In vivo study of probiotic effects on production of some cytokines (IL-4, IL-12 and IFN-γ) in Candida albicans infection.

Hamzia Ali Ajah and Sarah sadeq Al-Quraishi

Department of Biology, College of Science, AL- Mustansirya University, Baghdad, Iraq.

Address For Correspondence:
Hamzia Ali Ajah, Department of Biology, College of Science, AL- Mustansirya University, Baghdad, Iraq.

This work is licensed under the Creative Commons Attribution International License (CC BY).
http://creativecommons.org/licenses/by/4.0/

Received 23 August 2016; Accepted 1 November 2016; Published 20 November 2016

ABSTRACT
Background: The immune mechanisms of defense against candidiasis are numerous, and range from protective mechanisms that were present early in evolution (innate immunity) to sophisticated adaptive mechanisms that are induced specifically during infection and disease. And usually, for treatment of systemic fungal infections, azoles such as fluconazole are used, but one of the biggest problems faced in clinical practice is the emergence of resistance due to mutation for most of these azoles drugs currently used. Objective: Laboratory mice animals infected with Candida albicans isolate were used to study the treatment with probiotic cell, probiotic supernatant and probiotic cell & supernatant of probiotic type Lactéol® fort, and the current study focus on detecting the concentration of cytokines (IL-4, IL-12 and IFN-γ) of infected C. albicans mice and control by used sandwich ELISA to quantify these cytokines in serum. Results: The data suggests these cytokines were increased in all experimental groups in first and second week compared to the negative control (Immunological suppress) group, the results of statistical analysis showed different significant (P ≤ 0.05) for treatment groups (probiotic cell treatment, probiotic supernatant treatment and probiotic cell & supernatant treatment) compared with control positive groups and control negative, also compared with fluconazole treatment. Conclusion: The level of IL-4, IL-12 and IFN-γ were increased in the three groups but the highest increase was observed in probiotic cell & supernatant group, then probiotic supernatant group, lowest level was in the probiotic cell only with significant different (P ≤ 0.05) between three groups.

KEYWORDS: Candida albicans, cytokin, probiotics, IFN-γ, IL-12, IL-4

INTRODUCTION

Among species of the genus Candida, C. albicans is the pathogen most frequently isolated from the human body, including the oral cavity and gastrointestinal and genitourinary tract. This species capable of causing life-threatening opportunistic fungal infections. C. albicans is considered opportunistic pathogen can cause harmless under abnormal conditions [1,2]. When the immune system is suppressed, this yeast can multiply rapidly, penetrate the intestinal lining and move into the blood stream, Yeast population is controlled by probiotic or "friendly" bacteria[3].

Innate immunity is the first line of unspecific host defense against pathogens carried out by macrophages, neutrophils, and dendritic cells[4]. In adaptive immunity both CD4+ (T helper cells) and CD8+ (cytotoxic T cells) have been reported a play role in antifungal immunity, the nature of the T cell response is established by the cytokine of the T cells during encounter activation: IL-12/IFNγ for Th1 cells, IL-4 for Th2 cells, IL-1β/IL-6/IL-23 for Th17 cells, and IL-2/TGF-β for Treg cells[5].
Probiotics are "live microorganisms that grant a health benefit to the host when used in appropriate amounts[6]. The best and common studied probiotics are species of the bacteria Lactobacillus, Bifidobacteria, and Streptococcus, Enterococcus, nonpathogenic E. coli, Bacillus and the yeast Saccharomyces boulardii [1]. Probiotics have been extensively studies due to their remarkable ability to inhibit the growth of other organisms through bactericidal activity and by producing lactic acid as product of metabolism. Lactic acid production, production of bacteriocins, and the production of hydrogen peroxide have led to an abundance of search involving the ability of probiotic to inhibit pathogens[7]. This research was designed to Study the probiotic effects on production of some cytokines(IL-4, IL-12 and IFN-γ).

MATERIAL AND METHODS

Candida albicans isolate:

*Candida albicans* isolate was obtained from Mycology Lab, Biology Department, Al-mustansryah University. The isolate was grown on sabourand dextrose broth(SDB) until used.

**Preparation of probiotics:**

We used probiotic that posed in the markets under the trade name (Lactéol® fort) product by Company Rameda (Egypt), each gram of it contain the following microorganism:

- *Lactobacillus delbruekii* (10⁸)cfu/g and *L. fermentum* (10⁹)cfu/g.

The probiotic overnight cultures contained 1.5x10⁸ CFU/ml were grown in MRS broth at 37°C for 24 hrs. under anaerobic conditions. These cultures were centrifuged at 6000 rpm/min for 10 min at 4°C. The resulting supernatants were filtered through a 0.22 µm membrane filter to remove the remaining bacteria and debris, and then supernatants concentrated to two fold[8].

**Antagonistic activity of probiotic in C. albicans in vivo:**

Sixty Male *albino* mice aged 8-12 weeks, weighing 20-28 g were obtained from National Center for Drug Control and Research, housed under standard condition in animal house of biology department in College of science /AL-Mustansriyah University.

Fifty-four mice were injected, for three days, with dexamethasone 2.5mg/kg/day intramuscular [9], 30 mice were infected by injected with 0.1ml of *C. albicans* isolate that contain (1.5x10⁹) yeast/ml intraperitoneal .After three days, all mice were divided into 9 groups each group contain 6 mice, each group inoculated as a follow:

**Group 1** (none infected immunological suppress): inoculated orally by stomach tube (0.1ml/day) normal saline for 14 days consider as negative control.

**Group 2** (infected): inoculated orally by stomach tube (0.1ml/day) normal saline for 14 days consider as control positive group.

**Group 3** (none infected): inoculated orally by stomach tube probiotic cell (0.5ml/day) that contain 1x10⁸ cell/ml for 14 days consider as probiotic cell control group.

**Group 4** (infected): inoculated orally by stomach tube probiotic cell (S isolate) (0.5ml/day) that contain 1x10⁹ cell/ml for 14 days consider as probiotic cell treatment group.

**Group 5** (infected): inoculated orally by stomach tube with (0.06 ml/day) from fluconazole drug for 14 days consider as fluconazole treatment group.

**Group 6** (none infected): inoculated orally 0.5ml/day, by stomach tube, probiotic supernatant that concentrate for two times for 14 days consider as probiotic supernatant control group.

**Group 7** (infected): inoculated orally 0.5ml/day, by stomach tube, probiotic supernatant that concentrate for two time for 14 days consider as probiotic supernatant treatment group.

**Group 8** (none infected): inoculated orally by stomach tube probiotic cell and supernatant (0.5ml/day) that contain 1x10⁸ cell /ml for 14 days consider as probiotic cell and supernatant control group.

**Group 9** (infected): inoculated orally by stomach tube probiotic cell and supernatant (0.5ml/day) that contain 1x10⁹ cell /ml for 14 days consider as probiotic cell and supernatant treatment group.

**Group 10** (non-infected and non-immunological suppress): inoculated orally by stomach tube (0.1 ml/day) normal saline for 14 days consider as control negative 2.

**Collection of Blood:**

After 7 and 14 days’ post-infection and treatment, from each mouse about 2 ml of blood were collected from eye. The blood was dispensed in a plain tube to collect serum (after clotting, blood was centrifuged at 2000 rpm for 15 minutes at room temperature, and then serum was separated, put in tube and stored at -20°C until assayed.

**Serum Level of Cytokines:**
Serum level of three cytokines (IL-12, IL-4, and IFN-γ) were determined by ELISA method using KOMA Cytokine ELISA Kits (Korea), which were designed for the quantitative measurement of cytokine in mice.

Principles of KOMA Cytokine ELISA Kits:

The three kits were based on similar principles, in a sandwich ELISA format in which a monoclonal antibody specific for the cytokine was coated onto wells of microtiter plate. Samples are added into these wells, and during the first incubation period, the antigen of standards or cytokine in samples and a monoclonal antibody specific for these cytokine are simultaneously banded. After washing, Added Detection Antibody and after incubate and was then added the enzyme streptavidin- horseradish peroxidase (HRP) that binds the biotinylated antibody, incubated and washed. Then added (Tetramethyl benzidine) TMB substrate solution, which acts on the bound enzyme to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of cytokines present in the samples.

Fig. 1: Standard curve of IL-4 by using ELISA.

Fig. 2: Standard curve of IL-12 by using ELISA.
Fig. 3: Standard curve of INF-γ by using ELISA.

**Statistical analysis:**
Minitab software version 6 was used data analyzing. The ANOVA - test has been done to calculate the P value between the control and test groups IN THE previous studies. Least significant difference- LSD test was done also to compare means between groups in this study. The results were presented as mean ± SD. A P value equal or less than 0.05 was considered as the level of statistical significance.

**RESULTS AND DISCUSSION**

**A. Serum level of IL-4:**

Serum level of IL-4 was increased in all experimental groups in two weeks compared to the negative control (Immunological suppress) group (23.66 ± 4.04 pg/ml)(Table 1). In positive control (infected with *C. albicans*) group the lowest level was in the second week (39.33 ± 9.61 pg/ml).

Whereas the results of probiotic cell treatment group mice were observed increased the level of IL-4 after first week (28.33 ± 7.64 pg/ml), then increased in high levels at second weeks (40 ± 14.14 pg/ml) but remained lower than probiotic cell control group at first and second weeks (45 ± 7.11 and 52 ± 10.44 pg/ml) respectively.

While after treatment infected mice with probiotic supernatant the level of IL-4 was begin increased at first and second weeks (48.33 ± 23.11 and 53 ± 9.53 pg/ml) respectively compared to the probiotic supernatant control groups at first and second weeks (39.33 ± 16.9 and 31.66 ± 5.77 pg/ml) respectively.

In treatment infected mice with probiotic cell and supernatant groups, the level of IL-4 at first week was reached to 40.33 ± 4.5 pg/ml and then increased to highly level at second week (84 ± 17.69 pg/ml) compared to the control the level of probiotic cell and supernatant group at first and second weeks (38.33 ± 17.55 and 54.33 ± 5.51 pg/ml) respectively.

While in fluconazole treatment group the results have been showed high level occurred in first week (41.66 ± 14.34 pg/ml) and then got high level in the second week (55.33 ± 2.88 pg/ml) with significant difference (p ≤ 0.05) found within the same groups and with immunological suppress negative group.

**Table 1:** Serum level of IL-4 in experimental groups and control groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean (Pg/ml) ± SD</th>
<th>One week</th>
<th>Two week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic cell treatment</td>
<td>28.33 ± 7.64</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Probiotic cell control</td>
<td>45 ± 7.11</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Probiotic supernatant treatment</td>
<td>48.33 ± 23.11</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Probiotic supernatant control</td>
<td>39.33 ± 16.9</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Probiotic cell and supernatant treatment</td>
<td>40.33 ± 4.5</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Probiotic cell and supernatant control</td>
<td>38.33 ± 17.55</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Fluconazole treatment</td>
<td>41.66 ± 14.34</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Negative control (Immunological suppress)</td>
<td>23.66 ± 4.04</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Negative control (non-Immunological suppress)</td>
<td>42 ± 4.36</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Positive control (Infected)</td>
<td>43.66 ± 7.14</td>
<td>***</td>
<td>**</td>
</tr>
</tbody>
</table>

The stars * means significant difference (P ≤ 0.05) of IL-4 with experimental groups and negative control (Immunological suppress).
B. serum level of IL-12:

The IL-12 of appeared significantly (P ≤ 0.05) increased in all treatment group compared to immunological suppress negative control (98.66 ± 8.08 pg/ml) (table 4-9).

In the positive control group, the level of IL-12 in first week was (117.5 ± 28.99 pg/ml), while in second week reached to (193.66 ± 65.68 pg/ml).

In the probiotic cell treatment group mice, it was observed increased the level of cytokine after first week (129.67 ± 11.01 pg/ml) and reached to (133 ± 17.11 pg/ml) at second week, but remained higher than probiotic cell control group at two weeks (91.33 ± 53.59 and 128.33 ± 24.82 pg/ml) respectively. As well as in probiotic supernatant treatment group IL-12 level increased in the first week (141 ± 9.53 pg/ml) and in second week (153 ± 26.5 pg/ml), but remained lower than probiotic supernatant control second week (161 ± 6.1 pg/ml).

While after treatment infected mice with probiotic cell and supernatant the level of IL-12 was increased and reached to high level in the second week (158.33 ± 7.6 pg/ml) and there was no significant different (P ≥ 0.05) found between treatment probiotic cell and supernatant control group (158 ± 21 pg/ml).

In the treatment fluconazole group, the level of IL-12 was observed the highest level during the first week (162 ± 40.58 pg/ml), while the level in second week was more decreasing (90 ± 10 pg/ml).

Table 2: Serum level of IL-12 in experimental groups and control groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean (Pg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One week</td>
</tr>
<tr>
<td>Probiotic cell treatment</td>
<td>129.67 ± 11.01 **</td>
</tr>
<tr>
<td>Probiotic cell control</td>
<td>91.33 ± 53.59 *</td>
</tr>
<tr>
<td>Probiotic supernatant treatment</td>
<td>141 ± 9.53 ****</td>
</tr>
<tr>
<td>Probiotic supernatant control</td>
<td>125.66 ± 91.55 ****</td>
</tr>
<tr>
<td>Probiotic cell and supernatant treatment</td>
<td>128 ± 25.11 ***</td>
</tr>
<tr>
<td>Probiotic cell and supernatant control</td>
<td>157.66 ± 32.34 ****</td>
</tr>
<tr>
<td>Fluconazole treatment</td>
<td>162 ± 40.58 ****</td>
</tr>
<tr>
<td>Negative control (Immunological suppress)</td>
<td>98.66 ± 8.08 **</td>
</tr>
<tr>
<td>Negative control (non-Immunological suppress)</td>
<td>141 ± 10.26 **</td>
</tr>
<tr>
<td>Positive control (Infected)</td>
<td>117.5 ± 28.99 **</td>
</tr>
</tbody>
</table>

The stars * means significant difference (P ≤ 0.05) of IL-12 with experimental groups and control negative (Immunological suppress).

C. Serum level of IFN-γ:

Most of experimental groups showed significant (P ≤ 0.05) increased in the level of IFN-γ compared to negative control (78.3 ± 1.5 pg/ml) (table 4-10).

In positive control group highest level was in the first week (162.66 ± 56.19 pg/ml), then returned decreased in the second week (131.33 ± 37.52 pg/ml).

Whereas the results of probiotic cell treatment showed the increase of IFN-γ level has been gradual since the first week (107.67 ± 4.04 pg/ml) and reached to (136 ± 13.4 pg/ml) in second week compared to probiotic cell control, the IFN-γ level increased in first week (312 ± 181.01 pg/ml), then drop back to (172.33 ± 52 pg/ml) in second week.

In probiotic supernatant group it was observed the highest level after one week (174 ± 8.72 pg/ml) then gradually lowered to (154.66 ± 37.8 pg/ml) in the second week, compared to probiotic supernatant control the high level after one week and retinal to decreased in the second week (208.66 ± 111.93 and 176.33 ± 47.5 pg/ml) respectively.

When treatment infected mice with probiotic cell and supernatant, the level of IFN-γ was increased in first and second week (145.33 ± 12.8 and 173.33 ± 15.2 pg/ml) respectively, while the level in control group increased in first week and decreased in the second group (178.66 ± 128.46 and 171 ± 30.64 pg/ml) respectively.

The highest increase in the first week was showed when mice treated with fluconazole (223.66 ± 89.53 pg/ml) then decreased in the second week (81.33 ± 6.1 pg/ml).
This result agrees with result of study done by Elahi et al., [10]) who found IL-12 and INF-γ were higher produced at 7 days from starting treat, the levels of cytokines remained high before decline at 15 days from initiation treat.

Increasing IL-4 in our study partially agree with study done by Haghighi et al., (11) who found ability of probiotic to elevate IL-4.

Elevated IL-12 in our study partially related with result of study done by Ho et al., [12] found groups who consumed the probiotics that had the highest effect on the production of IFN-γ.

Our experiment result of elevated INF-γ by probiotics agree with study done be You and Yaqoob, (13) who found ability of probiotic to induce production of INF-γ.

Monachese et al., [14] mentioned that probiotic has capacity to increase many cytokines including IL-12, IFN-γ and IL-4 production, this result had close near with our study result. Also this result agrees with local study result done by Al-Kafaji, [15] who found Lactobacillus probiotics have able to stimulate IL-4, IL-12 and INF-γ production.

In other study done by Neau et al., [16] reported undetectable or very low levels of IL-4 were produced by all probiotic treat, whereas every treat produced significant high secretions of IL-12 and IFN-γ.

The IL-12 and IFN-γ have higher levels during all three treat of our study partially similar to study done by Ho et al., [12] who reported increase INF-γ may due to NK cell can release IFN-γ to stimulate the dendritic cells (DCs) to produce IL-12, and then IL-12 will amplify the IFN-γ production from NK cell. This positive feedback process establishes the increasing in cytokines response. Horinaka et al., [17] who demonstrated ability of lactobacillus bacteria to enhance INF-γ production.

Decreasing INF-γ level after one week from initiation treatment agree with study done by Eisenhut, [18] who reported ability of IL-4 interferes with TH 1 cell development and reduces the production of IFN-gamma. Hardy et al., [19] show the capacity range of probiotic strains live cells to induce of cytokines drive Th1 differentiation (IL-12), also hence INF-γ production and Th2 differentiation (enhance IL-4 production).

The results in this study was agreement with[20,21] who revealed that IFN-γ was induced by oral administration of Lactobacillus bacteria that referred to the critical role of these bacteria in enhancing immune response against disease in vaccinated mice. IFN-γ is an essential cytokine for both innate and adaptive immune responses. NK cells are the innate cells source of this factor and rabidly produce IFN-γ upon activation. Significant increase in IFN-γ expression by CD+4 T-cells is observed only after T-helper (Th1) differentiation, this cytokine secreted by Th1 cells, induces cell mediated and inflammatory immune responses[22].

Studies have partially agreement with result of our study in ability of probiotic cells, probiotics supernatant and both probiotic cells and supernatant to induce immune system, that reported Whole lactobacilli are able to induce both regulation and activation immune response in a species-specific manner[23,24,25]. Also, live and cells free supernatant of the same lactobacilli strain may have differently affect immune cells[26].

**Conclusion:**

In this present study, we can conclude that,The level of cytokines (IL-4, IL-12 and IFN-γ) were increased in the serum level of mice after treatment with probiotic cell, probiotic supernatant and probiotic cell & supernatant of probiotic type Lactéol® fort, but the highest increase was observed in probiotic cell & supernatant
group, then probiotic supernatant group, lowest level was in the probiotic cell only were increased in all experimental groups in two weeks.

ACKNOWLEDGMENTS

I would never have been able to finish my dissertation without the of my help from Dr. Sabaa Taher Mohammed

REFERENCES


