Original Article

Effect of Motiflor AS probiotic for oral health on cell viability in human gingival fibroblasts and human dental pulp stem cells

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Abstract

Objectives: In this study, it was aimed to investigate the possible effects of oral chewable probiotic tablets (PTs) produced to directly support the oral flora on the proliferation of human dental pulp stem cells (DPSCs) and human gingival fibroblast cells (HGFCs).

Materials and Methods: For analysis in this study, "Motiflor AS," a PT that dissolves in the mouth, containing 13.5mg *Lactobacillus helveticus* Rosell-52, *L. rhamnosus* Rosell-11, *L. halivarus* HA-118, and *Bifidobacterium longum* Rosell-175 was used. Cell survival and proliferation were analyzed by methyl-thiazole-diphenyl-tetrazolium (MTT) test and real-time cell analysis method (xCELLigence RTCA-DP) after 24-, 48-, and 72-h incubation periods.

Results: According to the data obtained with RTCA-DP software, there was a significant increase in the proliferation of human dental pulp stem cells (HDPSCs) and HGFCs in the 72-h incubation after PT application compared to the 24-h and 48-h incubations ($P < 0.0001$). After the MTT test, for HDPSCs, the cell proliferation rate was 62.8% and 85.6% in 24- and 48-h incubation, respectively, while HDPSCs cell proliferation rate in 72-h incubation was 135.2% ($P < 0.0001$). For HGFCs, the cell proliferation rate was 73% and 120.4% in 24- and 48-h incubation, respectively, while HDPSCs cell proliferation rate in 72-h incubation was 139.8% ($P < 0.0001$). When the results of the two tests applied were evaluated together, the results showed compatibility.

Conclusions: Based on the results, it has been concluded that PT will be useful for maintaining oral health and for dental and gingival patients who will/have undergone dental treatment. It should be keep in mind that protecting our oral and dental health is very important in terms of protecting our general health.

Keywords: Cell proliferation; dental pulp stem cell; gingiva fibroblast cell; oral health; probiotic

INTRODUCTION

Today, after food consumers understand the link between basic lifestyle, diet, and health, food manufacturers are trying to meet the increasing demand for products that can improve health. Nowadays, probiotics are becoming one of the fastest-growing categories of foods in light of scientific research.[1] Probiotics have a wide range of therapeutic applications such as the prevention of urogenital diseases, positive effects on the gastrointestinal system, reduction of hypercholesterolemia, protection against bladder cancer, prevention of osteoporosis, and food allergy.[2]

The use of probiotics is increasing day by day due to its proven effects such as preventing infections and strengthening immunity. The positive aspects of the use of

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probiotics in general come to the fore in studies. In recent years, in terms of gut microbiology and immunology, gut microbes have been shown to modulate the host environment and the composition and products of gut microbes have a major impact on the immune response.[3]

It has been reported that probiotic mechanisms of action in the oral cavity are similar to those described for the gut.[4] To date, oral colonization by probiotic bacteria has generally been considered necessary for oral action. However, there is not enough research on the positive or negative effects of orally dissolving probiotic tablets (PT) on oral health.[5] In this study, it was aimed to investigate the possible cytotoxic effects of PT that dissolve in the mouth, which are produced to directly support the oral flora, on the proliferation of human dental pulp stem cells (HDPSCs, CELPROGEN, 36086-01, USA) and human gingival fibroblast cells (HGFCs, ATTC. PCS-201-018, Manassas, Virginia, USA).

MATERIALS AND METHODS

Probiotic materials

In this study, PTs commercially available under the trade name Motiflor AS (13.75 mg *Lactobacillus helveticus* Rosell-52, 13.75 mg *L. rhamnosus* Rosell-11, 13.75 mg *L. salivarus* HA-118, 13.75 mg *Bifidobacterium longum* Rosell-175; Abfen Pharma, Canada) were evaluated for their effects on proliferation of HDPSCs and HGFCs. Motiflor AS was completely dissolved in 10 ml of DMEM by vortexing at low speed and was made sure that it was mixed so that there was no residue.

Cell culture and experimental design

HDPSCs and HGFCs were grown in a 25-cm^2 culture flask (Corning®, Sigma-Aldrich, St. Louis, MO, USA) containing 5 ml of Minimal Essential Medium (DMEM) (Gibco, Thermo Fisher Scientific, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific, Waltham, MA USA), nonessential amino acid (2mL), streptomycin (100 µg/mL), penicillin (100 U/mL), sodium bicarbonate, and L-glutamine (2mL) at 37°C gassed with 5% CO2. The media were refreshed every 2–3 days. When 80% confluence was reached, cells were routinely passaged, using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin + 0.02% EDTA, Thermo, Germany) for the enzymatic dissociation of the extracellular matrix. Subsequently, the cells were separated from the flask base and new fresh medium was added into the flask. After mixing, the solution was centrifuged at 15.000 rpm for 3 min (Hettich Zentrifugen, ROTOFIX 32A, Germany). After the supernatant was aspirated, the cells in the remaining pellet were homogenized in DMEM medium at 37°C. Subsequently, the HDPSCs and HGFCs were seeded into 96-well plates (20,000 cells/well; well volume: 250µL; base diameter of well: 5 mm) following which the culture vessels were checked daily and the media were renewed every 2 days cells between the 7^{th} and 9^{th} passages were used in the experiments. To examine the possible effect of PT on HGFCs and HDPSCs, according to each cell type, 9 wells were used for the control group (no PT added) and 9 wells for the experimental group (PT added). Again, 9 wells were used for contamination control.

Determination of cell viability using methyl-thiazole-diphenyl-tetrazolium and assay

Cells were treated with or without of the Motiflor AS in serum-free media for 24 h of between the weight of the Motiflor AS (13,75 mg, 0.75×10^7 CFU/mL per bacteries) and incubated at 37°C for 48 h in a 5% CO_2 environment. HDPSCs and HGFCs were prepared in DMEM. After an incubation period of 72 h, the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Abcam, ab211091, USA) was applied. Media was carefully aspirated from the wells for adherent cells. Each well added 50 µL of serum-free media and 50 µL of MTT reagent and incubated for 3 h at 37°C in a CO_2 oven. To dissolve the formazan crystals formed by living cells, 150 µl of MTT solvent solution was added to each well and left in the CO_2 incubator for 15 min. All procedures were carried out in a sterile cabinet (Microtest, Class II-A2, Turkey) to avoid the risk of contamination. The absorbance was measured at 590 nm using a microplate reader (SPECTROstar® Nano, Germany). The optical density of the cells cultured in the DMEM medium without any Motiflor AS was used as a negative control. Negative control media were attributed to 100% cell viability and used as a reference for determining the level of cytotoxicity. This test was repeated three times for each group. Using the values obtained from the optical reader and the formula presented by Vande Vannet *et al*., the cell proliferation percentages of each test material were calculated by the following formula:

Cell viability (%) = (OD of test group/OD of cellular control group) ×100

Subsequently, the classification of Ahrari *et al*. was applied to determine cell viability by defining:

- No cytotoxicity: More than 90% cell viability
- Mild cytotoxicity: 60 to 90% cell viability
- Moderate cytotoxicity: 30% to 59% cell viability
- Severe cytotoxicity: Cell viability less than 30%.

Determination of cell viability xCELLigence assay

The xCELLigence system (Roche Applied Science, and ACEA Biosciences) was used to assess the survival of HDPSCs and HGFCs upon exposure to Motiflor AS over time. By following the *xCELLigence assay* procedure, 200 µL of the cell suspensions was seeded into a 16-well E-plate (20,000 cells/well) in a laminar flow cabinet, placed in the incubator at 37°C and 5% CO_2, and monitored

using the RTCA-DP system at 15-min time intervals for up to 72 h with or without dental materials. The control samples received only medium and, in accordance to the xCELLigence technical manual, at least three repeats of each experimental condition were performed to facilitate statistical evaluation.

Statistical analysis

MTT analyses were performed using GraphPad Prism (version 9.1.1., GraphPad Software, San Diego, USA) and xCELLigence data were calculated using the RTCA-DP integrated software of the xCELLigence system. Data from the proliferation experiments were statistically evaluated using the two-way ANOVA test and whether there was a difference between the two groups was determined using Tukey's multiple comparisons *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Cell viability and possible cytotoxicity were monitored with xCELLigence RTCA-DP for up to 48 h. At the end of 48 h, an impedance graph was created that numerically expressed the living cells [Figure 1].

According to the data obtained with RTCA-DP software, there was a significant increase in the proliferation of HDPSCs and HGFCs in the 72-h incubation after PT application compared to the 24-h and 48-h incubations ($P < 0.0001$). For HGFCs, the cell proliferation rate was 73% and 120.4% in 24- and 48-h incubation, respectively, while HGFCs cell proliferation rate in 72-h incubation was 139.8% ($P < 0.0001$). When the results of the two tests applied were evaluated together, the results showed compatibility. HGFCs proliferation values obtained from the MTT in Table 1 and xCELLigence RTCA-DP in Figure 2 analysis for Motiflor AS are shown.

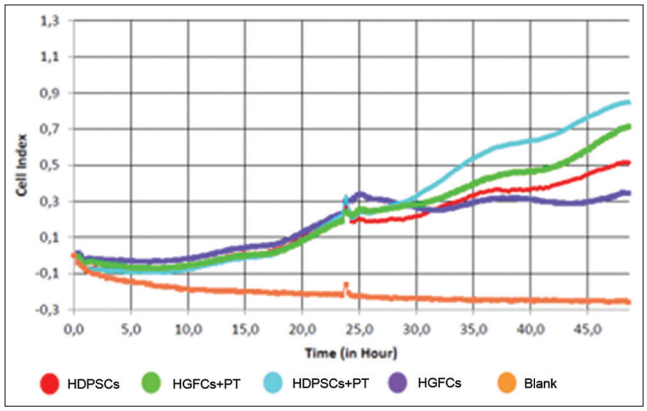

Figure 1: Effect of Motiflor AS (PT) on human dental pulp stem cells and human gingival fibroblast cell proliferation. Cell growth graph according to xCELLigence RTCA-DP analysis

After MTT test, for HDPSCs, cell proliferation rate was 62.8% and 85.6% in 24- and 48-h incubation, respectively, while HDPSCs cell proliferation rate in 72-h incubation was 135.2% ($P < 0.0001$). When the results of the two tests applied were evaluated together, the results showed compatibility. HGFCs proliferation values obtained from the MTT in Table 2 and xCELLigence RTCA-DP in Figure 2 analysis for Motiflor AS are shown.

DISCUSSION

Probiotics have recently entered clinical use in health-related fields such as dentistry, immunology, and nutrition, especially gastroenterology, with increasing concern for health.[6] While previous research on probiotics was mostly about the gastrointestinal tract, research on the effects of probiotics on oral health has gained importance in recent years.[7] However, the effects of beneficial bacteria and their metabolites on teeth and gingiva are still unclear. In the literature, there are studies investigating the effects of probiotics on dental caries, halitosis, and periodontal diseases.[8,9] In recent years, the positive effects of probiotic bacteria have been proven in the treatment of many diseases, and have also been shown to be very effective in the treatment of lots of oral and dental diseases (tooth decay, gingivitis, etc.).[10] In this study, we investigate the effect of Motiflor AS on the proliferation of HDPSCs and HGFCs, targeting the effect.

xCELLigence and MTT assay are commonly used to evaluate cytotoxicity and cell viability. Both assays are used to determine cell viability, but their fundamental working principles are very different. xCELLigence analysis was used to determine real-time cell proliferation and cytotoxicity. The functional unit of the xCELLigence RTCA-DP impedance

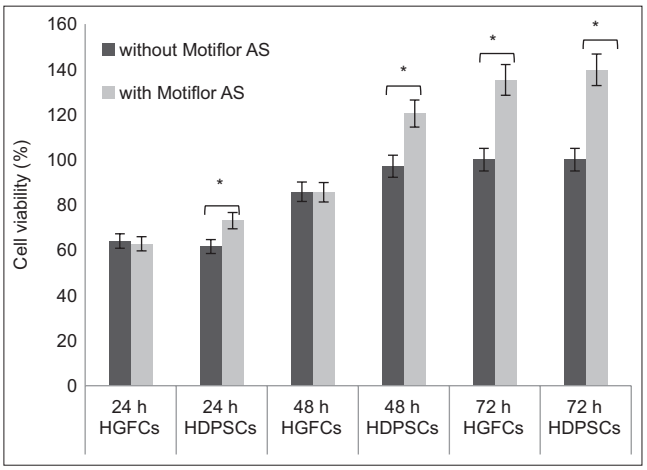

Figure 2: Comparison of proliferation rates of human dental pulp stem cells and human gingival fibroblast cells according to xCELLigence analysis results over time (24, 48, and 72 h) *$P < 0.05$

Table 1: Evaluation of the change in the proliferation of human gingival fibroblast cells with the methyl-thiazole-diphenyl-tetrazolium test according to the addition of probiotics (two-way ANOVA test)

Time (h)	Motiflor AS		HGFCs (without Motiflor AS)		HGFCs + Motiflor AS		*P*
	Total amount of cells/μm²	Cell viability (%)	Total amount of cells/μm²	Cell viability (%)	Total amount of cells/μm²	Cell viability (%)	
24	0.251	22.1	0.544	61.5	0.63	73	0.08
48	0.339	33.9	0.809	97.1	0.982	120.4	0.04*
72	0.36	36.7	0.83	100	1.126	139.8	0.009*

HGFCs: Human gingival fibroblast cells, *$p<0.05$

Table 2: Evaluation of the change in dental pulp stem cells proliferation according to the addition of probiotics by methyl-thiazole-diphenyl-tetrazolium test (two-way ANOVA test)

Time (h)	Motiflor AS		HDPSCs (without Motiflor AS)		HDPSCs + Motiflor AS		*P*
	Total amount of cells/μm²	Cell viability (%)	Total amount of cells/μm²	Cell viability (%)	Total amount of cells/μm²	Cell viability (%)	
24	0.251	25	0.642	64	0.63	62.8	0.08
48	0.339	33.7	0.861	85.8	0.859	85.6	0.06
72	0.427	42.5	1.003	100	1.358	135.3	0.0001*

HDPSCs: Human dental pulp stem cells, *$p<0.05$

analysis is an array of gold microelectrodes integrated into the bottom surface of a microtiter plate well. Physiologic changes in the cells were identified and measured by the electronic impedance of the sensor electrodes. This real-time monitoring system provides quantitative information on the biological status of cells.[1] MTT analysis is based on measuring the activity of succinate dehydrogenase, which converts the tetrazolium salt to the formazan derivative in the mitochondria of living cells.[11,28] We used these two validated tests in our study to investigate the proliferation of HDPSCs and HGFCs in the Motiflor AS PT.

The main principle of the use of probiotics is to replace pathogenic species with nonpathogenic species such as *Lactobacilli* or *Bifidobacteria* strains, which are the most common microorganisms used as probiotics.[12] Studies using *Lactobacilli* or *Bifidobacterium* species as probiotics to prevent dental caries have reported that decrease the number of *Streptococcus mutans* and the DMFT index score.[13] Ishikawa *et al*.[14] showed that the levels of *Porphyromonas gingivalis, Prevotella intermedia,* and *P. nigrescens*, three of the main periodontal pathogens, were significantly reduced after 4 weeks of oral administration of the probiotic containing *Lactobacillus salivarius.* A study reported that *L. reuteri* containing probiotic compounds has a positive effect on gingivitis.[15] In another study with *L. reuteri,* the effects of probiotic bacteria on oral wounds in mice were examined and it was shown that there was a significant increase in stem cell migration capacity, expression of stem cell markers, osteogenic differentiation, and proliferation of gingival fibroblast cells.[6] In another study examining the effect of *L. reuteri* on the viability of HGFCs, it was reported that this bacterium did not have a cytotoxic effect on HGFCs cells.[16] These results are consistent with our findings.

Motiflor AS, which is commercially available and whose effect we have investigated, contains 13.75 mg of each; *L. helveticus, L. rhamnosus, L. salivarius, and B. longum* bacteria species are included. *L. helveticus* is a type of lactic acid bacteria known to have healing effects on the gastrointestinal tract.[17] In the study of Yamashita *et al*.,[18] it was reported that *L. helveticus* can prevent and treat collagen-induced arthritis by upregulating anti-inflammatory factors. In recent years, its use in the treatment of *L. helveticus* periodontitis has been investigated. Khasenbekova *et al*.[19] confirmed that the probiotic mass containing *L. helveticus* can significantly reduce local inflammation in periodontitis.

Näse *et al*.[20] evaluated the effect of milk with added *L. rhamnosus* on dental caries in 594 children aged 1 to 6 years. According to the results of this study, they found that consumption of milk supplemented with *L. rhamnosus* in children aged 3–4 years significantly reduced the amount of *S. mutans* in saliva and caries formation. In another study examining the effects of a probiotic (*L. rhamnosus*) and a pathogenic (*P. gingivalis*) bacteria on gingival epithelial cells, it was reported that probiotic bacteria had no cytotoxic effect on cell viability and reduced gingival inflammation caused by pathogenic bacteria.[21] These results are consistent with our findings.

Bifidobacterium are probiotics commonly used in humans. Caglar *et al*.[22] investigated the effect of short-term consumption of yogurt-containing *Bifidobacteria* on the amount of *S. mutans* and *S. lactobacilli* in the saliva of young adults. They found that consumption of yogurt-containing *Bifidobacteria* significantly reduced the amount of *S. mutans* in saliva; however, they observed that it did not change the amount of *Lactobacilli*.

In order for probiotic bacteria to prevent and slow down tooth decay, they must be able to adhere to host surfaces and be included among the bacteria that form microbial dental plaque. However, probiotic bacteria must compete with cariogenic bacteria and be able to harm the bacteria and prevent the growth of bacteria. In addition, probiotic bacteria should be able to reduce acid production by affecting carbohydrate metabolism.[23]

Probiotics directly or indirectly affect oral health. Through their direct interaction, probiotics inhibit biofilm formation, compete with oral microorganisms for available substances, and produce chemicals (organic acid, hydrogen peroxide, and bacteriocin) that inhibit bacteria that harm oral hygiene. Through indirect interactions, probiotics play a role in eliminating harmful bacteria and stabilizing normal conditions, regulating systemic immune function and nonimmunological defense mechanisms.[24]

Although we can get rid of periodontitis with conventional oral cleaning therapy, it is known that periodontitis tends to recur after treatment. It has been reported that probiotics can effectively treat such oral diseases by improving the microbial environment in the oral cavity of periodontitis patients.[25] It is of great importance for clinical use to know that probiotics have a positive effect on the microenvironment as well as a beneficial effect on the microbial environment.[26,27] In our study, it was determined that Motiflor AS had a proliferative effect on HDPSCs and HGFCs, which will form the oral microenvironment, and did not show cytotoxicity.

The increase in cell proliferation in the 48–72 h range was greater than in the 24–48 h range. We think that with the addition of PT to the medium between 24 and 48 h, different living cells spend this time adapting to the microenvironment for mutualistic relationships.[28,29] Probably due to HDPSCs being incompletely differentiated cells, their proliferation was faster in the 24–48 h range compared to HGFCs.[30,31]

The ecological shift of the periodontal microbial community toward disease can be prevented by the intake of oral microenvironment-specific probiotics such as Motiflor AS, which contain beneficial bacteria known to have antimicrobial and immunomodulatory properties. Therefore, Motiflor AS may include a discussed ecological therapeutic approach to control periodontitis.

CONCLUSIONS

In addition to the positive effect of probiotic bacteria on inflammation, it is clinically valuable that they have a proliferation-increasing effect on HDPSCs and HGFCs, which will form the dental pulp and gingiva, respectively. In general, probiotics have been discussed in terms of their effects on regulating inflammation. The possible impact of Motiflor AS on the established oral microenvironment (teeth and gingiva) is discussed for the first time in this study. Comprehensive research on this subject will also reveal the different benefits of probiotics on human oral health.

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Ethics committee approval
Ethics committee approval is not required as the cell culture is obtained from a standard line.

Peer-review
Externally peer-reviewed.

Authorship contributions
Concept; T.E. -Design; T.E., Y.E.M; -Supervision; T.E. Funing; T.E., Y.E.M; -Materials; T.E., Y.E.M; -Data collection and/or processing; T.E., Y.E.M; - Analysis and/or interpretation; T.E., Y.E.M; - Literature search; T.E., Y.E.M; - Writing; T.E., Y.E.M; Critical review; T.E.

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Conflicts of interest
There are no conflicts of interest.

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