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Natural Oils Enhance IL-10 and IFN-γ Production by Human PBMCs Cultured with *Malassezia furfur*

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ABSTRACT

Background: *Malassezia furfur* is alipophilic yeast that causes skin disease. **Objective:** To evaluate the level of IL-10, IFN-γ and IL-12P70 in co-incubation of *M. furfur* grown on different forms of natural oils with PBMCs of healthy individuals. **Methods:** PBMCs were obtained from blood samples of normal volunteers. *M. furfur* was cultured in different culture media containing almond oil, fish oil, walnut oil, full-fat milk, and a fat-free medium; and the yeasts grown were harvested and used for co-incubation with PBMCs in vitro. The IFN-γ, IL-10, and IL-12P70 levels were measured at different time intervals using ELISA methods. **Results:** Generally, IFN-γ and IL-10 levels in the co-incubation of yeasts with walnut oil group (WOG) and fish oil group (FOG) were higher than those in the almond oil group (AOG) and full-fat milk group (FFMG). Although the IL-12P70 was higher in groups such as AOG, FOG, and WOG; the increase was not statistically significant. **Conclusion:** The results demonstrated that the type of fat used by *M. furfur* in the culture media can influence the immune response and increases IFN-γ and IL-10 levels in an early time point of the culture system.

Keywords: IL-10, IFN-γ, IL-12, *Malassezia furfur*, Natural Oil, PBMC

INTRODUCTION

Malassezia furfur (*M. furfur*) is a lipophilic fungus, which is present as a component of normal flora on human skin. The microorganism is mostly found on the skin of the skull, is responsible for development of several dermatological and systemic diseases

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including pityriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, and psoriasis (2,3). In some cases, the microorganism causes systemic infections such as septic arthritis, fungemia, meningitis, peritonitis, and pneumonia (4,5). From the cytological viewpoint, compared with other yeasts, *M. furfur* has a thick wall, approximately 0.12 μm, comprising 26-37% of the whole cell volume, and it is estimated that 15-20% of the cellular wall is made up of lipids. With regard to the metabolism, the organism can directly use lipid sources, and does not require vitamins and trace elements (6,7,8). The dependence of the growth of these species on lipids has been described. The lipid source used by the yeast during its growth is not consumed as an energy source; rather it is employed in the construction of a cell wall undergoing the least metabolism and modification (9). It has been demonstrated that in co-incubation of *M. furfur* with fibroblasts of natural human skin, the yeast attaches the fibroblasts within 4 hours and after 24 hours; the yeast enters the cytoplasm of the cells, finally leading to the destruction of these cells (10). Several studies have evaluated the effect of *Malassezia* strains on the host immune response. The results obtained indicate that the yeast has a modulating effect on the immune system response. For instance, when the yeast encounters leukocytes from normal volunteers in vitro, it causes a decrease in production and secretion of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (11,12,13). Lipids of *Malassezia* cell wall have anti-phagocytic effect; such that, the fungi in which the cell wall lipids are removed by solvents, undergo phagocytosis by neutrophils and macrophages more rapidly (14). It has been demonstrated that TLR-2 is an important receptor in the identification of peptidoglycans and some lipids of the cell wall of microbes and LPS of gram-negative bacteria. The studies indicate that TLR-2 is involved in diseases caused by these organisms, and plays an important role in skin and keratinocyte defense and the promotion of the innate immunity response against *Malassezia* (15).

As was mentioned, *M. furfur* is a lipophilic yeast, and the lipids present in its cell wall are one of the main factors effective on the host immune response. Considering these, the aim of this study is to evaluate the levels of IFN-γ, IL-10, and IL-12P70 in co-incubation of *M. furfur* grown with PBMCs of healthy individuals on different forms of natural oils.

MATERIALS AND METHODS

***M. furfur* Culture.** In this study, we used the clinical strain of *M. furfur* isolated from patients with seborrheic dermatitis. The *M. furfur* strain was identified according to macroscopic and microscopic properties and also by biochemical tests. The yeast was cultured on Leeming and Notman agar culture medium (LNA) containing 10.0 g of peptone, 5.0 g of glucose, 0.1 g of yeast extract, 8.0 g of ox bile, 1.0 mg of glycerol, 0.5 g of glycerol stearate (all obtained from Merck,Germany), 0.5 mg of tween 60, 10 ml of cow's milk, and 12.0 g of agar per liter (16,17).

Natural Oils. After preparation of the culture medium, different groups were prepared by adding specific amounts of sterile oils from walnut (Barij Essence Pharmaceutical Co, Iran), fish oil (omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, Swiss Herbal Remedies Ltd, Swiss), sweet Almond (Barij Essence Pharmaceutical Co, Iran), and full-fat milk (Pak dairy Co, Sanandaj, Iran) and were considered as walnut oil group (WOG), fish oil group (FOG), almond oil group (AOG), and full-fat

milk group (FFMG), respectively. Moreover, a culture medium was used without added fat and was considered as a fat-free group (FFG). In the next step, a suspension containing 2×10^7 *M. furfur cells* grown on sterile phosphate buffered saline (PBS) was prepared of which 100 µl was added to each plate. These media were incubated for four days at 28°C under humid conditions. At the end of the incubation period, sub-cultures were prepared in new media from single colonies grown. This process was repeated three times, and finally, the grown yeasts were used for the co-incubation assays (18).

Blood sampling and isolation of PBMCs. Blood samples were obtained from four healthy volunteers, who had a negative history of pityriasis versicolor, seborrheic dermatitis, dandruff, folliculitis, and psoriasis. Then peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Sigma, USA). Percent survival of the cells was determined using trypan blue staining. The mean acceptable rate of living cells was 95%. The number of PBMCs was counted using a hemocytometer. A suspension of 1×10^6 cell/ml of RPMI-1640 medium containing 60 µg/ml penicillin, 100 µg/ml streptomycin, 20 µM HEPES solution, and 5% heated FCS (Sigma, USA) was then prepared (13,14).

Co-incubation of PBMCs with *M. furfur*. Different suspensions were prepared from *M. furfur* grown on AOG, FOG, WOG, FFMG, and FFG media in RPMI-1640 medium and 100 µl of each suspension containing 2×10^7 CFU/ml was added to three wells of a 96-well plate (SPL, Korea). Then, 100 µl of PBMCs with a concentration of 1×10^6 cells /ml were added to each well. For the negative control group (NCG), we used only the PBMCs and the culture medium, and for the positive control group (PCG), we used 20 ng/well of LPS (Sigma, USA) and the PBMCs in each well. For the measurement of IFN-γ, 2.5 µg/ml phytohaemagglutinin (PHA) (Gibco, Denmark) was also used in the positive control. The plates were incubated at 37°C and 5% CO_2. Then after 12, 24, 48, 72, and 96 hours of incubation, the supernatants from the wells were collected and stored at -70°C until used for the measurement of cytokine levels. The MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method was employed for the evaluation of survival and the proliferation of the cells (13,14).

Cytokine Measurements Using ELISA Methods. After preparation, 100 µl antibody coating was added to each flat-bottomed well of the 96-well microplate. Then, the plate was incubated at 37°C for 2 hours (19,20). For blocking, the liquid in the wells were removed and wells were washed using phosphate buffer for six times and 200 µl of 1% BSA was added to the wells and the plate was incubated at 37°C for another 2 hours (19,20). The liquid in the wells was then removed and 100 µl of the standard concentrations of each cytokine (U-cyTech, Netherlands) along with the samples of culture supernatant were added to each well, and then the plate was incubated at 37°C for 2 hours. All wells were washed for five times (PBS-T) and then, 100 µl of the biotin-conjugated secondary antibody (U-cyTech, Netherlands) was added to each well and the wells were incubated at 37°C for one hour. After incubation, the wells were washed and 100 µl of HRP-streptavidin (U-cyTech, Netherlands) was added to the wells and the wells were again incubated at 37°C for one hour. After washing the wells, 100 µl of Tetramethylbenzidine (TMB) (U-cyTech, Netherlands) was added to all of the wells and incubation in darkness was performed until obtaining desirable color. Finally, the enzymatic reaction was stopped by adding 1M H_2SO_4, and the absorbance was read using ELISA reader at 450 nm. Using the standard curves, the amount of cytokines was measured in pg/ml (19,20).

Statistical Analysis. The data were analyzed by SPSS software, version 14, using the non-parametric Mann-Whitney test. p values less than or equal to 0.05 were considered statistically significant.

RESULTS

***M. furfur* Culture in Various Natural Oils.** Culturing of the yeast on LNA showed that the organism grew well on different natural oil containing media including WOG, AOG, FOG, and FFMG, and no growth inhibition was observed in such media. The yeast had a very poor growth on the fat free medium and the colonies were hardly observable (Figure 1). The time of colony appearance and the size of the colonies demonstrate the effectiveness of various oils on the growth of this yeast.

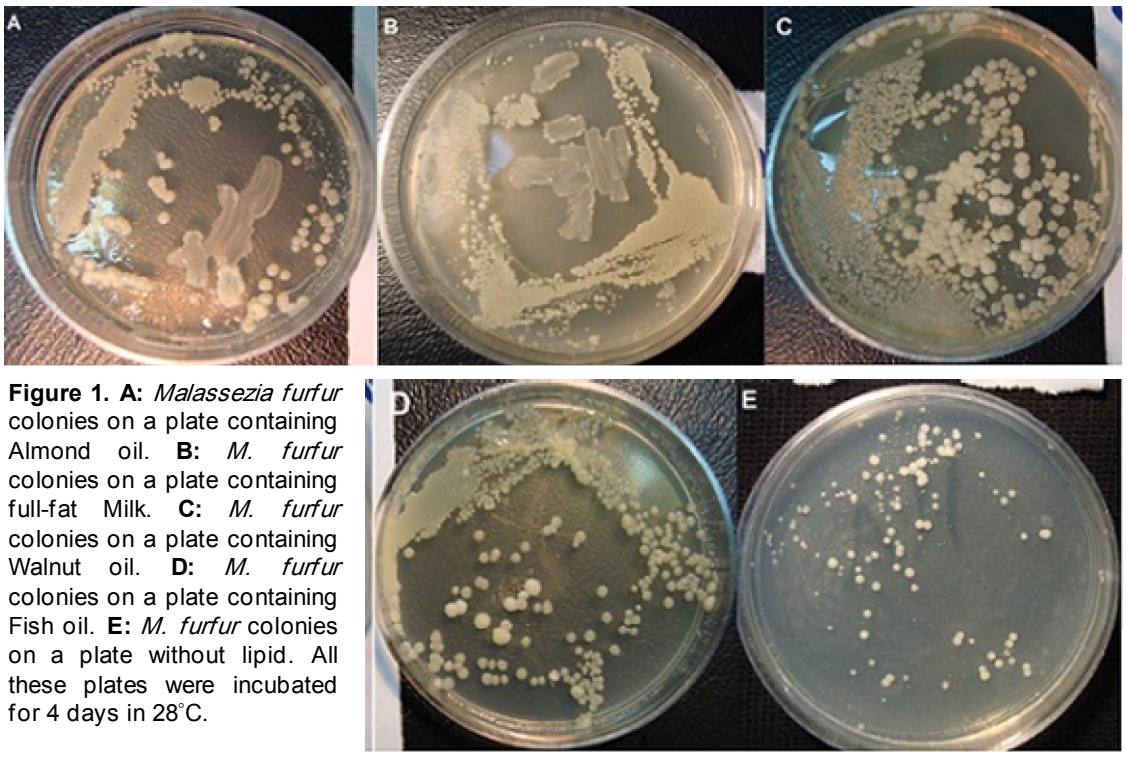

Figure 1. A: *Malassezia furfur* colonies on a plate containing Almond oil. **B:** *M. furfur* colonies on a plate containing full-fat Milk. **C:** *M. furfur* colonies on a plate containing Walnut oil. **D:** *M. furfur* colonies on a plate containing Fish oil. **E:** *M. furfur* colonies on a plate without lipid. All these plates were incubated for 4 days in 28˚C.

The Levels of IFN-γ, IL-10, and IL-12P70. The levels of IFN-γ after co-incubation of *M. furfur* with PBMCs of healthy individuals for different periods demonstrated that the increase in the secretion of these cytokines was different in various incubation times. For instance, the increase in the secretion of IFN-γ initiated 12 hours after co-incubation and continued till 96 hours. The highest level of IFN-γ was present in WOG followed by FOG (Figure 2).

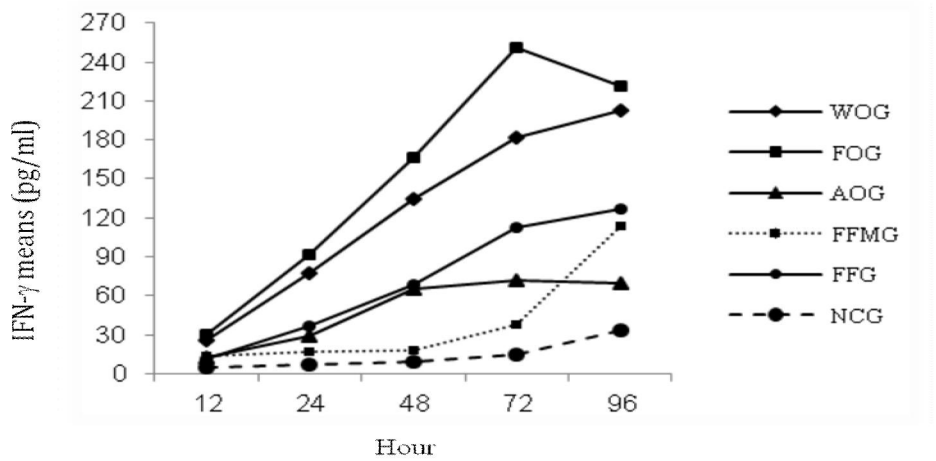

Figure 2. IFN-γ levels in the supernatant of co-incubation of *M. furfur* grew in the presence of different natural oils with PBMCs of healthy individuals. From the yeasts grew in presence of each oil evaluated, the concentration of 2×10^7 CFU/ml was prepared and then the sample were co-incubated with 1×10^6 PBMCs/ml of healthy individuals. The supernatant was extracted 12, 24, 48, 72, and 96 hours after co-incubation and the IFN-γ levels were measured using ELISA method. The mean values of IFN-γ in four healthy individuals at different hours are shown in pg/ml.

The level of IL-10 in the supernatant of PBMCs of healthy individuals co-incubated with *M. furfur* grown in media containing different natural oils did not show a statistically significant increase (Figure 3).

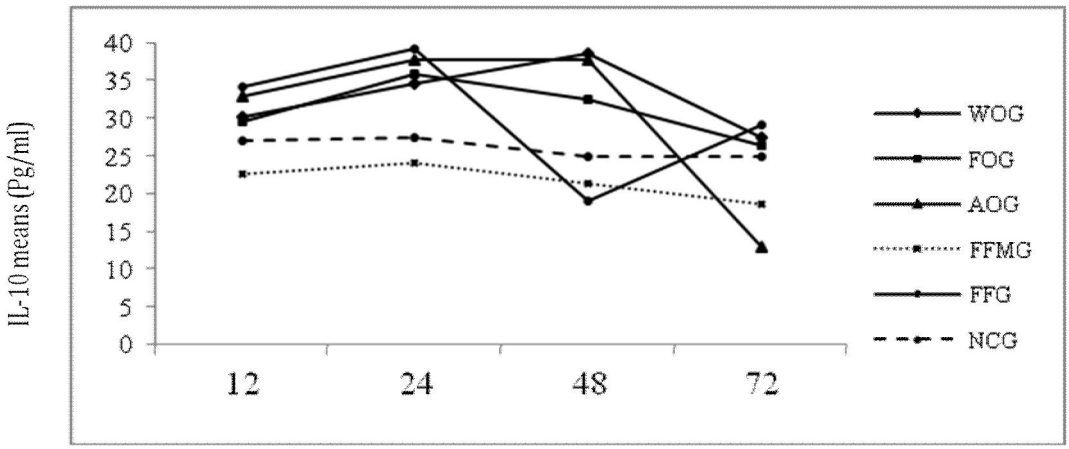

Figure 3. IL-10 levels in the supernatant of co-incubation of *M. furfur* from different groups with PBMCs of healthy individuals. Samples with the concentration of 2×10^7 CFU/ml were prepared from the yeasts grew in presence of each oil evaluated, and then the sample were co-incubated with 1×10^6 PBMCs/ml of healthy individuals. The supernatant was extracted 12, 24, 48, 72, and 96 hours after co-incubation and the IL-10 levels were measured using ELISA. The mean values of IL-10 in four healthy individuals at different hours are shown in pg/ml.

The mean level of IFN-γ in WOG in comparison with that of FFMG and AOG at 48 and 72 hours after co-incubation showed a statistically significant increase (p=0.02). The mean level of IFN-γ in WOG compared with NCG was significantly higher at all time periods (p<0.05) (Table 1). The mean IL-10 level in the WOG compared with AOG at 72 hours, with FFMG at 24, 48, and 72 hours, with FFG at 48 hours, and with NCG at 72 hours demonstrated significant differences (Table 1).

Table 1. Mean levels of IFN-γ and IL-10 in co-incubation of PBMCs of healthy volunteers (Four individuals) with *M. furfur* grown in the medium containing walnut oil (WOG) compared with those of the other groups.

Group / Hours	FOG		AOG		FFMG		FFG		NCG	
	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10
12	p=0.4	p=1	p=0.2	p=0.3	p=0.3	p=0.3	p=0.3	p=0.6	p=0.02	p=0.3
24	p=0.4	p=0.6	p=0.3	p=0.3	p=0.057	p=0.02	p=0.2	p=0.6	p=0.02	p=0.1
48	p=0.4	p=0.4	p=0.3	p=0.4	p=0.02	p=0.02	p=0.2	p=0.02	p=0.02	p=0.057
72	p=0.2	p=0.6	p=0.2	p=0.2	p=0.02	p=0.02	P=0.3	P=0.6	p=0.02	p=0.02
96	p=0.6	p=-	p=0.2	p=-	p=0.3	p=-	p=0.3	p=-	p=0.02	p=-

p≤0.05 was considered statistically significant

The mean IFN-γ levels in the culture supernatant in FOG was significantly higher than AOG at all hours of measurement, FFMG at all hours except for hour 12, FFG at hour 72, and NCG at all hours of measurement (p<0.05) (Table 2).

Mean IFN-γ levels in the AOG was higher than FFMG at all hours of measurement, FFG at hours 72 and 96, and NCG at all hours of measurement (p=0.02) (Table 3). Furthermore, the mean IL-10 levels in the AOG compared with FFMG at all hours of measurement, with FFG at hours 48 and 72, and with NCG at all hours 12, 24, and 48 showed a statistically significant difference (p=0.02) (Table 3).

Comparison of mean values of IFN-γ in the FFMG with FFG at hours 48 and 72 and with NCG at all hours of measurement, and also comparison of FFG with NCG at all hours of measurement showed a significant difference (p=0.02) (Table 3). Also, comparison of mean level of IL-10 in the FFMG with FFG at hours 48 and 72, and with NCG at hour 12, and comparison of FFG with NCG at hour 72 demonstrated a statistically significant difference (p=0.02) (Table 3).

Evaluation of IL-12P70 levels in culture supernatant in co-incubation of *M. furfur* with PBMCs of healthy individuals indicated that the increase in the level of this cytokine was not detectable in most cases. Therefore, comparison of the groups with regard to the mean levels of this cytokine was not possible.

Table 2. Mean levels of IFN-γ and IL-10 in co-incubation of PBMCs of healthy volunteers (Four individuals) with *M. furfur* grown in the medium containing fish oil (FOG) compared with those of the other groups.

Group / Hours	AOG		FFMG		FFG		NCG	
	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10
12	p=0.02	p=0.3	p=0.3	p=0.3	p=0.057	p=0.6	p=0.02	p=0.3
24	p=0.02	p=0.3	p=0.02	p=0.1	p=0.2	p=0.6	p=0.02	p=0.2
48	p=0.021	p=0.3	p=0.02	p=0.02	p=0.057	p=0.02	p=0.02	p=0.2
72	p=0.02	p=0.02	p=0.02	p=0.1	p=0.02	p=0.4	p=0.02	p=0.02
96	p=0.021	p=-	p=0.02	p=-	p=0.057	p=-	p=0.02	p=-

p≤0.05 was considered statistically significant

Table 3. Mean levels of IFN-γ and IL-10 in co-incubation of PBMCs of healthy volunteers (Four individuals) with *M. furfur* grown in the medium containing almond and milk oil compared with those of the other groups.

Groups / Hours	AOG with FFMG		AOG with FFG		AOG with NCG		FFMG with FFG		FFMG wit NCG		NCG with FFG	
	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ
12	p=0.021	p=0.02	p=0.3	p=0.3	p=0.021	p=0.02	p=0.3	p=0.3	p=0.02	p=0.02	p=0.02	p=0.3
24	p=0.021	p=0.02	p=0.2	p=0.3	p=0.021	p=0.02	p=0.3	p=0.3	p=0.02	p=0.3	p=0.02	p=0.3
48	p=0.021	p=0.02	p=0.5	p=0.02	p=0.021	p=0.02	p=0.02	p=0.02	p=0.02	p=0.3	p=0.02	p=0.3
72	p=0.0 2	p=0.02	p=0.02	p=0.02	p=0.021	p=0.4	p=0.02	p=0.02	p=0.02	p=0.057	p=0.02	p=0.02
96	p=0.02	p=-	p=0.021	p=-	p=0.021	p=-	p=0.3	p=-	p=0.02	p=-	p=0.02	p=-

p≤0.05 was considered statistically significant

DISCUSSION

The current study evaluated the effect of *M. furfur,* grown on culture media containing different natural oils, on the response of PBMCs of healthy individuals to production of IFN-γ, IL-10, and IL-12P70 under in vitro condition for the first time. The results obtained indicate that there are some differences in the levels of cytokines induced by *M. furfur* grown on media containing different natural oils. According to these results, IFN-γ levels in the FOG at different hours of measurement were higher than those of the other groups. Furthermore, FFMG followed by AOG had the lowest levels of IFN-γ at different time intervals. In general, the level of IFN-γ in groups grown on media containing natural oils was higher than those grown on NCG. Moreover, the highest levels of IFN-γ in different groups were observed 72 hours after the co-incubation of PBMCs with *M. furfur*. With regard to IL-10 levels, the highest levels were observed in

FFG, WOG, and AOG, and the lowest levels were observed in FFMG. It can be stated that the highest levels of IL-10 production were observed almost 24 hours after the co-incubation of the yeast with PBMCs. Furthermore, it should be mentioned that the growth of *M. furfur* after adding different oils to the LNA culture medium led to some differences in the yeast growth quality and quantity, including the time of appearance of the first colonies, and the size and the number of colonies. *Malassezia* directly obtains the lipids required for the synthesis of its cell wall from the culture media and is not able to synthesize these lipids (21,22). Thus, it seems that the yeasts that grew in the presence of various forms of lipids, present different types of lipids in their cell wall. This consequently may lead to some changes in the antigenic profile of the organism. In the study carried out by Kesavan et al., the effect of lipid cell wall of *Malassezia* in modulating the immune response was evaluated in vitro. According to their results, the yeasts with a lipid cell wall in comparison with the yeasts without a lipid cell wall caused a decrease in the production of pro-inflammatory cytokines during co-incubation with PBMCs under in vitro conditions. In this respect, it was observed that PBMCs produced lower amounts of IL-6, TNF-α, and IL-1β, when co-incubated with *Malassezia* that had its wall removed. In general, the Kesavan et al. Study demonstrated that lipids present in *Malassezia* cell wall can modulate the immune response (14). Consistently, the results obtained in the current study showed that the IFN-γ and IL-10 levels in groups with different oils did not have a significant difference with those from FFG at different hours. Moreover, it was observed that the levels of IFN-γ and IL-10 at different hours in groups exposed to PHA mitogen and LPS were significantly higher than those in other groups of the study. Furthermore, the lowest level of IFN-γ was observed in the AOG, 96 hours after co-incubation. The difference observed in the level of this cytokine compared with other groups that contained oils in their culture media probably shows that the type of oil used by *M. furfur* can influence the immune system response and finally leads to stimulation or modulation of the response. In another study conducted by Savolainen, the cytokine response of PBMCs in atopic dermatitis patients exposed to *Pityrosporum ovale* and *Candida albicans* were evaluated. Their results indicated that *Pityrosporum ovale* caused the stimulation of PBMC proliferation. Moreover, stimulation of IFN-γ and IL-4 production by *Pityrosporum ovale* in atopic dermatitis patients was higher than in patients infected with *Candida albicans*. It was concluded that the cellular immunity response plays an important role in the defense against *Pityrosporum ovale* in atopic dermatitis patients (23).

In the current study, there was a statistically significant differencein the level of IFN-γ between the yeast groups and NCG, which indicates the stimulation of IFN-γ production by *Malassezia*. Previous studies have shown that IFN-γ is an important factor in the stimulation of cellular immunity. In this study, it was observed that the groups with different types of natural oils acted differently with regard to stimulation of IFN-γ production, showing the effect of different lipids in the type of cellular immunity response. In the study carried out by Valli et al., the cytokine response of PBMCs from healthy dogs in exposure to *Malassezia* and other factors were investigated. According to their results, the IFN-γ level increased 4, 24, and 48 hours after culture. Moreover, they evaluated the level of IL-10, as a cytokine involved in Th2 response, and a decrease in IL-10 level was observed by 4, 24, and 48 hours after culture (24). We also observed that IFN-γ levels in different groups increased by 12, 24, 48, 72, and even 96 hours after co-incubation. However, contrary to what was reported by Valli et al., the IL-10 level increased by 12 and 24 hours after co-incubation, and diminished at 48 and

72 hours after co- incubation. To explain the observation, it can probably be stated that during the response of the immune system to an infectious factor, the levels of various cytokines increase, and then depending on the pathogen and its antigens, the levels of more specific antigens increase and certain cytokines will diminish. In 2001, Watanabe carried out a study to evaluate the effect of *Malassezia* species on cytokine production by human keratinocytes. It was reported that after co-incubation of different strains of *Malassezia*, the levels of IL-6, IL-8, and TNF-α increased from 1 to 24 hours after co-incubation, while *M. furfur* strain caused the least increase in the levels of these cytokines. These results demonstrate the immunomodulatory effect of *M. furfur* (11). Compared with the study in which different strains of *Malassezia* were used, in the current study we used one strain, which was grown on culture media containing different types of natural oils. In the current study, various yeast groups had different potentials in stimulating the production of IFN-γ and IL-10. In a study carried out by Kanda et al., the effect of *M. furfur* co-incubation with PBMCs from psoriasis and atopic dermatitis patients and healthy individuals in stimulating the cytokine profile of Th1 and Th2 pathways was investigated (25). According to their results, *M. furfur* stimulates production of IL-4 and increases the cytokine level in atopic dermatitis patients. Moreover, co-incubation of PBMCs from psoriasis patients with *M. furfur* led to an increase in the level of IFN-γ. It was also demonstrated that the IFN-γ level did not significantly increase in co-incubation of PBMCs from healthy individuals with the yeast.

Our results showed that the type of oil added to the culture medium of *Malassezia furfur* led to a change in the cytokine response. It seems that the growth of *Malassezia furfur* in culture media containing various natural oils can alter antigenic profile and these variations cause an elevation in the levels of IFN-γ and IL-10 during co-incubation of this yeast with PBMCs in the first 24 hours. The level of IL-12P70 did not change by such a co-incubation. Since the types of oils used in this study are highly consumed in general, further investigation on the effect of diet on the immune response to infections could be of prime importance.

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