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Exopolysaccharide of *Lactobacillus rhamnosus* E9 Strain Improves Dental Pulp Mesenchymal Stem Cell Proliferation, Osteogenic Differentiation, and Cellular Collagen Production

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HIGHLIGHTS

- The effective EPS concentration was determined by xCELLigence analysis method.
- EPS was able to accelarate ostegenic differentiation.
- Cellular collagen was increased in the EPS gropus.
- EPS reduced the calcium granules that occurs due to long term cultivation.

Abstract: Among mesenchymal stem cell (MSCs) sources, the most interesting ones in tissue engineering and regenerative medicine are oromaxillofacial tissue. Of these, dental pulp derived (DP-MSCs) MSCs are part of interest. The substantial expansion of MSCs *in vitro* is a requirement site to achieve adequate cell numbers for cell-based therapy. However, the limited proliferation of MSCs diminishes with long term cell culture amplification. Therefore, natural agents are being investigated to increase proliferation and/or differentiation. *Lactobacillus rhamnosus* draws attention with the expolysaccharides (EPSs) it produces. EPSs have been attributed to have a significant role in probiotic activity; including immunomodulatory, antitumor, cholesterol lowering, biofilm reducing, antioxidant, antiallergic, and wound healing effects. However, there is incompetent knowledge discussing their effect on DP-MSCs. In this study we aimed to demonstrate the induced proliferation, differentiation and cellular collagen secretion of DP-MSCs in response to the *L. rhamnosus* E9 EPSs. 1000 µg/mL EPSs was determined as the effective concentration by using a real-time monitoring system. *L. rhamnosus* E9 EPS was able to accelarate ostegenic differentiation and secretion of cellular collagen. Indeed, EPS was able to decreased the calcium granules that occurs due to long term cultivation. By consideration of inducing proliferation, osteogenic differentiation and secretion of cellular collagen, *L. rhamnosus* EPS could be considered as good candidate for preconditioning agent and/or scaffold material.

Keywords: *Lactobacillus rhamnosus*; exopolysaccharide; dental pulp mesenchymal stem cells; collagen; osteogenic differentiation.

INTRODUCTION

Today, several factors, including the aging, genetic factors, food habits, and lifestyle behaviours have caused diseases related to bone [1]. Osteoporosis, osteogenesis imperfecta, osteodystrophy, osteosarcoma, bone fractures in craniofacial area and long extrremities are the main diseases lead to clinical, economical and psyhcological issues [2]. Although the current therapeutic strategy involves bone grafting via autogenic, allogenic or xenogenic origins, each of them has many limitations [3]. For this reason, cell therapy techniques using mesenchymal stem cells is part of interest [4, 5].

Among the different mesenchymal stem cell (MSCs) sources, the most remarkable ones in tissue engineering and regenerative medicine are those originate from oromaxillofacial tissue [6]. Due to their similarity to other MSCs, their ease of obtainability and easy processing, MSCs from dental pulp tissue (DP-MSCs) are of particular interest [7]. The potential of DP-MSCs has been studied for bone regeneration, however the therapeutic efficiency of DP-MSCs needs further consideration and examinations through *in vitro* and *in vivo* studies [8].

It is important to expand DP-MSCs *in vitro* to achieve in desired cell number, and directed acelerated differentiation for clinical therapy [9]. Over 10 years, different physical conditions (scaffolds, membranes), pharmacological agents, and biological approaches (e.g. Cytokines) are used. However, undesirable side effects were seen and many of them may cause malignant formation in healthy cells [10-14]. Considering these limitations of synthetic drugs, probiotics and/or their metabolites could be a new perspective in cellular therapies or regenerative medicine [15, 16].

Probiotics are non-pathogenic, live organisms, when administered in sufficient amounts, beneficially affect the health of the host [17]. Among the probiotics, lactic acid bacteria (LAB), have been widely used in animal husbandry and food additives since they are declared as safe by The American Food and Drug Administration (FDA) [18]. A various set of benecifial properties have been ascribed to probiotics involving modulation of immunity, reduction in colorecteral cancer, promoting intestinal epithelial growth and survival, alleviating symptoms of lactose intolerance antagonizing the growth of pathogenic bacteria, enhancing mucosal barrier function, and wound healing [18-27]. Probiotics comprise a wide genera, involving *Bifidobacterium*, *Propionibacterium*, *Escherichia*, and *Sacchoromyces*, and particularly, *Lactobacillus* sp. [27].

In the genera of *Lactobacilli*, *Lactobacillus rhamnosus* GG is a well known strain which probiotic effects are attributed to the ability of exopolysaccharide (EPS) production [17, 28-31]. EPSs, are a polymer secreted on cell surface and/or in the microenvironment and, have been used as a natural biothickener owing to its smoothing and stabilizing effects on fermentation products [32-35]. Nevertheless its immunostimulatory, antitumor, cholesterol lowering, antibiofilm, antioxidant [36] and wound healing effects have also received attention [27, 36- In our previous studies, we reported that *L. rhamn*osus GG (ATCC95905) have the ability to modulate immune response by suppressing TLR-4 and TLR-2 expression [29]. Also another strain of *L. rhamnosus* (strain GD11) with high EPS production ability showed protective effect through its antioxidant activity on gingival fibroblasts exposed to oxidative damage by hydrogen peroxide [36]. Tukenmez and coauthors (2019) demonstrated the monomer composition and structure of *L. rhamnosus* EPSs (strain E9). They showed that E9 strain EPSs is mannose rich heteropolysaccharide has a low molecular weight leading easy entry to cell [27]. There was no data describing the effect on DP-MSCs which are promising cell therapy agents in bone and pulp regeneration. From this point of view, we aimed to investigate the effect of *L. rhamnosus* EPS on DP-MSC proliferation, osteogenic differentiation and cellular collagen production in this study.

MATERIAL AND METHODS

Culture of bacterial strain

L. rhamnosus E9 was isolated and identified from healthy infant feces previously [41] 16S rRNA sequence analysis was used to confirm identification and the universal primers (Uni27F, 5′ AGAGTTTGATCCTGGCTCAG 3′ and Uni1492R, 5′ GGTTACCTTGTTACGACTT 3′) are used. MRS broth was used to maintain stock cultures in −20 °C with 10% (v/v) glycerol (Oxoid, Istanbul, Turkey) and stored at Gazi University, Biotechnology Laboratory Culture Collection. Frozen stocks were used to prepare working

samples by two sequential transfers to MRS broth and, subsequently incubated aerobically at 37 °C for 18 h.

Isolation and lyophilization of exopolysaccharide

EPS of E9 was isolated according to the method of [32]. The growth culture was adjusted to 0.6 optical density at 600 nm (~8.5 log) and boiled in a water bath for 15 min to restrict the viability of bacteria. The sample was cooled and subsequently treated with trichloracetic acid solution (17% of 85%) and centrifuged at 15000 rpm for 20 min. The EPSs was precipitated in absolute ethanol (1:1) and centrifuged at 13000 rpm for 15 min. The fluid was discarded and the pellet containing EPS was dissolved in deionized water. Total carbohydrate in the samples were measured by phenol-sulfuric acid method which the glucose was used as standard [42]. The isolated EPSs were stored at −80 °C. Lyophilization was conducted in Christ Alpha 2–4 freeze dryer (Marin Christ Co. FL, USA). The freeze-dried EPS powder was stored at 4 °C [43].

Cell culturing

Commercially available DP-MSCs were purchased from Lonza (Treviglio, Italy) (PT5025, and Lot: 0000361150) and cultured in DMEM-LG (minimal essential medium alpha Eagle low glucose) with L-glutamine (2nM), penicillin (100 U/mL), streptomycin (100 µg/mL), and fetal bovine serum (10 %, FBS) (Invitrogen/GIBCO, Grand Island, NY, USA) (hereafter referred to as the MSC culture medium, CULT). The DP-MSCs were cultured in a concentration of $1x10^5$ cells/mL in CULT, and the cell growth were followed regularly with an inverted microscope (Olympos CKX41, Tokyo, Japan). The CULT was changed every three days. Trypsin/EDTA (0.05%) (SigmaAldrich, St. Louis, MO, USA) was used to harvest the cells, when 70–80% confluence was obtained and, sub-cultured for further experiments. Passage 4-5 (P4-5) cells were used in the the experiments. Throughout the study, the experimental conditions and protocols of cell culture handling and monitoring were followed according to ATCC guidelines.

xCELLigence Analysis

The system, consists of 4 segments: (i) an impedance-based real-time cell analyzer, (RTCA), (ii) E-plate 16, (iii) computer and, (iv) cell-culture incubator (Roche Applied Science, Mannheim, Germany) (Roche Diagnostic, 2008). The E-plate 16 was connected to the RTCA, subsequently checked in the incubator to ensure that concurrent electrical contacts were established. Then the background impedance was measured. Impedence values recorded by xCELLigence instrument were reported using the unitless parameter cell index, which is defined as (Zn -Zb)/15, where Zn and Zb are the impedance values in the presence and absence of cells, respectively [44].

100 µl of an CULT involving $1x10^4$ DP-MSCs /well were addded to each E Plate16 well and, incubated until the cell indexes reached to 1,00 (±0,02). When the targeted cell index was reached (at 15 hours), the media was replaced with new media containing different concentrations of *L. rhamnosus* E9 EPS (25, 50, 100, 500, 800, 1000 µg/mL). Cell indexes were normalized in the system software and followed up for 24 and 48 hr 15 min intervals for viability and proliferation.

The Effect of L. rhamnosus E9 EPS on DP-MSCs Osteogenic Differentiation

Pittenger and coauthors (1999) method's was used to demonstrate the osteogenic differentiation. Selected EPS concentration was added to the osteogenic medium (OST) only at the days of 1 and 14 (OST-1,14) and continously for 21 days (OST-CONT) [45]. The growth medium (CULT) was the control group for OST. Also, CULT-1, 14 and CULT-CONT were used as the control group for OST-1, 14 and OST-CONT. The media was replaced on days 1, 4, 7, 10, 14, 17. In groups OST-1, 14 and CULT-1, 14, EPS was added to the medium on days 1 and 14 only. In the OST-CONT and CULT-CONT groups, EPS was included in each medium change. The images were obtained at day 21 (Olympus CKX41, Tokyo, Japan). The discarded culture supernatant was stored in -80°C. The secreted Osteonectin (between the limits 3,12-200 ng/mL) and Osteocalcin (between the limits 0,2-40 ng/mL) protein levels in the culture supernatants were determined through ELISA kit (Cloud-Clone Corp, USA). The concentration of calcium ion in the DP-MSCs OST, OST-1,14, OST-CONT and CULT, CULT-1,14, and CULT-CONT supernatant was measured also (DICA 500, BioAssay Systems, Hayward, USA).

Quantification of the Cellular Collagen of EPS-treated DP-MSCs

Collagen quantification in the EPS-treated DP-MSCs were examined according to the manufacaturer's instructions (Sircol Collagen Assay, Biocolor). The cellular collagen was studied in CULT and OST in 12 well culture plate. Analyzes were performed on day 21. In brief, EPS-treated and nontreated cells were incubated in acetic acid (0,5 M containing 0,1 ng/mL pepsin) for 48 h at 48°C. The Sircol dye reagent was used to form collagen-dye complex. After centrifugation, the pellet was washed in the Acid-Salt Wash reagent and then resuspended in the alkali reagent. The absorbance was read at 555 nm. The amount of cellular collagen (n=3) was calculated based on a standard curve obtained with the bovine type I collagen supplied with the kit.

Statiscal Analysis

All calculations were carried out using the xCELLigence system The cell index, IC50 and doubling time data are presented as the mean (µg/mL) ± SD (n=3). Differentiation and cellular collagen quatintification (n=3) results are presented as the mean ± standard deviation (SD). For the experiments, a statistical analysis was performed using a one-way analysis of variance (ANOVA) (p<0.05).

RESULTS

xCELLigence assay

Up to 48 hours of cell viability were monitored in the study. It was seen that a growth curve graph consisting of plateau, logarithmic and stationary phases is formed. At the end of the duration, an impedence graph which shows viable cells was created. Cell index and doubling time (DT) were analyzed separately as 24 and 48 hours. Data were normalized at the 15th hour when the cell index reached 1.00±0,02. Figure 1.a shows the proliferation graph of DP-MSCs treated with different concentrations of EPS. DP-MSCs division appeared to be the highest at 1000 µg/mL EPS concentration. It was also observed that DP-MSCs entered the stationary phase later than the control group at 1000 µg/mL EPS concentrations. Figure 1.b (24 hours) and Figure 1.c (48 hours) show the cell indexes of DP-MSCs. It was determined that the cell index increased in paralell with concentration compared to the control group. Doubling time (DT), which indicates the amount of time to double of size of cells, was found short at 25 µg/mL and 1000 µg/mL concentrations until 24 hours (shown in Figure 1.d). At 48 hours (shown in Figure 1.e) the DT slowed due to the increase in cell confluency. Half maximal effective concentration, EC50, was also determined as 19µg/mL at 48 hours (shown in Figure 1.f).

1000 µg/mL EPS concentration was selected for differentiation and ECM quantification assays since the concentration increased cell index and reduced doubling time of DP-MSCs.

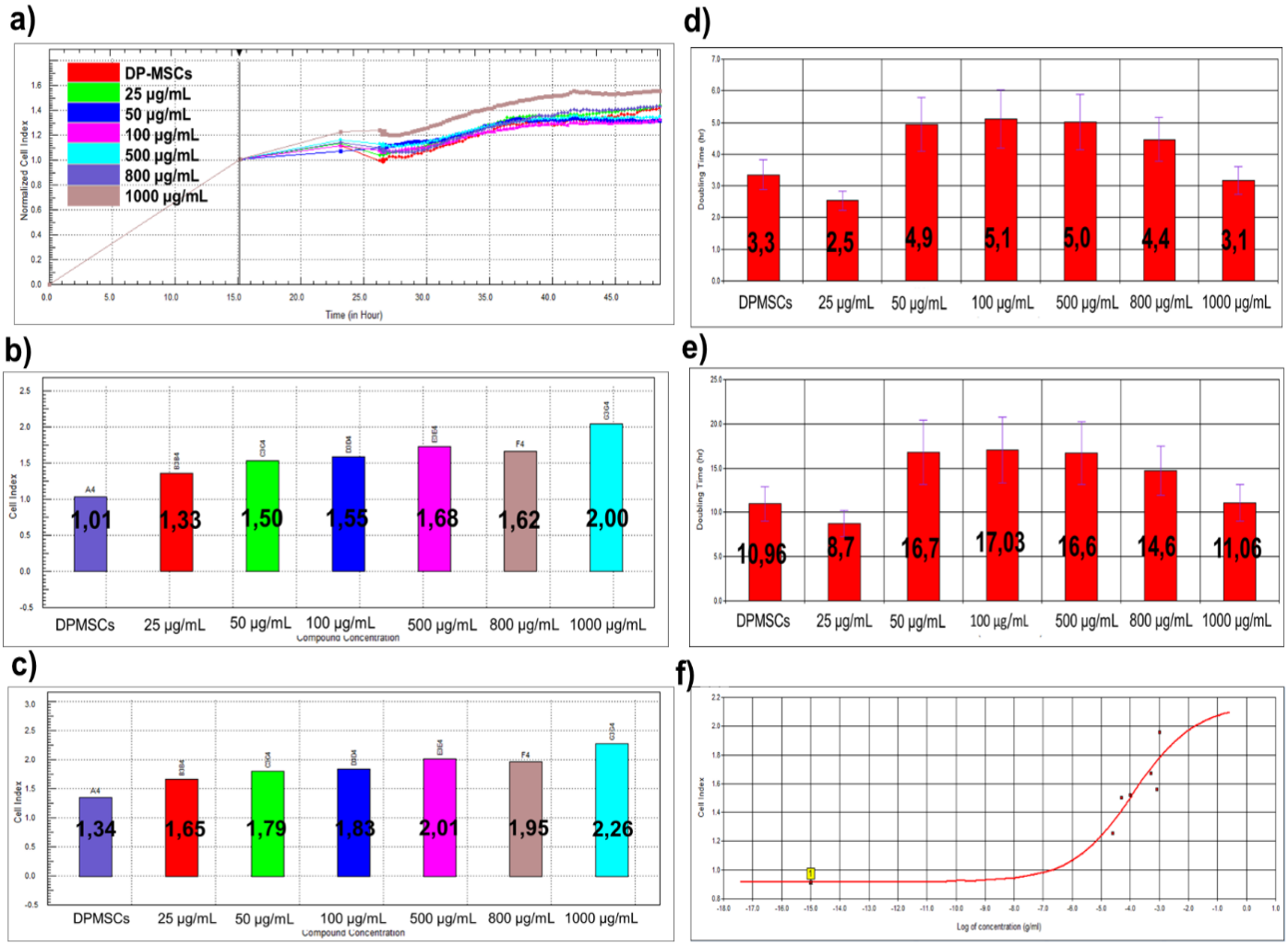

Figure 1. Effect of EPSs on DP-MSCs proliferation. a. Cell growth graph. The cell index was normalized at the 15th hour. Cell viability was monitored with measurements taken every 15 minutes for 48 hours. b, c. Vitality values for 24 and 48 hours, respectively. d, e. Doubling time values for 24 and 48 hours respectively. f. IC50 value.

The Effect of the E9 strain EPS on DP-MSCs osteogenic differentiation

1000 µg/mL EPS was added to osteogenic differentiation (OST) and CULT as control. Media were changed every 3 days for 21 days. Alizarin red staining showed that calcification was occured in the CULT (shown in Figure 2.a). Interestingly, calcification was seen decreased in the CULT- 1.14, and no calcification was observed in the CULT-CONT. In the ostogenic differentiation group, mineralization was observed as the highest in the group in OST-CONT, and the ECM was strongly stained. The levels of the osteogenic markers (osteonectine and osteocalcine), and calcium concentration were also examined (shown in Figure 3.a-c). The ON (osteonectin) level was decreased in the OST-1,14 and OST-CONT, whilst the levels of OCN (osteocalcine) and calcium concentration increased when compared with the OST (p<0,05).

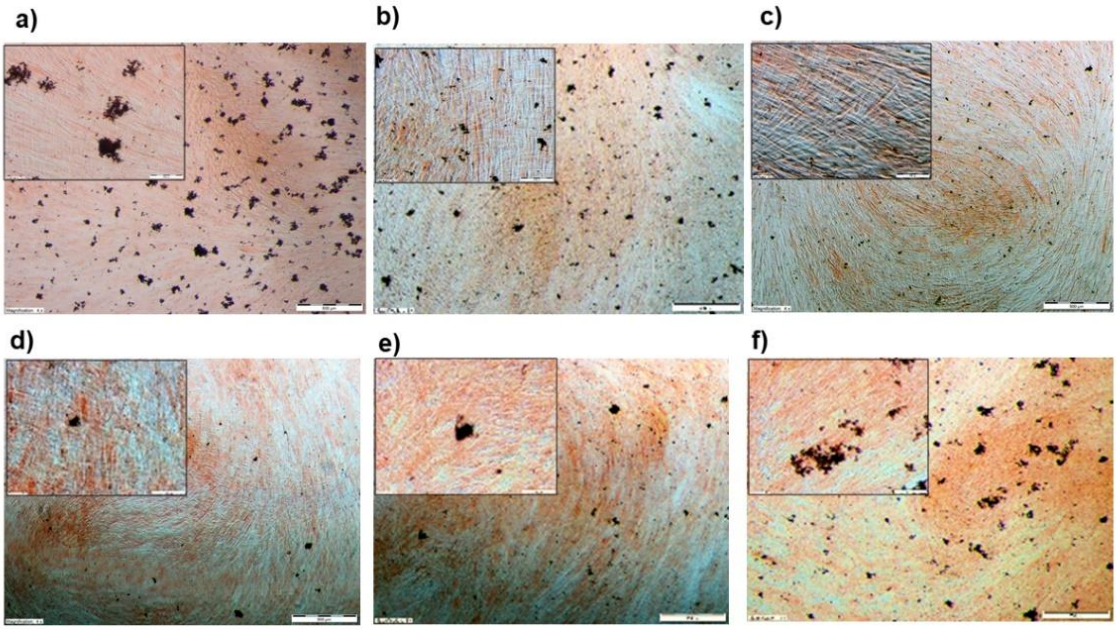

Figure 2. Follow-up of EPS effect in osteogenic growth (a, b, c) and differentiation (d, e, f) culture medium at 21st day (Olympos CKX41) a) CULT, b) CULT-1,14, c) CULT-CONT, d) OST, e) OST-1,14, f) OST-CONT. CULT: MSCs culture medium; CULT-1, 14: EPS added culture medium at day 1 and day 14; CULT-CONT: Continuously EPS added culture medium; OST: Osteogenic medium, OST-1,14: EPS added osteogenic medium at day 1 and day 14; OST-CONT: Continously EPS added osteogenic medium.

Cellular collagen quantification

The amount of cellular collagen was shown in Figure 3.d. Both in growth and osteogenic media collagen quantification was increased in CULT-CONT (p<0,05) and OST-CONT (p<0,05).

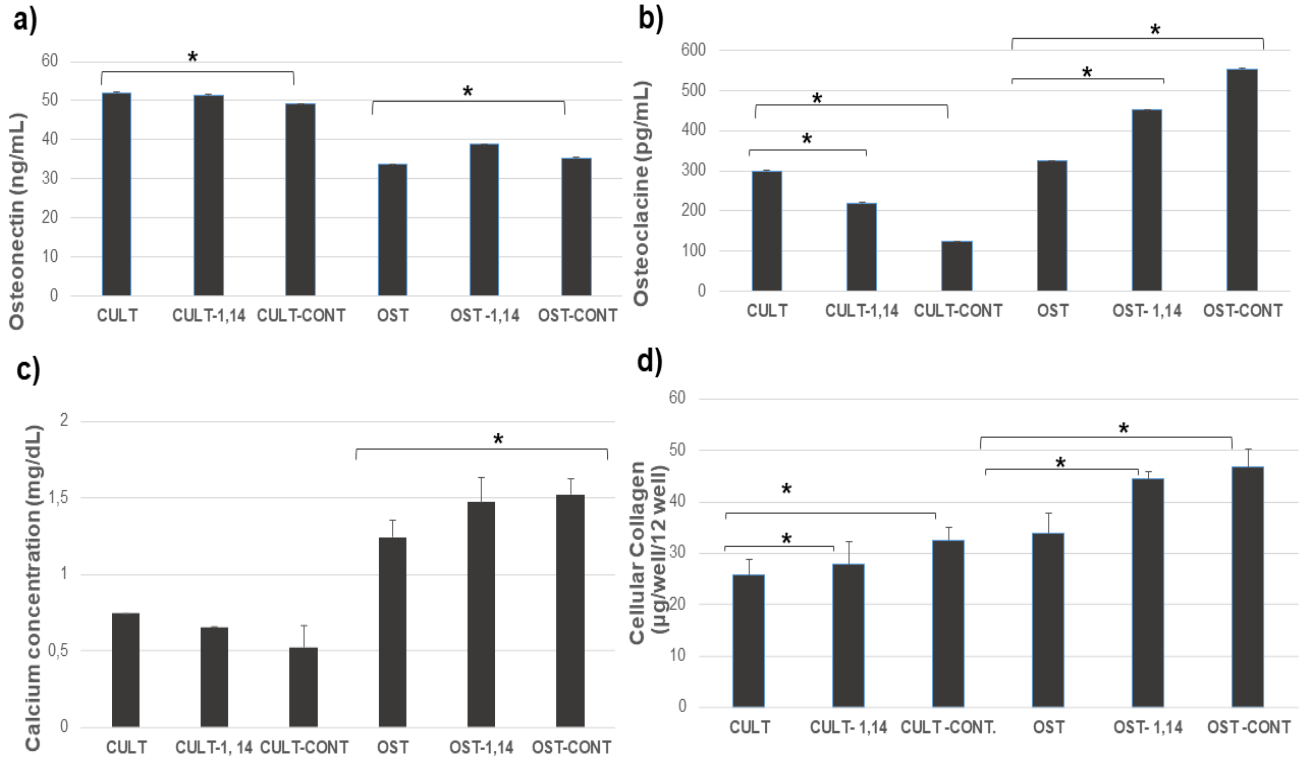

Figure 3. a) Early osteogenic differentiation marker in culture supernatants Osteonectin levels, b) Late osteogenic differentiation marker Osteocalcin levels in culture supernatants, c) Calcium concentration in osteogenic differentiation and growth culture, d) Cellular collagen amounts. CULT: MSCs culture medium; CULT-1, 14: EPS added culture medium at day 1 and day 14; CULT-CONT: Continuously EPS added culture medium; OST: Osteogenic medium, OST-1,14: EPS added osteogenic medium at day 1 and day 14; OST-CONT: Continously EPS added osteogenic medium.

DISCUSSION

Probiotics have been newly clinically introduced into the field of dentistry, immunology, gastroenterology with the improvement of health concerns [46]. However, the effects of beneficial bacteria and their metabolites, on the function of MSCs is still unclear [47, 48]. In this study, targeting *L. rhamnosus* EPS effect, we investigate the proliferation, osteogenic differentiation, and cellular collagen production of DP-MSCs. To the best of our knowledge, this study is the first to describe that EPS stimulates DP-MSC viability in long term culture conditions, accelarates osteogenic differentiation, and stimulates the secretion of the amount of cellular collagen.

L. rhamnosus is a well-known probiotic that could be isolated from different origins such as milk, infant feces and the oral cavity [49]. We prefer to study with *L. rhamnosus* since the strain was well known for the ability to secrete high amounts of EPS [27, 29, 50]. Furthermore, in our previous studies we showed significant protective effect on gingival fibroblasts against hydrogen peroxide stimulated oxidative stress and prevents *Streptococcus mutans* to adhere to fibroblasts from gingiva and mesenchymal stem cells from bone marrow [21, 36].

xCELLigence analysis used in this study allowed to show label free, the real-time cell proliferation monitoring. The functional unit of xCELLigence RTCA impedance assay is a set of gold microelectrodes fused to the bottom surface of a microtiter plate well. When submersed in n electrically conductive solution such as growth medium, the application of a weak electric potential across these electrodes causes electric current to flow between them. Since this phenomenon is dependent upon the elecctrodes interacting with solution, the presence of adherent cells at the electrode-solution interface impedes current flow. The magnitude of this impedence is dependent on the number of cells, the size of the cells, and the cell substrate attachement quality. Thus, the simultaneous proliferation of cells can be monitored accurately. In oır study, it was seen that cell index was increased depending on EPS concentration. 1000 µg/mL EPS managed to shorten doubling time (DT) and increased cell index. This results were in agreement with the report which describes EPSs may have a esssential role in organizing the signallig pathways of fibroblast growth factor [52]. In studies conducted with the use of probiotics and/or metabolites in cells or animals, dose determination is based on personal experiences. There are several reports on neoplastic cell lines determining the lethal doses [52]. This is the first to describe the effective dose in mesenchymal stem cells. There is no definitive result reporting EPS and/or probiotic concentration. Shirzad and coauthors (2018) selected a 2000 mg/mL EPS as effective concentration by using MTT analysis where it is calculated as IC50 value in our study [41]. In this study, as in our other studies, it is thought that reliable data are used in real-time cell proliferation monitoring method for effective concentration selection [8, 53]. We found a 1000 µg/mL concentration induced the proliferation of DP-MSCs. In paralel with our study a significant increase in the proliferation of stromal cells was observed by the probiotic culture supernatant; where the EPSs are secreted [53, 54].

We designed an *in vitro* model to exhibit whether the EPSs induce osteogenic differentiation of DP-MSCs in which markers of osteogenesis, concentration of calcium ions and determination of collagen secretion were assesed. During osteogenic differentiation preosteoblasts secrete ON and bind to collagen with a less affinity. Additionally, ON has a short half-life (>24 hours) and quite stable in the culture [56, 57]. This is why the ON, was reduced in OST-CONT and OST-1.14. EPSs accelerated the osteogenic differentiation of DP-MSCs and ON decreased in the culture supernatant. Indeed OCN, late marker of osteogenic differentiation, was higher in OST CONT and OST-1.14. According to our research, our study is the first to show that EPS promotes the osteogenic differentiation of dental pulp mesenchymal stem cell in the *in vitro* model. There are several *in vivo* animal models which declare that probiotics could prevent/attenuate systemic bone diseases [57, 58]. Maekawa and coauthors (2014) demonstrated that topically used *L. brevis* inhibits alveolar bone loss [59]. Garcia and coauthors (2016) reported that a combination of *Saccharomyces cerevisiae* administration reduced alveolar bone loss [61]. In another study, orally administered *L. rhamnosus* protected mandibular bone loss [62]. Increased osteogenic differentiation in DP-MSCs with EPS, has given us hope for its potential for use as a topical or scaffold in dentistry and / or other bone injury therapies.

Surprisingly, spontaneous mineralization of DP-MSCs in CULT group was detected. During culture, MSCs may age owing to exposure to physical or chemical conditions. In this study, even though the medium was changed every 3 days, 21 days was still a challenging environment for the culture to continue as the cells spread fully in the plate well. It was reported that spontaneous calcification of bone marrow derived MSCs from rats, which were under normoxia condition (20% O_2) without osteogenic medium after continous culture for 21 days [54]. In similar with our study, mineralized nodules and extracellular matrix were detected by Alizarin Red Staining. It is known that granularity increase is considered as aging signs [62, 63]. Although senescence assay did not conducted in this study we propose that *L. rhamnosus* EPS may alter the aging and/or senescence of MSCs. Antioxidant, antiinflammatory and anticancer properties of *L. rhamnosus* may

lead to change the pathway of senescence since *L. rhamnosus* is able to recognize the difference between neoplastic cells and fibrolasts or stem cells [64].

Also, we examined the cellular collagen concentration of DP-MSCs in osteogenic and growth culture media treated with EPS. It was observed that the amount of cellular collagen in the control and osteogenic differentiation groups increased in DP-MSCs treated with continuous EPS. The ECM is a dynamic microenvironment in which its components allow structural support to the cells. In addition ECM has regulatory effects in terms of directing cellular signalling, shape, migration, and differentiation [66]. Collagen is one of the ECM proteins that play a part in cell activity. Collagen also plays essential roles in adhesion, migration, and ostoegenic differentiation [67-68]. Increased cellular collagen in growth and ostegenic culture of DP-MSCs with *L. rhamnosus* EPS is suggested to be a pomising regenerative inducer for preconditioning studies. It was reported that function of gingival and palatal MSCs was impaired in oral microbiological imbalance [68-71]. Up to now, it was discussed that delayed wound healing processed was attributed to the result of imbalanced flora [72-75]. We suggest that EPSs of *L. rhamnosus* could accelerate wound healing via inducing collagen secretion and well known competitive adhesion ability to the cells [21, 37].

The role of EPSs in probiotic activity may be polymer and strain dependent [54]. EPSs show diversity in their sugar composition. Most of EPSs are heteropolysaccharides those consist of different sugars for instance glucose, galactose, rhamnose, N-acetylglucoseamine, N-acetylgalactosamine, and mannose [54]. *L. rhamnosus* E9 EPS was found to be a mannose rich heteropolysaccharide [27]. Increased proliferation, osteogenic differentiation and, collagen production can be explained by publications in which EPSs containing more than 50% mannose are reported as biologically active [75]. On the other hand, in parallel with our study, glycated collagen with mannose improved the cell proliferation of pancreatic islet stem cells [77]. There are several studies reporting the anticancer activity of EPSs has thought to be related with high amount of mannose in sugar composition also [78]. Mannose is widely used as an inexpensive backbone for the synthesis of immunostimulatory and antitumor agents, in novel non-viral gene therapy approaches, and as a mediator in natural killer cell function [79]. Also, it was shown that polysaccharides consisiting of glucose and mannose can easily interact with Toll like receptors (TLRs) and activate host immunity [80]. The response of MSCs to known TLR activators, and the ability of a TLR2 and TLR4 ligand to regulate MSC proliferation and differentiation has been reported [81]. We know from our previous study that *L. rhamnosus* could activate *in vitro* both TLR-2 and TLR-4 through increasing IL-6 while downregulate IL-8 [29].

CONCLUSION

In conclusion, we suggest that EPSs of *L rhamnosus* E9 accelerates the proliferation and differentiation of DP-MSCs via heteropolysaccharide composition, especially mannose sugar. Reducing the calcification in long term growth culture justify further investigations on the MSCs senescence and aging studies. EPSs may be used as monotherapy, a component in scaffolds or culture media, and filling materials since they found to be effective at proliferation, differentiation, and extracellular matrix component production.

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Conflicts of Interest: The authors declare no conflict of interest.

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