 40.33 pg/ml) than control (8.45 ± 0.83 pg/ml) P≤0.01 (Table 6). Mastitis treated mice with (bifidocin 9mg\ml, 18 mg/ml) and (cazacin extracts 11mg/ml, 22 mg/ml) showed no significant difference P>0.05 in TNF-α concentration than mastitis group, except in group of mice treated with low dose of *Bifidobacterium spp*.(9mg/ml) at first week only showed significant decrease P≤0.05 in TNF-α level (Table,7,8,9,10) The gram-negative bacteria commonly caused many clinical cases of mastitis. Among these bacteria, *P. aeruginosa* considers one of the most intramammary infections characterized by strong resistance to several antibiotic therapies. Despite this, little is known about the immune response to *P. aeruginosa* (1,10). Following the establishment of infection and treatment with by bifidocin and cazacin, the current study descript the immune response to the intramammary infection by *P. aeruginosa* in mice through the increased level of IFN-γ and TNF-α in the mastitis group experimentally infected by *P. aeruginosa* compared to the healthy control (340.22 ± 41.61 pg/ml and 320.11 ± 40.3

pg/ml, 8.45 ± 0.83 pg/ ml). In the inflammatory response, the mammary gland immune system is activated to eradicate the pathogen, this defense mechanism comprised cellular, anatomical, and soluble subjects that act in coordination, such these subjects are critical to the modulation the mammary gland confrontation and susceptibility to infection. The migration of Neutrophil from the bloodstream to mammary gland tissue occurs as a response to the proinflammatory cytokines, such as TNF-α. In addition, several cytokines might also increase the phagocytic bactericidal activity; bacteria are able to modify the production of cytokines in the mammary gland immune system cells (3, 24, 29), *P. aeruginosa* slime-glycolipoprotein (slime-GLP) is the most potent stimulant substance for the production of TNF-α and NF-kB activation in human monocytes, the secretion of TNF-α by human monocytes induced by *P. aeruginosa* slime-GLP, LPS or viable bacteria, was paralleled by the phosphorylation and/or the activation of Mitogen-activated Protein Kinases (MAPKs) (17) .IFN-γ facilitates the direction of the leukocytes to infection sites through expression regulation of the chemokines and some adhesion molecules. On the other hand, IFN-γ antimicrobial effector activates are demonstrated by increasing receptor-mediated phagocytosis as well enhancing microbial killing in neutrophils and macrophages (5,15,23). Also, it appeared that the mice suffering lacking IFN-γ response have been decreased their natural resistance to the viral, bacterial, parasite infections (25),the immune response may differ according to the host's defence system and bacterial isolates, with the observation of varied individual variation (26).For bifidocin and cazacin treated mice groups, the level of IFN-γ was in high concentration, but it remained significantly higher in mastitis animals than bifidocin and cazacin treated groups, while a non-significant high level of TNF-α was observed among the mastitis and the treated mice with bifidocin and cazacin groups. The pathogen inhibition facilitated through nutrient competition, production of inhibitory substances (organic acids, bacteriocins, $H_2O_2$), removal/degradation of toxin, adherence sites competition (mucus, receptors of cell), co-aggregation and virulence modulation and induction the immune responses of the host. So that the ability of pathogens inhibition is one of the three master probiotics mechanisms, the enhancement of barrier function and immune ability and interactions being the other two mechanisms (21).

**Conclusion**

*IFN*-γ and TNF-α *level* was higher in *Pseudomonas aeruginosa* mastitis mice compared to control. For bifidocin and cazacin treated mice the level of IFN-γ decrease compare to mastitis animals, high level of *TNF*-α was observed in bifidocin and cazacin treated groups as in mastitis. The extracts of bifidocin and cazacin can treat mastitis infections and change level of immunity in nursing mice

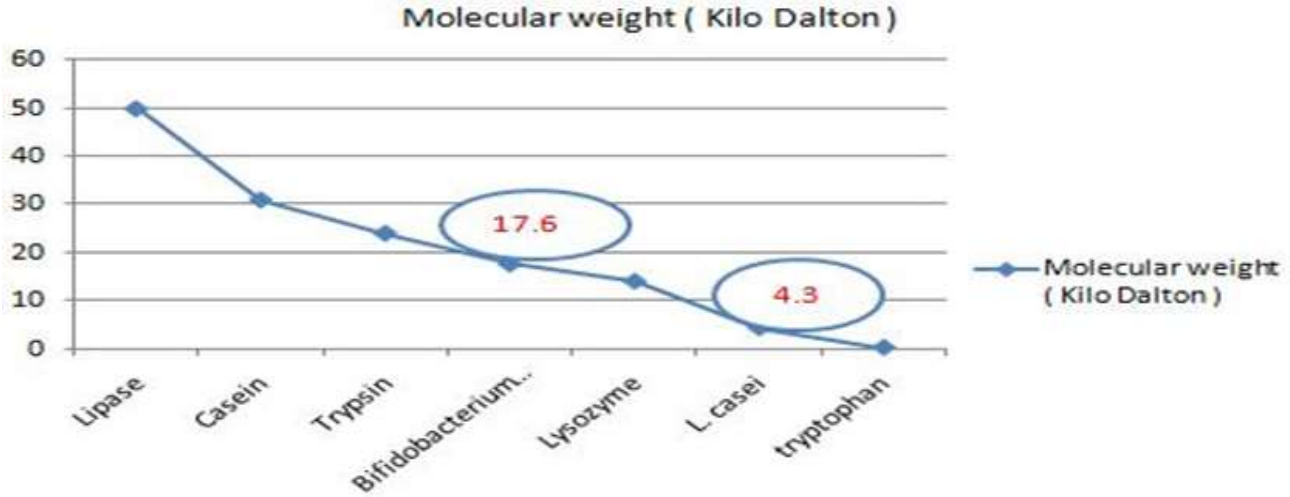


**Figure1. The molecular weight of Cazacin and Bifidocine by gel filtration chromatography methods using standard protein of different molecular weight**

**Table1. IFN- γ serum level in *Pseudomonas aeruginosa* infected group comparison with control group**

| Groups | Mean ± SE of conc.(pg/ml) of *IFN-γ* |
|---|---|
| Infected mice (5) | 340.21 ± 41.62 a |
| Control (5) | 8.45 ± 0.83 b |
| LSD value | 95.995 ** |
| P-value | 0.0001 |
| Means having with the different letters in column differed significantly. ** (P≤0.01). | |

**Table 2. INF- γ level in infected group with *Pseudomonas aeruginosa* comparison with treated mastitis group with Bifidocin extracts after one weeks of treatment**

| Groups | No. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 340.22 ± 41.61 a |
| Treated mice with low dose of Bifidobacterium spp.9 mg\ml, | 5 | 51.86 ± 18.35 c |
| Treated mice with high dose of Bifidobacterium spp.18 mg\ml | 5 | 162.43 ± 36.55 b |
| LSD value | | 103.81 ** |
| P-value | | 0.0002 |
| Means having with the different letters in column differed significantly. ** (P≤0.01). | | |

**Table 3. Level of blood *IFN-γ* in *Pseudomonas aeruginosa* treated group with cazacin after one week of treatment.**

| Groups | NO. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 340.22 ± 41.61 a |
| Treated mice with low dose of cazacin extracts 11 mg \ml | 5 | 104.04 ± 30.55 b |
| Treated mice with high dose cazacin extracts 22 mg \ml | 5 | 78.75 ± 4.08 b |
| LSD value | | 92.135 ** |
| P-value | | 0.0001 |
| Means having different letters in column differed significantly. ** (P≤0.01). | | |

**Table 4. IFN-γ Level in infected mice with *Pseudomonas aeruginosa* comparison with treated mastitis group with Bifidocin extracts after two weeks of treatment**

| Groups | NO. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 340.22 ± 41.61 a |
| Treated mice with low dose of *Bifidobacterium spp*. (9 mg\ml) | 5 | 51.74 ± 17.07 b |
| Treated mice with high dose of *Bifidobacterium spp*. (18 mg\ml) | 5 | 143.09 ± 35.57 b |
| LSD value | | 102.03 ** |
| P-value | | 0.0002 |
| Means having different letters in column differed significantly. ** (P≤0.01). | | |

**Table 5. Level of blood *IFN-γ* in *Pseudomonas aeruginosa* treated group with cazacin after two weeks of treatment.**

| Groups-2 | No. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 340.22 ± 41.61 a |
| Treated mice with low dose of cazacin extracts 11 mg \ml | 5 | 107.07 ± 33.99 b |
| Treated mice with high dose of cazacin extracts 22 mg \ml | 5 | 77.50 ± 7.69 b |
| LSD value | | 96.571 ** |
| P-value | | 0.0001 |
| Means having different letters in column differed significantly. ** (P≤0.01). | | |

**Table 6. TNF-α serum level in *Pseudomonas aeruginosa* infected group comparison with control group**

| Groups | Mean ± SE of conc.(pg/ml) of *TNF-α* |
|---|---|
| Infected mice (5) | 320.11 ± 40.33 |
| Control (5) | 8.45 ± 0.83 b |
| LSD value | 95.995 ** |
| P-value | 0.0001 |
| Means with different letters in column differed significantly. ** (P≤0.01). | |

**Table 7. TNF- α level in infected mice with *Pseudomonas aeruginosa* Comparison with treated mastitis group with Bifidocin extracts after one week of treatment**

| Groups-1 | No. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 320.11 ± 40.33 |
| Treated mice with low dose of *Bifidobacterium spp*.( 9 mg\ml) | 5 | 209.48 ± 6.33 b |
| Treated mice with high dose of *Bifidobacterium spp*.( 18 mg\ml) | 5 | 295.97 ± 24.13 a |
| LSD value | | 86.329 * |
| P-value | | 0.0188 |
| Means with different letters in column differed significantly. * (P≤0.05). | | |

**Table 8. Level of blood** *TNF*-α in ***Pseudomonas aeruginosa* treated group with** cazacin **after one week of treatment**

| Groups-1 | NO. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 320.11 ± 40.33 a |
| Treated mice with low dose of cazacin extract 11 mg \ml | 5 | 272.36 ± 8.07 a |
| Treated mice with high dose cazacin extracts 22 mg \ml | 5 | 271.33 ± 12.89 a |
| LSD value | | 78.833 NS a |
| P-value | | 0.202 |
| NS: Non-Significant. | | |

**Table 9. TNF- α level in infected mice with *Pseudomonas aeruginosa* comparison with treated mastitis group with Bifidocin Extracts After Two Weeks of Treatment**

| Groups-2 | No. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 320.11 ± 40.33a |
| Treated mice with low dose of *Bifidobacterium spp*.(9 mg\ml) | 5 | 211.28 ± 49.73 a |
| Treated mice with high dose of *Bifidobacterium spp*.(18 mg\ml) | 5 | 322.38 ± 43.97 a |
| LSD value | | 139.40 NS |
| P-value | | 0.134 |
| NS: Non-Significant. | | |

**Table 10. Level of blood** *TNF*-α in ***Pseudomonas aeruginosa* treated group with** cazacin **after two weeks of treatment**

| Groups-2 | No. | Mean ± SE |
|---|---|---|
| Infected mice | 5 | 340.22 ± 41.61 a |
| Treated mice with low dose of cazacin extracts 11 mg \ml | 5 | 273.86 ± 12.55 a |
| Treated mice with high dose of cazacin extracts 22 mg \ml | 5 | 297.43 ± 24.94 a |
| LSD value | | 89.166 NS |
| P-value | | 0.295 |
| Non-Significant. | | |

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