

Overview of the angiogenic effect of probiotics (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) at human umbilical vein endothelial cells

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ABSTRACT

Introduction: Angiogenesis (neovascularization), which means new vessel construction, is normal and physiologically, wound healing, embryogenesis, a necessary menstrual cycle it's a mechanism. When taken in appropriate amounts together with or separately with nutrients, mucosal and by regulating systemic immunity, ensuring nutritional and microbial balance in the intestines living nonpathogenic microorganisms that positively affect the health of the host it is called "probiotics". Lactic acid bacteria, the most probiotic microorganisms it constitutes its important group. Where probiotics have an effect on angiogenesis, and it is thought to help heal wounds through the road. With this research indicated that roles of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on angiogenesis if present to demonstrate in vitro methods and the gene expression responsible for the formation of these effects it is intended to reveal.

Material and Method: This study is an experimental study conducted in vitro human umbilical cord vein endothelial cell (HUVEC) MTT test in cell culture with (3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) evaluation of viability and proliferation wound healing model, tube formation method and gene expression with real time-polymer chain reaction (RT-PCR) methods of appointment were used.

Results: HUVEC cells *L. acidophilus* 10⁹ CFU/ml after extract application statistical of mRNA expression of VEGF and FGF genes by control group 24 per hour it was found to increase significantly. *L. rhamnosus* 10⁶ CFU/ml and 10⁹ CFU/ml after application of extracts VEGF gene mRNA by control group 24 per hour its expression was found to be statistically significantly increased. Also *L. rhamnosus* extracts cell proliferation and migration of in vitro wound model it was found to increase statistically significantly.

Conclusion: In this study, in vitro *L. acidophilus* 10⁹ CFU/ml extract and 10⁶ CFU/ml and 10⁹ CFU/ml extract of *L. rhamnosus*, VEGF gene mRNA revealed to be effective on angiogenesis in HUVEC cells by increasing expression it is.

Keywords: Probiotics, angiogenesis, HUVEC, wound healing model

INTRODUCTION

Angiogenesis (neovascularization), which means new vessel construction, is actually a necessary mechanism for normal and physiological wound healing, embryogenesis, menstrual cycle. Angiogenesis is a very complicated process that progresses with endothelial cells proliferation, migration and new vessel formation. Angiogenesis as a dynamic event occurs as a result of releasing proteolytic enzymes to the environment in certain proportions, breaking the extracellular matrix and reconstructing the endothelial cells by migration

from the microvascular field. Provided to all these processes, new capillaries and blood vessels are formed from the existing microvascular bed by proliferation of endothelial cells in the capillaries. The quality of all angiogenic interactions has not yet been clarified. The greatest possibility is that the balance between angiogenic stimulants and angiogenesis inhibitors ensures that the vascular components normally remain silent. Increased angiogenic stimuli and decreased angiogenesis inhibitors initiate angiogenesis (1).

Stimulation of endothelial cells by some factors also initiates a series of events that cause angiogenesis. The occurrence of pathological angiogenesis is responsible for many diseases such as diabetic retinopathy, hemangioma, psoriasis and collagen tissue diseases, as well as tumor growth and metastasis development. In triggering angiogenesis, which plays an important role in cell proliferation; by gaining importance again, especially in the field of industrial microbiology, it has brought probiotic bacteria to the agenda, which take part in many biological processes such as wound healing. Although vascular endothelial cells in adult humans are typically at low turnover rate, they have the capacity to proliferate to form new blood vessels throughout their lifetime (2-3).

Angiogenic stimulation activates endothelial cells shortly after proteolytic destruction. Endothelial cells migrate to the extracellular matrix and multiply. The most effective angiogenic factor in this process is vascular endothelial growth factor (VEGF). Angiogenesis begins with the formation of hemostatic buffer with the release of PDGF, TGF- β and FGF from platelets. VEGF is released in combination with other cytokines. Thus, neovascularization begins by increasing endothelial cells. When the angiogenesis process progresses, a rich vascular network forms from the healthy vessels to the wound area. The oxygen levels in the tissues regulate the angiogenesis process by interacting with oxygen proteins that regulate the transcription of angiogenic and anti-angiogenic genes.

Wound healing is a natural physiological process and microflora is one of the important factors that can affect this process both negatively and positively. In the case of wounds or burns, bacterial colonization occurs due to disruptions in the skin barrier and an infection-prone condition occurs. The wound is a stressful condition and causes the release of neuroendocrine and stressors such as cortisol, epinephrine, norepinephrine, acetylcholine, catecholamine, substance P, α -melanotropin. These molecules increase the risk of infection and complicate wound healing (4-5).

Probiotics are thought to be effective in the expression of various growth factors such as ornithine decarboxylase (ODC), VEGF, fibroblast growth factor (FGF), B-cell lymphoma 2 (Bcl-2) and epidermal growth factor receptor (EGF receptor) by affecting these mechanisms. These molecules activate mechanisms such as chemotaxis, cell proliferation, angiogenesis, extracellular matrix deposition and reconstruction (6-9).

Probiotic bacteria can survive in the gastrointestinal environment without being damaged. When taken with food, probiotics can maintain up to 1-4 hours in an stomach with an pH between 2.0 and 3.0 in an enzymatic environment. Probiotic bacteria can grow in the mucous substance secreted from the mucosa. Can use mucin in this secretion as an energy source (10-12).

L. acidophilus is naturally present in the human intestinal microflora. These microorganisms, which have a facultative anaerobic feature, develop in the colonic intestinal epithelial tissue they form, and prevent them from adhering to the surface and show antagonistic effect. In particular, dairy products containing *L. acidophilus* have been found to decrease serum cholesterol levels and increase antibody responses, and have effects to increase the number of fecal lactobacilli (13-16).

Lactobacillus rhamnosus is a type of beneficial bacteria that produces L (+) lactic acid and ethanol in an oxygen-free environment, is naturally found in the human intestinal flora, resistant to low pH environments, and can attach to the wall of the gastrointestinal tract. Some studies show that *L. rhamnosus* can stimulate natural barrier mechanisms in patients with atopic dermatitis and food allergy and can be an effective therapy method in the treatment of diseases such as food allergy (17,18).

The aim of our study is to show the effects of lactic acid bacteria commonly used in the field of industrial microbiology such as *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* in vitro methods and to analyze the gene expressions responsible for the occurrence of these effects.

MATERIAL AND METHOD

Ethics committee approval is not required for cell culture research in preclinical studies. All procedures were performed adhered to the ethical rules and principles of the Helsinki Declaration.

Cell Culture

HUVEC cell line (supplied by ATCC) was used and in vitro analysis were performed at vascular biology lab in the study. HUVEC is in stem cell structure, it has the basic features of vascular endothelial cells, and are frequently used in angiogenesis studies the reason for preferred.

The medium for HUVEC cells was prepared as 90% DMEM (L-glutamine), 10% FBS. Cells were produced in 25 cm² and 75 cm² flasks containing this nutrient medium by keeping them in an incubator with a 5% CO₂, 95% air mixture and humidity inside the 37°C and routine passage 3 times a week cell model in studies related to angiogenesis.

After the cells were evaluated cytotoxicly with the MTT test, it was decided to study the bacterial extracts with the most appropriate proliferation property on HUVEC cells in the wound model. In order to evaluate the effects on wound healing, 4 test groups and a control group were created for HUVEC cells.

Would Healing Assay

Petri dishes (35 mm, high), which are physically suitable for cell adhesion and proliferation, with a highly hydrophobic unbedded surface, have used. In addition, a silicone structure was placed on the graders at the base of these flasks in order to mimic the shape of the wound edges just before cultured the cells. After the cells were expected to completely cover the bottom of the wells, the previously placed silicone structures were carefully removed. Then, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were treated which has determined optimum concentrations and 0, 8, 24 h incubation period. In the in vitro mimic wound healing model, the effects of bacteria species on cellular activities were compared with the control group.

Tube Formation Model

M-Slide Angiogenesis and M-Plate Angiogenesis Tube Formation plates, which were placed in a 37°C incubator with BD Matrigel gel solution 12 hours before, were removed from the incubator and 10 microliter gel solution was added to each in middle of well. Bacterial extracts and cell suspensions were prepared and cultivated on the matrigel stabilized in the wells. Within 12-24 hours, the results were visualized by phase contrast microscope and scoring was taken considering the tube length, number and density.

Gene Expression by RT-PCR

In order to perform gene expression study mRNA molecule (QIAGEN Cat.No.74104 RNeasy Mini Kit) from each cell application group, the Transcriptor First Strand cDNA synthesis kit (ROCHE-Germany) was used primarily for obtaining cDNA. Quantitative RT-PCR method followed by cDNA synthesis used Light Cycler FastStart DNA Master SYBR Green I kit (Roche-Germany) in gene expression analysis. "Betaactin" gene was studied as a reference gene. Target mRNA normalization rates were determined using the Lightcycler software 4.0 program version. Normalization rates were calculated automatically from the amplification curves of the program samples. ROCHE Lightcycler 480 device was used in the study. Statistical evaluations were made using target mRNA primers, FGF, VEGF, whose expression level want to be measured.

RESULTS

MTT cell viability test was performed to determine the effect of different concentrations (10^6 - 10^9 CFU/ml) of bacterial extras on the HUVEC cell line. Significant results were obtained after 24-hour incubation of bacterial extras on the HUVEC cell line. Changing cell viability effects at different concentrations and different incubation points are shown in **Figure 1**.

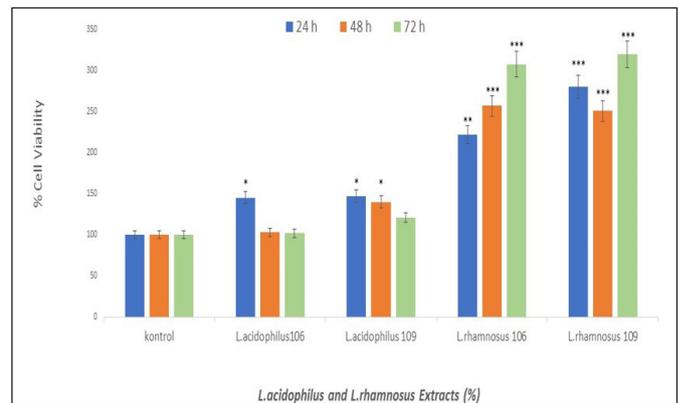


Figure 1. MTT cytotoxic assay of *L. acidophilus* and *L. rhamnosus* on HUVEC cells (different time point)

According to datas, 10^9 of *L. acidophilus* concentration had more effective. Compared to *Lactobacillus acidophilus*, *L. rhamnosus* has more dominant role on endothelial cell functions.

Images of the cell culture wound model created with *L. acidophilus* 10^9 CFU/ml extract were taken under the inverted microscope at 0, 8, and 24 hours. No statistically significant effect on cell migration compared to the control group. However, *L. rhamnosus* 10^9 CFU/ml extract were taken under an inverted microscope at 0, 8 hours and observed that it increased the cell migration statistically significantly compared to the control group and closed the wound model at 8 hour time point (**Figure 2**).

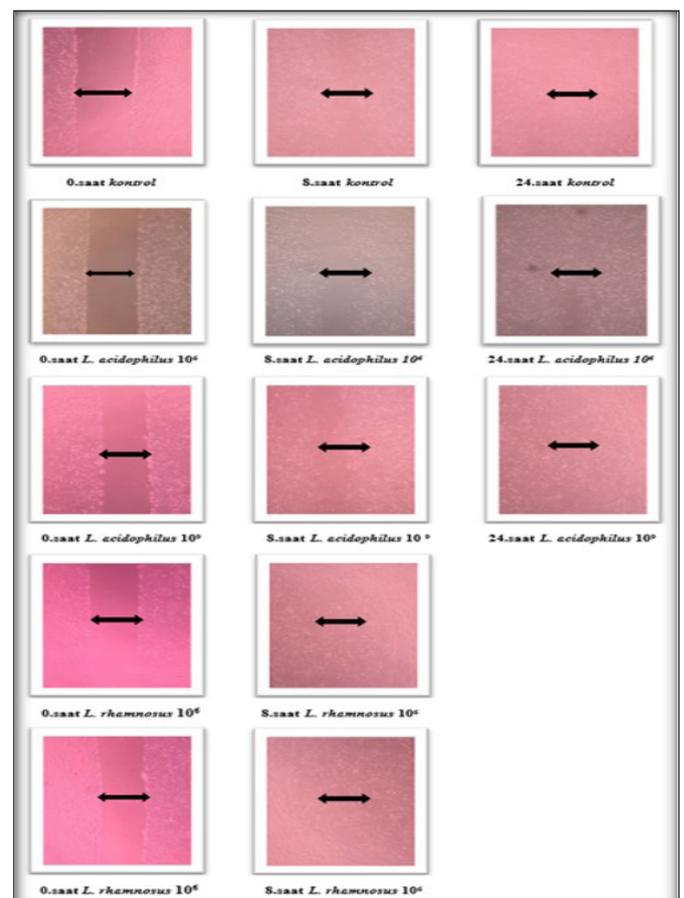


Figure 2. Wound healing cell migration rate on HUVEC cells

Genes involved in angiogenesis and wound healing in HUVEC cells were evaluated by RT-PCR method. While *L. acidophilus* 10^6 did not show a significant effect at the dose, in the 10^9 CFU/ml group, 5 times more gene expression was observed among VEGF gene expressions compared to the control group, and FGF gene expression increased 2-fold in this group (Table 1). For *L. rhamnosus* 10^9 CFU/ml group, there was a statistically significant difference in VEGF gene expression 2 times more than in the control group, but no statistically significant difference was found for FGF gene expression.

Table 1. RT-PCR results of VEGF and FGF on *Lactobacillus* application group

	Control group	<i>L. acidophilus</i> 10^6	P Value	<i>L. acidophilus</i> 10^9	P Value
VEGF	0.105±0.001	0.125±0.022	p>0.05	0.525±0.021	p<0.05
FGF	0.040±0.012	0.040±0.223	p>0.05	0.095±0.011	p<0.05
	Control group	<i>L. rhamnosus</i> 10^6	P Value	<i>L. rhamnosus</i> 10^9	P Value
VEGF	0.105±0.001	0.250±0.022	p<0.05	0.019±0.021	p<0.05
FGF	0.040±0.012	0.040±0.012	p>0.05	0.045±0.013	p>0.05

The effect of *L. acidophilus* and *L. rhamnosus* bacteria on tube formation was investigated by using tube formation analysis (Wimasis) on matrigel, which was stabilized in the wells by preparing bacterial extracts and cell suspensions. It could not be determined tube formation effect that *L. acidophilus* bacteria, which applied 10^6 CFU/ml dose. While a slight effect of *L. rhamnosus* bacteria administered 10^6 CFU/ml dose was observed on tube formation, a statistically significant difference was found for *L. acidophilus* and *L. rhamnosus* bacteria at 10^9 CFU/ml dose (Figure 3)

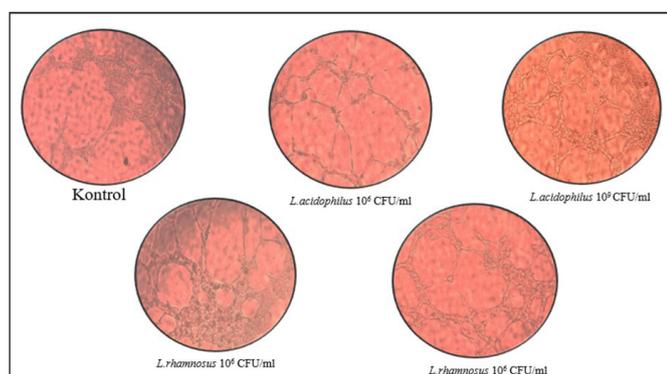


Figure 1. MTT cytotoxic assay of *L. acidophilus* and *L. rhamnosus* on HUVEC cells (different time point)

DISCUSSION

In recent studies in the field of angiogenesis and wound healing, it is aimed to find new molecules that increase cell proliferation, support migration and regulate cellular activities. In this respect, biological molecules, namely probiotics, which do not show cytotoxic

properties, do not damage other organs and systems of the body, come to the fore. Live non-pathogenic microorganisms that provide microbial balance in the intestines and positively affect the health of the host in this way by regulating mucosal and systemic immunity when taken together with the nutrients or separately, are called "probiotic" (19-21). According to the increasing number of scientific research results, it is pointed out that living microorganisms can be used in the prevention and even treatment of some diseases. In this context, the importance of probiotics is increasing day by day. It has been proven that human and animal model studies that probiotics have many beneficial effects in the organism, especially in the GI system. Therefore, the place of probiotic bacteria has become indisputable in the treatment of a healthy life and diseases (22,23).

Lactobacillus and *Bifidobacterium* are the most important and most frequently used group of probiotic cultures. Factors such as delivery method, antibiotic use, age, nutrition, genetic factors, stress and pregnancy affect the richness of microflora. Because probiotics are sensitive to factors such as pH, gastric and pancreatic fluids, bile, and intestinal mucosa, studies are often geared towards stabilizing bacteria. It is thought that probiotics may also have an effect on angiogenesis and in this way can help wound healing (24).

In our study, the effect of probiotics on migration on HUVEC cells was investigated by in vitro wound model. According to the studies in the literature, Halper et al. (25) reported that *Lactobacillus* supernatants have angiogenesis and wound healing properties in vitro and in vivo. In another study, *L. acidophilus* was reported to form reepithelialization in HaCaT cells. According to the study of Eunok et al. (26) probiotic *B. polyfermenticus* increases migration and proliferation on endothelial cells. Studies using *L. acidophilus* encapsulated with ginger extract have shown that gastric ulcer heals in rats without signs of mucosal damage. In our study, it was found that *L. rhamnosus* 10^6 CFU/ml and 10^9 CFU/ml extracts significantly increased cell proliferation and migration compared to the control group. While the proliferative effect of *L. acidophilus* extracts in HUVEC cells was observed, no effect on migration was observed in the in vitro wound model (27). In a study, it was reported that probiotic *B. polyfermenticus* is effective in tube formation in human intestinal endothelial cells. Besides our study, a statistically significant difference was found in tube formation at 18 hours after application of *L. acidophilus* 10^9 CFU/ml and *L. rhamnosus* 10^9 CFU/ml extracts to HUVEC cells. These results are also consistent with the findings of VEGF and FGF gene expression at the final stage of the study (28,29).

VEGF is an effective substance, from early vascular development to tube formation. After this stage, angiopoietin (Ang) interferes with the endothelium for vascular stabilization, collects periendothelial cells and vascular stabilization is achieved. In the literature, *L. acidophilus* has been shown to significantly accelerate wound healing and provide a significant increase in collagen content, which is one of the markers that show wound healing. In a study, it has been reported that probiotic culture [*Lactobacillus acidophilus*, bulgaricus, casei, plantarum, Bifidobacteria breve, infantis, longum and Streptococcus], which increase the angiogenesis by VEGF, have a statistically positive effect on wound healing in gastric ulcers (30).

It is stated that probiotics act on angiogenesis in the inflammatory process by VEGF receptor signaling in the gastrointestinal tract. By angiogenesis, VEGF stimulates new microvesel formation and granule formation. Simulation of angiogenesis has a wound healing effect. In our study, the fact that VEGF and FGF genes expressing a role in angiogenesis in the experimental group where *L. acidophilus* 10⁹ CFU/ml extract was applied to HUVEC cells compared to the control group supports the literature findings (31-33).

Although many studies have been conducted on probiotics for the treatment of gastrointestinal infections and cancer prevention, the results of these studies cannot be compared due to differences in many factors such as the type of probiotic microorganism used, its dosage, or whether the study was in vitro or in vivo. There are also significant differences between the in vitro effects of two different *L. acidophilus* and *L. rhamnosus* probiotic microorganisms we use. In addition, this shows that not only the variety but also the concentration changes applied may affect the results.

CONCLUSION

These findings support that probiotic microorganisms may differ depending on the type and nature. It is thought that it would be beneficial to support the data in this area and to reach new data by using different probiotics. It is also considered that it would be appropriate to try a variety of combinations including probiotics, prebiotics or their relationships with each other. After our research, realized that evaluate the probiotic effect of angiogenesis more detailly, it must be treated with other strains of *Lactobacillus*. The results of this in vitro study should be also shown in vivo and must be enriched with clinical applications.

Where the effects of probiotics on angiogenesis and human health are examined, we think that the results will provide an important data for the more comprehensive and more detailed studies to be conducted.

In addition, human studies are currently underway that strongly support the use of probiotics. After all these studies, it seems possible to benefit from probiotics in the diagnosis and treatment of diseases in the near future.

ETHICAL DECLARATIONS

Ethics Committee Approval: No interventional procedure was performed with the method and study protocol infrastructure of the study. Due to the absence of clinical studies, ethics committee approval is not required for cell culture research in invitro study.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest: The authors have no conflicts of interest for declaration.

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