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The Protective Effects of Long-Term Probiotic Application on Experimental Sepsis-dependent Inflammation Process

Uzun Dönem Probiyotik Uygulamasının Deneysel Sepsis Modeline Bağlı Oluşan Enflamasyon Süreci Üzerine Olan Koruyucu Etkilerinin Araştırılması

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ABSTRACT

Objective: Probiotics are defined as live microorganisms that provide beneficial effects on the host when applied in appropriate amounts. The immun-modulatory effects of some probiotics are one of these. We aimed to investigate the effects of probiotics on some inflammatory cytokine levels in sepsis.

Methods: The mixture including 12 different live-probiotic bacteria was used in this study. Group 1 and 2 were evaluated as control and sepsis groups. Sepsis was developed in groups 2, 3, 4, 7 and 8 using cecal ligation and puncture. The probiotic mixture was given to rats in groups 3 and 4 for 21 days before sepsis, groups 7 and 8 a single dose after sepsis at 10^{10} and 10^{11} doses, respectively. Rats in groups 5 and 6, the same doses of probiotics were administered without sepsis. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-10 and transforming growth factor (TGF)-β levels were measured by ELISA method.

Results: There was a serious increase on the level of TNF-α and IL-1β levels in sepsis group, and a serious decrease in 21 days of protective treatment groups. In accordance with IL-10 and TGF-β levels, although the cytokine levels of 21 days protective treatment groups were not reached in control group, a serious increase was observed compared to the sepsis group.

Conclusion: According to our findings, probiotics can be used for supportive purposes in addition to classical medical treatment of sepsis. However, we believe that more studies should be performed in order to standardize the effects of probiotic and determine its effects with different experimental groups of volunteers as well as experimental animal studies.

Keywords: Inflammation, sepsis, cytokine, probiotic

ÖZ

Amaç: Probiyotikler, yeterli miktarlarda alındığında konakta yararlı etkiler sağlayan canlı mikroorganizmalar olarak tanımlanır. Bazı probiyotiklerin sahip olduğu immünomodülatör etki bunlardan biridir. Biz bu çalışmada, probiyotiklerin sepsiste bazı enflamatuvar sitokin düzeyleri üzerindeki etkilerini araştırmayı amaçladık.

Yöntemler: Çalışmamızda 12 farklı canlı probiyotik bakteri içeren karışım kullanıldı. Grup 1 ve grup 2 sırasıyla kontrol ve sepsis grubu olarak değerlendirildi. Grup 2, 3, 4, 7 ve 8'de yer alan deney hayvanlarında çekal bağlama ve delme yöntemi kullanılarak sepsis geliştirildi. Grup 3 ve 4'te yer alan deney hayvanlarına sepsis öncesi 21 gün süreyle, grup 7 ve 8'dekilere ise sepsis sonrası tek doz olmak koşuluyla sırasıyla 10^{10} ve 10^{11} dozlarında hazırlanan karışımdan uygulandı. Grup 5 ve 6'da ise aynı dozlarda sepsis oluşturulmadan probiyotik uygulaması yapıldı. İnterlökin (IL)-1β, tümör nekrozis faktör (TNF)-α, IL-10 ve büyüme faktörlerini dönüştürme (TGF)-β seviyeleri ELISA yöntemiyle ölçüldü.

Bulgular: Sepsis grubunda, TNF-α ve IL-1β seviyeleri bakımından ciddi derecede artış ve 21 gün koruyucu tedavi uygulanan gruplarda ise ciddi derecede azalış tespit edilmiştir. Anti-enflamatuvar sitokinler olan IL-10 ve TGF-β seviyeleri detaylı şekilde incelendiğinde 21 gün koruyucu tedavi uygulanan gruplarda sitokin seviyeleri kontrol grubu seviyesine erişememiş olsa da, sepsis grubuna nazaran ciddi derecede artış tespit edilmiştir.

Sonuç: Bulgularımıza göre probiyotikler, sepsisin klasik medikal tedavisine ek olarak destek amaçlı kullanılabilir. Ancak, probiyotiklerin etkilerinin standardize edilebilmesi ve hayvan deneylerinin yanı sıra gönüllü donörlerde de muhtemel probiyotik etkilerin gösterilebilmesi için daha kapsamlı çalışmaların yapılması gerektiğine inanmaktayız.

Anahtar Sözcükler: Enflamasyon, sepsis, sitokin, probiyotik

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Introduction

The sepsis and septic shock are clinical pictures that may be very serious. They develop due to the spread of microorganisms or the toxic products of these microorganisms with blood circulation. The clinical features and findings of sepsis are results of "the fight" between the immune system of the host and the microorganism and its products. Sepsis stimulates the immunological, inflammatory and endocrine systems of the host, and the severity of these responses determines the clinical course (1).

Researches on the pathogenesis of sepsis have shown that; infection and traumatic injury in tissues activate the immunological mechanisms and cause inflammatory responses by releasing some mediators (especially some cytokines). In recent studies, the physiology of sepsis has been better understood. The metabolic and physiological changes continuously developed in the body have become clearer after the cytokines and other mediators and their mechanisms of action have been identified. As a result of these studies, it was understood that sepsis syndrome is a group of response sequences in which the released cytokines are largely responsible (1,2).

Under the name of "cytokine"; there are various groups of interleukins, monokines, lymphokines, growth factors, interferons and chemokines. Cytokines are produced by a wide variety of cell types (3,4). Cytokines are divided into two groups as pro-inflammatory and anti-inflammatory cytokines according to their mechanisms of action. While pro-inflammatory cytokines induce inflammation, anti-inflammatory cytokines prevent inflammation and promote healing (5,6). The pro-inflammatory cytokines necessary for the initiation and maintenance of the immune response are released at the onset of inflammation. The most important pro-inflammatory cytokines that play a key role in immune response are tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), IL-6 and IL-8. TNF-α and IL-1β are the first secreted cytokines and allow secretion of other cytokines that support migration and activation of the immune cells. Secondary or assisted cytokines are IL-6 and IL-8. Anti-inflammatory cytokines are released in later stages of inflammation, regulating the inflammatory response and down-regulation. The most important anti-inflammatory cytokines are IL-4, IL-10, IL-11, IL-13, and transforming growth factor (TGF)-β. Some cytokines may have both pro-inflammatory and anti-inflammatory effects (1,4).

Experimental studies on sepsis are continuing with a great acceleration. A variety of drugs and treatment protocols involving immune-modulatory substancesare being developed. One of the immune-modulators investigated and used for these purposes is probiotic bacteria (7).

The probiotics in the human gastrointestinal tract flora are located on the surface of the intestine and prevent pathogenic microorganisms from clinging, and the antimicrobial agents they produce control the growth of these bacteria. *Lactobacillus, Bifidobacterium, Streptococcus, Pediococcus, Leuconostoc* and *Enterococcus* species have been used as probiotics. There are significant differences between probiotic bacteria species and strains in terms of their effect, however the most effective probiotic bacteria are *Bifidobacterium* and *Lactobacillus* species according to the scientific studies (7,8).

Nowadays, probiotics can be used in many diseases and pathological conditions. They have beneficial effects such as strengthening the immunity of the host. Probiotics were shown to regulate immunological responses by stimulating host defense mechanisms both in *in vivo* and *in vitro* studies. It was shown in recent years that probiotics have anti-inflammatory, anti-oxidant, anti-bacterial, anti-parasitic, anti-fungal, apoptotic, anti-carcinogenic and many other immunological effects (7-9).

In our study, the aim was to evaluate the positive and/or negative effects of long-term probiotic use before sepsis and single/high dose probiotic administration after sepsis on TNF-α, IL-1β, IL-10 and TGF-β inflammatory parameters.

Methods

Experimental Procedure and Animals

This study was approved by the Local Ethics Committee of Animal Experiments with the date and approval numbers of 19.04.2016/48. Animal protocols were performed according to the local ethical rules and the rules of the Declaration of Helsinki. Eighty female Wistar-albino rats, 12-week-old and weighing 220-300 g, were purchased. Rats were kept in polycarbonate boxes with a standard 12-hour light-dark cycle. The rats were randomly divided into 8 groups. Each group contained 10 rats (Table 1).

Probiotic Mixture and Preparation

A probiotic mixture (Enzibody®, Kenz BioTech, USA) that contained 12 different living probiotic bacteria strains was used in current study (Table 2). All bacteria were mixed in a 1:1 ratio. Twelve different probiotic bacteria (7 strains of *Lactobacillus* spp., 3 strains of *Bifidobacterium* spp., 1 strain of *Leuconostoc* spp. and 1 strain of *Lactococcus* spp.) were grown on de Man, Ragasa and

Table 1. Number of animals in each group and group names

Group	Properties	Number
Group 1	Control	10
Group 2	Sepsis	10
Group 3	Septic group with 21-day 10^{10} cfu probiotic application	10
Group 4	Septic group with 21-day 10^{11} cfu probiotic application	10
Group 5	Healthy group with 21-day 10^{10} cfu probiotic application	10
Group 6	Healthy group with 21-day 10^{11} cfu probiotic application	10
Group 7	Septic group with single dose 10^{10} cfu probiotic application	10
Group 8	Septic group with single dose 10^{11} cfu probiotic application	10

cfu: Colony-forming unit

Sharpe Broth overnight at 37 °C. Bacteria were separated from the culture supernatant by centrifugation (15 min at 3000 xg), washed three times with ice-cold phosphate saline buffer (PBS) (pH=7.2) and re-suspended in PBS. The final concentration of mixture contained $1x10^{13}$ colony-forming unit (cfu) lactic acid bacteria. The final concentration of 10^{11} and 10^{10} doses was obtained by 1/100 and 1/1000 dilution series, respectively. The mixtures were stored at 4 °C after the preparation till the day of experiment.

Experiments Details and Surgical Process

Group 1 was the "control group" and was consisted of ten rats. No surgical or treatment process was applied to group 1. Rats regularly consumed water and nutrients and blood samples were collected on the day of experiment.

Group 2 was the "septic group" and was consisted of ten rats. The rats were euthanized with 25 mg/kg dose of thiopental sodium anesthesia. After shaving the abdomen, the peritoneum was opened. The cecum was isolated and ligated with 3.0 silk ligature. The ligated part of the cecum was then punctured twice with a 16-gauge needle, and a small amount of cecal contents was expressed thought the punctures. After repositioning of cecum, the abdominal incision was closed with a 4.0 sterile absorbable suture. Blood was drawn from the apex of the cardiac ventricle, and collected into sterile blood collection tubes containing ethylenediaminetetraacetic acid after the rats were killed. Then, the blood samples were centrifuged at 4000 rpm for 10 minutes. After centrifugation, the serum supernatant to be used for further analysis was aliquoted into microcentrifuge tubes, and stored at -80 °C.

Group 3 and 4 were the "septic groups with 21 day probiotic application" (10^{10} cfu and 10^{11} cfu, respectively) and each was consisted of ten rats. 10^{10} cfu and 10^{11} cfu probiotic mixture were administered via oral gavage on a regular basis for 21 days to 100 mg/kg of each animal. Then, cecal-ligation and puncture (CLP) sepsis procedure was applied on 20 rats in these groups.

Table 2. Probiotic strains in the mixture

Probiotic bacteria	Strain
Bifidobacterium infantis	ATCC-15697
Bifidobacterium thermophilum	ATCC-25867
Bifidobacterium longum	ATCC-1570
Lactobacillus acidophilus	ATCC-43121
Lactobacillus casei	ATCC-393
Lactobacillus paracasei	ATCC-25302
Lactobacillus helveticus	ATCC-15009
Lactobacillus plantarum	ATCC-14917
Lactobacillus bifidus	ATCC-11863
Lactobacillus brevis	ATCC-14869
Leuconostoc mesenteroides	ATCC-8293
Lactococcus lactis	ATCC-19435

ATCC: American Type Culture Collection

Group 5 and 6 were the "healthy groups with 21 day probiotic application" (10^{10} cfu and 10^{11} cfu, respectively) and each was consisted of ten rats. 10^{10} cfu and 10^{11} cfu probiotic mixture were administered via oral gavage on a regular basis for 21 days to 100 mg/kg of each animal. Nothing was applied on 20 rats in these groups.

Group 7 and 8 were the "septic groups with single dose probiotic application" (10^{10} cfu and 10^{11} cfu, respectively) and each was consisted of ten rats. Firstly, the CLP procedure was applied for these groups. Six hours later, single and high dose (500 mg/kg) of 10^{10} cfu and 10^{11} cfu probiotic mixtures were administered to evaluate the post-sepsis effectiveness of probiotic mixtures.

ELISA Procedure

To test the probiotic immunomodulation, the cytokine levels were detected by commercially available ELISA kits:

- Rat TNF-α ELISA kit (pg/mL) (Cat No: CSB-E11987r, Cusabio, ABD).
- Rat IL-1β ELISA kit (pg/mL) (Cat No: CSB-E08055r, Cusabio, ABD).
- Rat IL-10 ELISA kit (pg/mL) (Cat No: CSB-E04595r, Cusabio, ABD).
- Rat TGF-β ELISA kit (pg/mL) (Cat No: E-EL-R0084, Elabscience Biotechnology Ltd, ABD).

All of the chemicals and 96-well ELISA microplates were brought to room temperature before use. The first 7 wells were used for the standards; the 8th was used for the blank, and 100 µL of serum was added to each of the other wells. After incubation (at 37 °C, for 90 min), a 100 µL biotinylated detection antibody was added to each well, and then the plate was replaced in the incubator (at 37 °C, for 60 min). After a 3 step-washing, 100 µL of horseradish peroxidase conjugate was added to each well, placing the plate in the incubator again (at 37 °C, for 30 min). After the final 5 step-washing, 90 µL of substrate reagent was added, with 15 min incubation under darkened conditions. Finally, a 50 µL stop solution was added to the wells and the plate was read at a wavelength of 450 nm.

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 statistical software (IBM, SPSS, Inc., Chicago, IL, USA). Continuous variables were analyzed using the one-way ANOVA and Kruskal-Wallis tests. The differences between the groups were defined as statistically significant when the p-value was less than 0.05.

Results

Pro-inflammatory Cytokine Results

The level of IL-1β, a pro-inflammatory cytokine, showed statistically significant differences in some levels among experimental groups. IL-1β levels were significantly increased (146 pg/mL) in sepsis group and significantly decreased in

the groups treated with probiotics for 21 days before the development of sepsis ($p<0.05$). In addition, the IL-1β level in the group treated with high-dose probiotic (10^{11} cfu) increased significantly ($p<0.05$) when compared with the control group (Figure 1). There were no statistically significant differences between sepsis and single-dose post-sepsis groups ($p>0.05$).

The level of TNF-α, which is another pro-inflammatory cytokine, in all experimental groups are presented in Figure 2. TNF-α levels were significantly increased (581 and 470 pg/mL, respectively) in sepsis groups compared with (621 pg/mL) probiotic groups ($p<0.05$). TNF-α levels in healthy group significantly decreased when compared to the control group in a dose-dependent manner ($p<0.05$). A single dose probiotic application did not cause any significant decrease in IL-1β and TNF-α levels in post-sepsis groups ($p>0.05$).

After the evaluation of pro-inflammatory cytokine levels; it can be said that the present probiotic mixture suppresses excessive release of pro-inflammatory cytokines, which play an important role in the onset and permanency of the inflammatory process.

Anti-inflammatory Cytokine Results

The level of IL-10, an anti-inflammatory cytokine, showed statistically significant differences in some levels among experimental groups (Figure 3). In sepsis group, the level of IL-10 was the lowest among all groups (161 pg/mL), however; IL-10 levels were significantly increased in sepsis + probiotic treatment groups ($p<0.05$). In healthy groups, significant differences were observed depending on the dose manner of probiotic application. The level of IL-10 did not differ between the 10^{10} cfu healthy group and the control group ($p>0.05$), however; the level of IL-10 was significantly higher in the 10^{10} cfu healthy group than in the other groups except the control group ($p<0.05$).

TGF-β levels of all groups are shown in Figure 4. TGF-β level was 512 pg/mL in the control group and there was a significant decrease in TGF-β level (395 pg/mL) in sepsis group ($p<0.05$). This probiotic mixture, which we considered to be effective on anti-inflammatory cytokines in the process of inflammation caused by sepsis, resulted with an increase in TGF-β levels (414 and 456 pg/mL, respectively) in a dose-dependent manner ($p<0.05$).

When the test results were evaluated, the probiotic application used for therapeutic purposes (after sepsis) did not cause any changes, the probiotic application used for protective purposes showed differences in pro- and anti-inflammatory cytokine levels between the control group and the other groups.

Discussion

Sepsis is a serious infectious disease. Despite the developed intensive care conditions, high mortality rates have made it necessary to elaborate the mechanism of sepsis. Cytokines, which play a major role in the pathogenesis of sepsis, can be classified roughly as pro-inflammatory and anti-inflammatory mediators (5,6). The cytokines that are frequently studied in experimental

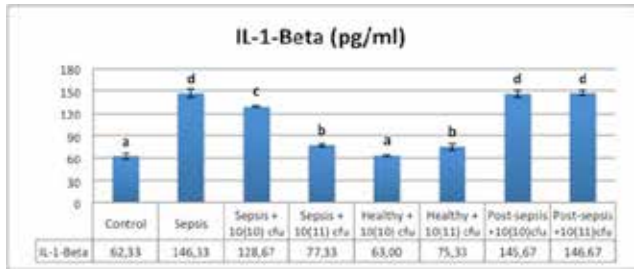


Figure 1. Interleukin-1-beta levels in each experimental group. The letters represent the difference between the groups ($p<0.05$)

IL-1: Interleukin-1, cfu: Colony-forming unit

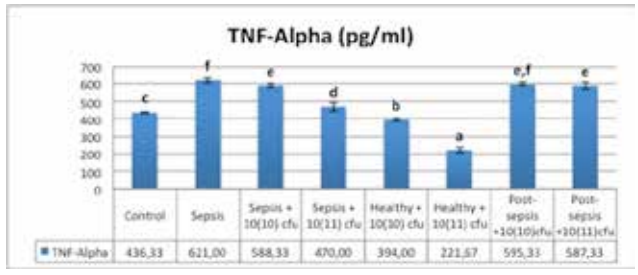


Figure 2. Tumor necrosis factor-alpha levels in each experimental group. The letters represent the difference between the groups ($p<0.05$)

IL-10: Interleukin-10, cfu: Colony-forming unit

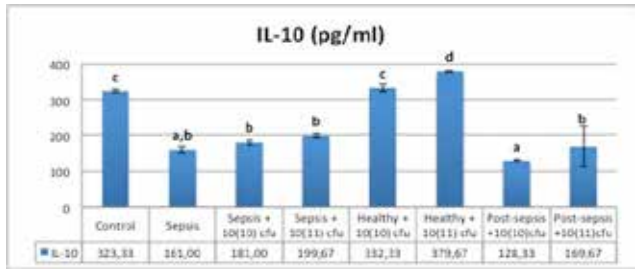


Figure 3. Interleukin-10 levels in each experimental group. The letters represent the difference between the groups ($p<0.05$)

TNF-10: Tumor necrosis factor-10, cfu: Colony-forming unit

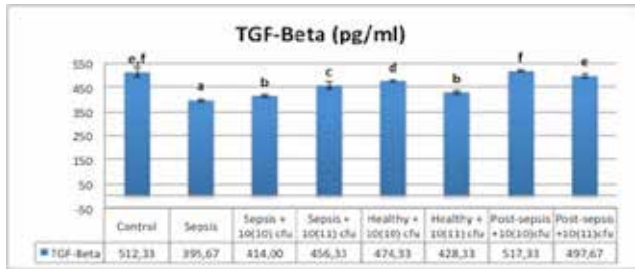


Figure 4. Transforming growth factor-beta levels in each experimental group. The letters represent the difference between the groups ($p<0.05$)

TGF-Beta: Transforming growth factor-beta, cfu: Colony-forming unit

studies on sepsis and inflammation are TNF-α, IL-1, IL-10 and TGF- β (10-13).

There is excessive and irregular production of sepsis-mediated inflammatory mediators. Cytokine imbalance is seen in these inflammatory events as an increase in plasma pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines (reduction in the amount of IL-10 by an increase in the amount of IL-1 and TNF-α) (14). In the light of these data, we decided to evaluate the critical pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10 and TGF-β) in our study.

Today, increasingly prevalent and intensive studies on probiotics show the effects of direct or indirect stimulation of the endogenous flora and the immune system (9). Animal models and human clinical trials show that probiotics affect cytokine release and reduce inflammation (15,16). The application doses of probiotics are another important point in clinical trials. Probiotic activity requires high intakes of bacteria, such as 10^9-10^{11} cfu/mL per day, so that they can be measured significantly in humans. In our study, we benefited from available literature for probiotic doses (17).

During inflammation, macrophages and lymphocytes cause production of TNF-α and IL-1 which are pro-inflammatory cytokines. Lots of scientific studies reported higher levels of TNF-α and IL-1 in patient groups when compared with the healthy donors (18-20). Our results are parallel with the current literature with regards to TNF-α and IL-1 levels. There are some important studies that have reported the possible positive effects of probiotic usage in patients with severe and multiple traumas (21). On the other hand, a study showed that IL-10 and IL-4 levels increased in patients with severe brain trauma by using probiotic mixture containing *Bifidobacterium longum, Lactobacillus bulgaricus, Streptococcus thermophilus* in $3x10^9$ cfu/mL dose (22).

Probiotics were proven to be effective in inflammation. In experimental studies, probiotic supplementation was found to have a potential to reduce TNF-α expression. *Lactobacillus* and *Bifidobacterium* species were especially used in these studies same as our studies (23-25). In another studies, Lammers et al. (26) and Mènard et al. (27) showed that probiotic usage can reduce the IFN-γ, IL-1β, TNF-α and IL-8 levels. We also reported in the current study that the probiotic mixture reduced TNF-α and IL-1β levels in sepsis groups when compared with the control group.

On the other hand, the anti-inflammatory cytokine levels, IL-10 and TGF-β, are at least as important as pro-inflammatory cytokines in inflammation process. Same as pro-inflammatory cytokine results, probiotic application affects IL-10 levels in some severe conditions such as colitis. Some studies confirmed these probiotic effects and added that especially Bifidobacterium spp., Lactobacillus spp., Leuconostoc spp., and Streptococcus thermophilus were responsible of IL-10 reduction (28-30).

In our study, IL-10 level was detected at the lowest level (161 pg/mL) in the sepsis group among all experimental groups, however IL-10 levels significantly increased in sepsis groups treated with probiotic mixture. When the levels of anti-inflammatory cytokines, IL-10 and TGF-β, were examined in detail, a significant increase in the level of cytokines was observed in the sepsis-treated group. The present probiotic mixture increases the release of anti-inflammatory cytokines, which play an important role in the inflammatory process in accordance with the literature.

Study Limitations

More detailed experimental procedures should be considered. Extra methods should be used to get better results. Further studies should be carried out by performing more detailed methods. Additionally, a specific probiotic bacterium effects should be shown in further studies.

Conclusion

According to our findings, the use of probiotics decreased the levels of pro-inflammatory cytokines associated with the deterioration of sepsis pathology and increased the anti-inflammatory cytokine levels which limits the inflammatory process. We believe that probiotic support can be used in addition to classical medical treatments in the treatment of sepsis, which affects all organs and systems. However, in this regard, we believe that more studies should be performed in order to standardize the effects of probiotics and to determine their effects in different experimental groups of volunteers as well as experimental animal studies.

Ethics

Ethics Committee Approval: This study was approved by the Local Ethics Committee of Animal Experiments with the date and approval numbers of 19.04.2016/48.

Informed Consent: As it was an animal experiment, only ethics committee approval was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.K., S.A., Ü.A., Design: M.K., S.A., S.A.K., Data Collection or Processing: M.K., S.A., S.A.K., Analysis or Interpretation: S.A., Ü.A., Literature Search: S.A., Ü.A., Writing: M.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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