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An *in vitro* coculture approach to study the interplay between dental pulp cells and *Streptococcus mutans*

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Abstract

Aim: To develop a new coculture system that allows exposure of dental pulp cells (DPCs) to *Streptococcus mutans* and dentine matrix proteins (eDMP) to study cellular interactions in dentine caries.

Methodology: Dental pulp cells and *S. mutans* were cocultured with or without eDMP for 72 h. Cell proliferation and viability were assessed by cell counting and MTT assays, while bacterial growth and viability were determined by CFU and LIVE/DEAD staining. Glucose catabolism and lactate excretion were measured photometrically as metabolic indicators. To evaluate the inflammatory response, the release of cytokines and growth factors (IL-6, IL-8, TGF-β1, VEGF) was determined by ELISA. Non-parametric statistical analyses were performed to compare all groups and time points (Mann–Whitney U test or Kruskal–Wallis test; $\alpha = .05$).

Results: While eDMP and especially *S. mutans* reduced the number and viability of DPCs ($p \leq .0462$), neither DPCs nor eDMP affected the growth and viability of *S. mutans* during coculture ($p > .0546$). The growth of *S. mutans* followed a common curve, but the death phase was not reached within 72 h. *S. mutans* consumed medium glucose in only 30 h, whereas in the absence of *S. mutans*, cells were able to catabolize glucose throughout 72 h, resulting in the corresponding amount of L-lactate. No change in medium pH was observed. *S. mutans* induced IL-6 production in DPCs ($p \leq .0011$), whereas eDMP had no discernible effect ($p > .7509$). No significant changes in IL-8 were observed ($p > .198$). TGF-β1, available from eDMP supplementation, was reduced by DPCs over time. VEGF, on the other hand, was increased in all groups during coculture.

Conclusions: The results show that the coculture of DPCs and *S. mutans* is possible without functional impairment. The bacterially induced stimulation of proinflammatory and regenerative cytokines provides a basis for future investigations and the elucidation of molecular biological relationships in pulp defence against caries.

KEYWORDS
bacteria, coculture techniques, dental pulp, dentin, glucose, lactic acid

INTRODUCTION

Dental caries is one of the most prevalent chronic diseases worldwide (Bagramian et al., 2009) and is caused by oral biofilms. These biofilms contain saccharolytic bacteria that metabolize sugars and produce organic acids, which demineralise enamel and dentine. Here, the microflora is dominated by Gram-positive bacteria such as streptococci, lactobacilli and actinomycetes, which can change depending on environmental factors, such as diet, saliva or the age of the lesion (Hahn & Liewehr, 2007; Hoceini et al., 2016; Love & Jenkinson, 2002). In particular, saccharolytic bacteria, such as mutans streptococci and lactobacilli, play an important role in the development and progression of caries (Zheng et al., 2019).

One of the Gram-positive bacteria that has been consistently associated with caries in both traditional culture studies and culture-independent techniques is *Streptococcus mutans*. Clinically, elevated levels of *S. mutans* have been found in the oral environment of patients prior to the development of caries and in patients with active lesions (Leverett et al., 1993; Tanner et al., 2011). Clinical studies have also reported that the reduction of mutans streptococci is associated with the prevention of caries and its recurrence with the development of new caries (Featherstone et al., 2012) demonstrating its role in the microbial dysbiosis associated with caries. Notably, its specific virulence factors are also characteristic of carious decay. For example, *S. mutans* is able to synthesize large amounts of extracellular matrix from sucrose and can also convert carbohydrates into organic acids. In this way, it creates the acidic environment necessary for the demineralization of enamel and dentine without being restricted in its function (Baker et al., 2017).

As the dental hard tissue continues to deteriorate, bacteria and their metabolites diffuse through the dentinal tubules and eventually reach the dental pulp. Here, odontoblasts are the first to encounter pathogenic stimuli, before more centrally located dental pulp cells (DPCs) and immune cells become involved (Izumi et al., 1995; Veerayutthwilai et al., 2007). DPCs are the most abundant cell type in the dental pulp and have been described to play a key role in both inflammatory and regenerative processes (Staquet et al., 2008). Pattern recognition receptors (PRRs) located in their cell membrane can recognize bacterial molecules or pathogen-associated molecular patterns (PAMPs) and elicit an antigen-unspecific response (Galler et al., 2021). For example, in the context of deep caries, Gram-positive microorganisms such as *S. mutans* can interact with DPCs via the PAMP lipoteichoic acid (LTA), which is part of their cell wall (Staquet et al., 2008). LTA binds to the corresponding PRRs of DPCs, Toll-like receptor 2 (TLR-2), which are organized in heterodimers with either Toll-like receptor 1 (TLR-1) or Toll-like receptor 6 (TLR-6), triggering cellular responses (Galler et al., 2021; Long et al., 2009). Activation can lead to the production of various chemokines and cytokines with the aim of modulating pulp inflammation and restoring tissue homeostasis (Galler et al., 2021). Thus, pulp cells are described to interact with immune cells through the expression of cytokines and prostaglandins, leading to the sequential attraction of inflammatory cells such as T cells, macrophages, neutrophils, and B cells, as well as an increased CD4/CD8 ratio of T lymphocytes (Galler et al., 2021), and to modulate the immune response following the activation of TLR-2 by LTA (Galler et al., 2021; Shayegan et al., 2021).

In addition to bacterial interactions with the dental pulp, bioactive proteins are released from the extracellular dentine matrix during acid demineralisation (Widbiller, Eidt, Lindner, et al., 2018). Among the proteins released are various signalling molecules such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF) or nerve growth factor (Widbiller et al., 2019, Widbiller, Eidt, Wölflick, et al., 2018), which can act on bacteria and, more importantly, on the cells of the dental pulp (Smith et al., 2012). Their molecular biological effects on DPCs are well described and include stimulation of their migration (Suzuki et al., 2011; Widbiller, Eidt, Lindner, et al., 2018) and induction of their differentiation into odontoblast-like cells and mineralisation (Tziafas et al., 1992; Widbiller, Eidt, Lindner, et al., 2018). Therefore, the bioactive components of the dentine matrix cannot be ignored when considering the interaction between bacteria and pulp cells.

While it is possible to study cellular responses to extracted dentine matrix proteins (eDMP) *in vitro*, microbiological cues can only be simulated by recombinant proteins, antigens extracted from bacteria or inactivated microorganisms (Schweikl et al., 2016; Widbiller, Eidt, Wölflick, et al., 2018). However, it can be assumed that pulp cells will respond more specifically to viable bacteria than to model substances, as these may differ structurally from the native antigens (Kang et al., 2016) or undergo post-translational modification.

To overcome these limitations, an *in vitro* coculture system would be desirable, allowing the cultivation of viable bacteria alongside cells and thus the study of specific signalling and crosstalk between the two organisms. Cocultures between cells and bacteria have been successfully used in other medical fields to study the pathogenesis and treatment of various diseases (Rashwan et al., 2018; Zhang et al., 2021). Therefore, cocultures between pulp cells and cariogenic bacteria such as *S. mutans* may have great potential for basic research to understand pulp pathology in dental caries.

To date, research groups have investigated interactions between *S. mutans* and peripheral blood mononuclear cells with regard to cytokine induction (Hahn et al., 2000). Another study evaluated the effects of *S. mutans* and heat-inactivated *S. mutans* on monocytic cell lines and human umbilical vein endothelial cells (Takahashi et al., 2008). Both studies reported stimulation of pro-inflammatory cytokines in immune cells and activation of lymphocytes, highlighting the importance of microbial stimuli in initial lesioning (Hahn et al., 2000; Takahashi et al., 2008). However, to the best of our knowledge, there is no coculture system that allows the simultaneous investigation of DPCs and viable cariogenic bacteria.

Thus, the aim of this study was to develop a coculture system in which human DPCs can be directly exposed to *S. mutans* in order to study the interaction between cells, bacteria and eDMP, as might occur in dental caries. The specific aims were (i) to verify the viability of both organisms in coculture, (ii) to confirm functional glucose metabolism of *S. mutans* and (iii) to investigate signalling of pulp cells confronted with bacteria and eDMP.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines.

Bacterial culture

Streptococcus mutans (ATCC 25175) was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and handled as recommended. Bacteria were expanded in Tryptic Soy Broth (TSB; Sigma-Aldrich) under aerobic conditions with 5% CO_2 at 37°C and stored in a 1:1 solution of TSB and glycerol (Merck) at −80°C. Planktonic cultures were established by resuspending 200 μL frozen *S. mutans* stocks in 10 mL TSB and culturing overnight. Subsequently, suspensions were harvested by centrifugation and resuspended in TSB yielding an optical density (OD) of 0.188 (10^8 CFU/mL) as measured by means of a spectrophotometer at 600 nm (Ultrospec 3300 pro; Amersham Biosciences) and then diluted 1:10 with PBS (10^7 CFU/mL).

Bacterial growth in different media and impact on pH

Bacteria (10^7 CFU/mL) were added at a 1:100 ratio to either TSB or Minimum Essential Medium alpha (MEM α) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL L-ascorbic acid 2-phosphate, 100 mg/dL (5.5 mmol/L) glucose and free from any antibiotic additives. All reagents for cell culture were purchased from Gibco (Thermo Fisher Scientific). Bacterial growth was verified by plate counting after 0, 6, 12, 24, 36, 48, 72, 96 and 120 h. After each time point medium was carefully removed and growth was determined by dilution plating technique on Columbia Agar with 8% sheep blood (Institute of Clinical Microbiology and Hygiene, University Hospital Regensburg, Regensburg, Germany). Two experiments have been performed in triplicates ($n = 6$) and summarized as median values with 25%–75% percentiles.

To assure a neutral pH of the medium (pH 7.4), the pH value was determined over 72 h using a pH meter (inoLab pH 7110; Xylem Analytics Germany). *S. mutans* was cultured in TSB as described above. Therefore, suspensions were resuspended with PBS, resulting in an OD of 0.188 and diluted 1:10 with PBS. Subsequently 5 mL MEM α were infected with 50 μL bacteria suspension (1:100), or kept as sterile control. Every 24 h, pH values of test tubes and controls were measured. All measurements ($n = 6$) were summarized as median values with 25%–75% percentiles.

Isolation of pulp cells and dentine matrix proteins

Human DPCs were isolated from extracted third molars of patients at age 15–20 with informed consent approved by the Ethics Committee (16-101-0022; Faculty of Medicine, University of Regensburg, Regensburg, Germany), and cultured as previously described (Galler et al., 2006). 2×10^4 cells of passage 3 were seeded into 48-well plates and cultured in MEM α deprived of antibiotics at 37°C with 5% CO_2.

Dentine matrix proteins were extracted from human teeth according to a protocol described before (Widbiller, Eidt, Lindner, et al., 2018). TGF-β1 was quantified (Quantikine ELISA Kit; R&D Systems) and eDMP was added to MEM α in a final concentration of 250 pg/mL TGF-β1.

Coculture and experimental groups

When DPCs reached 70% confluence in 48-well plates, media were exchanged to establish experimental groups as follows: DPCs (2×10^4/well); eDMP (250 pg/mL TGF-β1); *S. mutans* (10^5 CFU/mL); DPCs and eDMP; *S. mutans* and eDMP; DPCs, *S. mutans* and eDMP. Cultures and cocultures were maintained for 72 h at 37°C and 5% CO_2.

Proliferation and viability of cells

The cell number was determined at 0 and 72 h using a cell counter (TC20; Bio-Rad). Therefore, medium was removed and wells were rinsed with PBS. Subsequently, 500 μL of trypsin–EDTA was added and incubated at 37°C and 5% CO_2 for 5 min. After stopping the reaction, cells were collected and counted. Three experiments were performed with 10 replicates each ($n = 30$) and summarized as median values with 25%–75% percentiles.

Cell viability was analysed after 0, 24, 48 and 72 h of culture. Here, medium was replaced by 500 μL/well of 0.5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT; Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) and cells were incubated at 37°C and 5% CO_2 for 60 min. The converted dye was dissolved by 400 μL/well dimethyl sulfoxide (DMSO; Thermo Fisher Scientific), and optical density was measured on a microplate reader at $\lambda = 570$ nm (Infinite 200; Tecan). Median and 25%–75% percentiles were calculated based on five independent experiments in six replicates ($n = 30$).

Proliferation and viability of bacteria

The growth of *S. mutans* was quantified by plate counting after 0, 24, 48 and 72 h. Therefore, after each time point supernatants were removed gently and growth was determined by dilution plating technique on Columbia Agar plates as described above. Medians with 25%–75% percentiles were calculated from three experiments performed in duplicates ($n = 6$).

To get insight into the viability of all bacteria in culture, the LIVE/DEAD Backlight Bacterial Viability Kit (Molecular Probes) was applied at 0 and 72 h. At both time points, media of groups containing *S. mutans* were harvested and centrifuged ($250 \times g$) for 5 min. Pellets were resuspended in 1 mL NaCl (0.9%; Carl Roth). Additionally, 1 mL bacterial suspension was added to either 19 mL NaCl or 19 mL isopropyl alcohol (≥99.8%; Merck) and left for 1 h on an orbital shaker (20 rpm; Miniature Shaker KM-2; Edmund Bühler) at ambient temperature to create samples with specific proportions of live bacteria (100%, 90%, 50%, 10% and 0%). Subsequently, 100 μL of BacLight solution, which contained 15.3 μL SYTO 9, 3.83 μL PI and 3.4 mL *aqua dest.*, were mixed with 2 mL of solution of each group or standard, and stained for 15 min in a dark chamber. Fluorescence was measured on a microplate reader at $\lambda = 485$ nm/530 nm and $\lambda = 485$ nm/630 nm for SYTO 9 and PI, respectively. Median and 25%–75% percentiles were calculated on basis of three independent experiments performed in triplicate ($n = 9$).

Imaging

Images were taken of cultures with an inverted light microscope and a corresponding camera system (Zeiss AXIO LAB A1 and ZEN core v2.0.66.1000).

For a better understanding of cell–cell- and cell-bacteria-interactions, cells (1×10^4/well) were cultured on coverslips (Sarstedt) in 24-well plates and infected with 10^5 CFU/mL *S. mutans*. After 24 h, the coverslips were transferred into fresh 24-well plates and gently washed twice with PBS. Cells were fixed with 2.5% glutaraldehyde (SERVA Electrophoresis) followed by washes with 0.1 mol/L Sørensen's phosphate buffer. Regions of interest were visualized in high vacuum mode at a working distance of 6–7 mm (FEI Quanta 400 FEG; Thermo Fisher Scientific, FEI Deutschland).

Glucose degradation and lactate formation

To evaluate the categorization of glucose by DPCs and *S. mutans*, the glucose concentration was determined by a Glucose Colorimetric Detection Kit (EIAGLUC; Thermo Fisher Scientific) after 0, 6, 24, 30, 48, 54 and 72 h. Simultaneously, the formation of lactate was evaluated after 0 and 72 h using both L-Lactate Assay Kit (ab65331; Abcam) and D-Lactate Assay Kit (ab83429; Abcam).

Glucose concentrations were measured by first diluting samples in assay buffer. Samples were then mixed with 25 μL horseradish peroxidase concentrate (HRP), 25 μL substrate and 25 μL glucose oxidase and incubated for 30 min at room temperature. A standard was established with 15 μL glucose standard and 135 μL assay buffer. Optical density was determined on a microplate reader at $\lambda = 560$ nm. Five experiments have been conducted in triplicates ($n = 15$) and summarized as median values with 25%–75% percentiles.

To quantify lactate concentrations, medium was gently removed and deproteinized using the Deproteinization Sample Preparation Kit – TCA (ab204708; Abcam). First, media containing bacteria were centrifuged ($250 \times g$) for 5 min. Then, 1000 μL medium of each group was mixed with 150 μL cold trichloroacetic acid (TCA) and incubated on ice for 15 min. Samples were centrifuged for 5 min ($12\,000 \times g$) and 1000 μL supernatant was supplemented by 105 μL neutralization solution yielding a neutral pH. Samples were then stored at −80°C.

Afterwards, 50 μL of each deproteinized sample was mixed with 50 μL reaction mix, which contained 46 μL lactate assay buffer, 2 μL lactate substrate mix and 2 μL lactate enzyme mix, respectively. A sample background control was established with 50 μL sample and 50 μL background reaction mix which comprised 48 μL lactate

assay buffer and $2\,\mu L$ lactate probe. For quantification, a standard was set up with $50\,\mu L$ standard dilutions and $50\,\mu L$ reaction mix. Samples were then incubated for 30 min at 37°C protected from light and optical density was measured on a microplate reader at $\lambda = 450\,nm$. Three experiments have been conducted in duplicates ($n = 6$) and summarized as median values with 25%–75% percentiles.

Release of inflammatory cytokines

To determine the release of inflammatory cytokines, supernatants were collected at 0 and 72 h and stored frozen at −20°C. Interleukin-6 (IL-6), interleukin-8 (IL-8), TGF-β1 and VEGF were determined by enzyme-linked immunosorbent assay (Quantikine ELISA Kit; R&D Systems). ELISAs were performed with $100\,\mu L$ of sample or standards according to the manufacturer's instructions. Optical density was measured at a wavelength of 450 nm. Medians with 25%–75% percentiles were calculated from two experiments performed in four replicates ($n = 8$).

Statistical analysis

Data were treated non-parametrically and analysed at a significance level of $\alpha = .05$. Mann–Whitney U tests were performed for situations with two unpaired groups and p-values were adjusted for multiple comparisons by the Holm-Šídák method ($\alpha = .05$). Kruskal–Wallis test followed by Dunn's multiple comparison test was applied for cases of three or more unpaired groups. Significant differences between the test and control groups were indicated in the figures by asterisks. All statistical analyses were computed with GraphPad Prism 9 (GraphPad Software), and detailed records of all comparisons are provided in Tables S1–S10.

RESULTS

Bacterial growth in different media and impact on pH

The growth pattern of *S. mutans* in TSB under aerobic conditions at 37°C showed an exponential phase, a stationary phase and a death phase with no lag phase (Figure 1a). The growth maximum was observed after 24 h (10^9 CFU/mL). However, the culture of *S. mutans* in MEM α did not show typical bacterial growth phases. After a slower increase, a broad stationary phase was reached with a growth maximum close to that of TSB. A clear death phase could not be distinguished during the culture period. The growth curves in both media were significantly different except for the starting time point ($p \le .007937$). Despite bacterial growth, there were no significant differences ($p > .489177$) in the pH of the media (Figure 1b). Both groups were within the buffer capacity of MEM α at all time points.
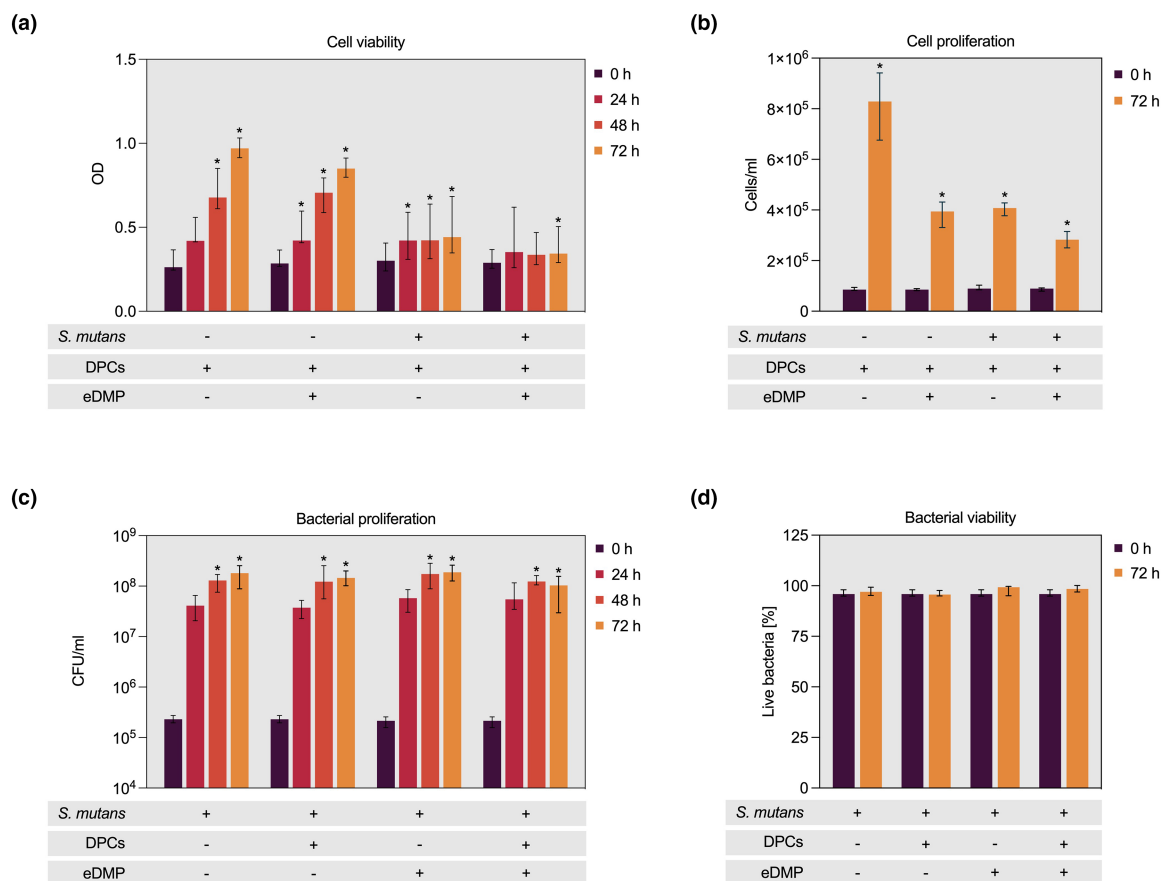
Proliferation and viability of cells and bacteria

As shown in Figure 2a (Table S1), the viability of DPCs increased significantly during 72 h of culture ($p \le .0197$). Supplementation with eDMP only affected viability after 72 h ($p = .0462$), whereas coculture with *S. mutans* resulted in stagnation of cell viability ($p \le .0001$). The number of cells increased significantly ($p \le .0001$) in all groups over the incubation period (Figure 2b; Table S2). However, the addition of eDMP or *S. mutans* affected DPC proliferation separately and most significantly when added in combination ($p \le .0008$).

In contrast, the growth of *S. mutans* was not affected by DPCs or eDMP at any time, but consistently reached the high counts previously observed (Figure 2c; Table S3).
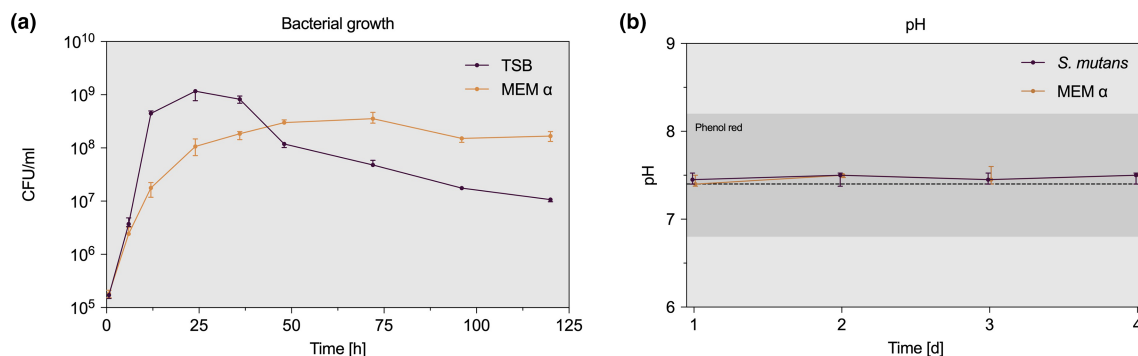
FIGURE 1 (a) Bacterial growth in cell culture media (MEM α) and bacterial culture media (TSB) over a period of 120 h. (b) pH values were measured in MEM α with or without *Streptococcus mutans*. The dark grey area represents the buffer capacity of phenol red in MEM α.

FIGURE 2 (a) Viability and (b) proliferation of dental pulp cells (DPCs) cultured with or without *Streptococcus mutans* and dentine matrix proteins (eDMP). (c) Proliferation and (d) viability of *Streptococcus mutans* cultured with and without DPCs and eDMP. Asterisks indicate significant differences from baseline (0 h).

As plate counts only represent culturable bacteria and the proportion of live and dead *S. mutans* in the culture was unknown, bacterial viability was determined. In all groups, a high proportion of live bacteria (>94%) was observed throughout the culture period (Figure 2d; Table S4) without significant differences throughout the coculture ($p > .0546$).

Imaging

Light microscopy images showed intact cell monolayers with spindle-shaped pulp cells at the bottom and floating aggregates of *S. mutans* organized in a matrix in the culture medium (Figure 3a).

In addition, SEM magnification showed direct interaction of *S. mutans* with the DPCs. The streptococci are seen in a chain formation on top of the confluent cell layer (Figure 3b). They are in direct contact and many even infiltrate the cell layer or are covered by cells (Figure 3c,d). The DPCs appear to be largely vital, but in some places

show blebbing (Figure 3e) or typical apoptotic morphology (Figure 3f).
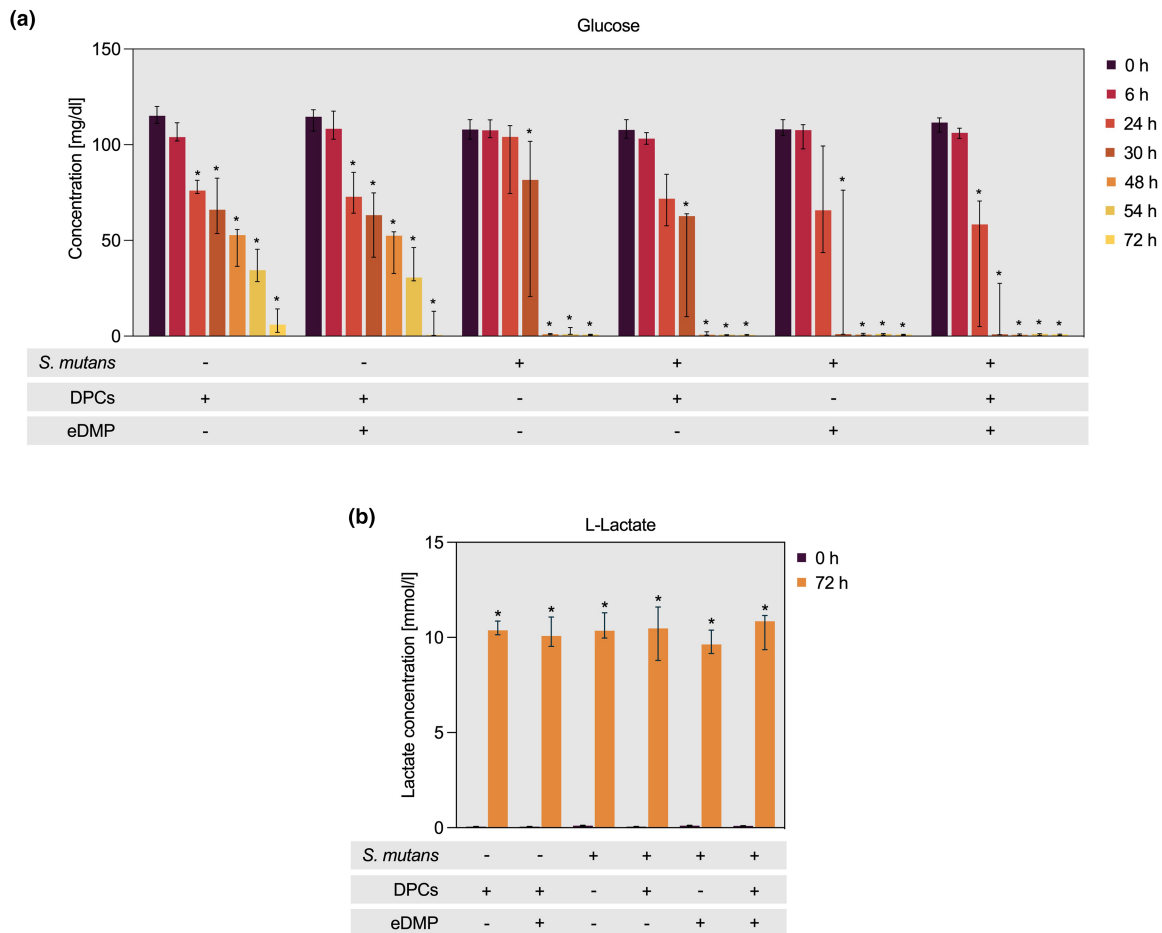
Glucose degradation and lactate formation

Initial glucose concentrations in cell culture media decreased continuously over 72 h in the presence of DPCs or *S. mutans* (Figure 4a; Table S5). After 72 h, only minute amounts of glucose were found in the supernatant. While the DPC culture alone or with eDMP showed a significant reduction in glucose levels already after 24 h ($p \leq .0236$), a significant reduction was observed in the bacterial cultures and cocultures only after 30 h ($p \leq .0249$). While the glucose catabolism process in cells appeared to be linear from the start, *S. mutans* showed a pattern consistent with exponential bacterial growth, characterized by a later onset but accelerated glucose degradation.

During culture, all the glucose present was converted into a stoichiometric amount of L-lactate (Figure 4b;
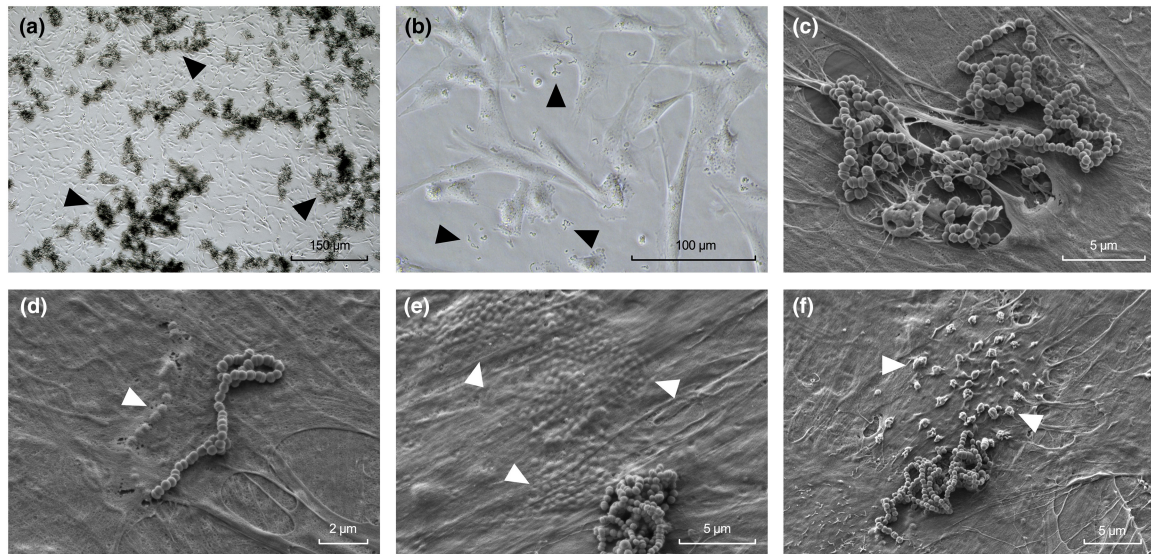
FIGURE 3 (a) Spindle-shaped dental pulp cells (DPCs) in monolayer and bacterial aggregates floating in culture medium (s. arrowheads). (b) Magnified view of DPCs in association with *Streptococcus mutans*, which is assembled like a chain of pearls. (c) *S. mutans* organized in typical chains on DPCs. (d) Streptococci located beneath the cell layer (s. arrowhead) either by invasion or overgrowth. (e) Numerous spherical elevations of the cell bodies (s. arrowheads) show a large number of mutans streptococci below the cell body. (f) DPCs with blebbing (s. arrowheads) of the cell membrane in course of apoptosis. Light microscope: (a) and (b). SEM: (c) to (f).

FIGURE 4 (a) Glucose degradation by dental pulp cells (DPCs) and *Streptococcus mutans* alone or in coculture with or without dentine matrix proteins (eDMP). (b) Lactate formation by DPCs and *S. mutans* alone or in coculture with and without eDMP. Asterisks indicate significant differences from baseline (0 h).

Table S6), while D-lactate was undetectable under the culture conditions described ($p \leq .0095$).

Release of inflammatory cytokines

The release of growth factors and cytokines was normalized to cell number to account for the different growth of DPCs between groups.

The cytokines IL-6 and IL-8 were not detected in any of the groups at baseline (0 h). After 72 h, IL-6 was found in the culture of DPCs (Figure 5a; Table S7), with a significant increase in coculture with *S. mutans* ($p \leq .0011$). IL-8 was also detected at 72 h (Figure 5b; Table S8), but there were no significant differences between the experimental groups ($p > .1511$).

As TGF-β1 is a natural component of the human dentine matrix, it was detected at the supplemented levels in the eDMP groups at 0 h (Figure 5c; Table S9). It was shown that DPCs led to a significant degradation of the initially present TGF-β1 after 72 h ($p \leq .0004$), which was lower in the coculture of DPCs and *S. mutans*. However, no relevant levels of TGF-β1 were detected in the experimental groups without eDMP either at baseline or at 72 h. VEGF, which was present in eDMP at low concentrations, was detected at high levels in all groups after 72 h ($p \leq .0444$). Compared to the culture of DPCs alone, VEGF production (Figure 5d; Table S10) was significantly increased when
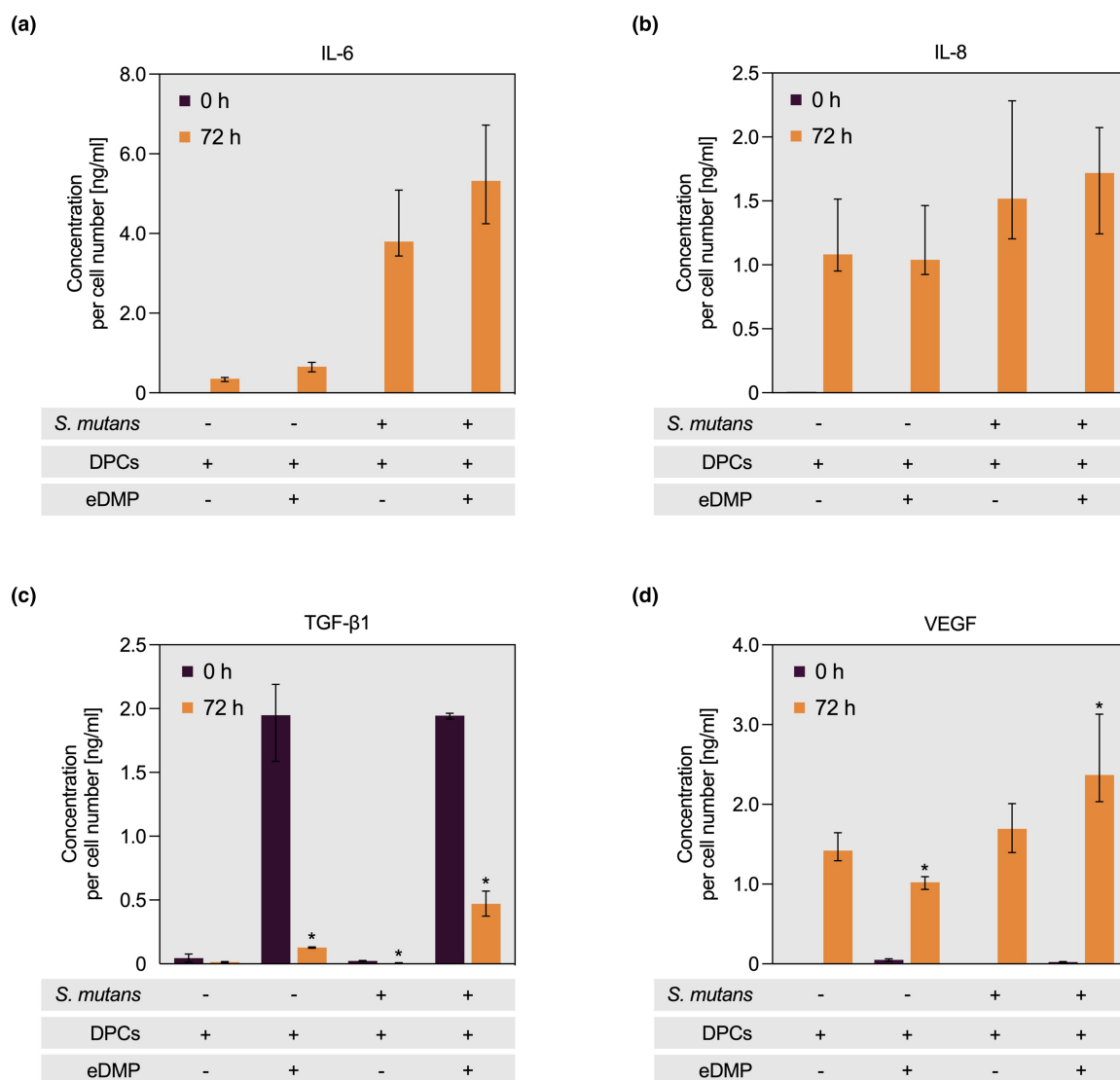
FIGURE 5 (a) Production of interleukin-6 (IL-6), (b) interleukin-8 (IL-8), (c) transforming growth factor-β1 (TGF-β1) and (d) vascular endothelial growth factor (VEGF) by dental pulp cells (DPCs) cultured with or without *Streptococcus mutans* and dentine matrix proteins (eDMP). Asterisks indicate significant differences from baseline (0 h).

DPCs were cultured with *S. mutans* and eDMP simultaneously ($p \leq .0035$).

DISCUSSION

During the progression of carious lesions, both released eDMP and bacteria with their toxins interact with the dental pulp, leading to immune responses (Galler et al., 2021). In particular, toxins such as LTA, which are found in the membrane of cariogenic Gram-positive bacteria such as *S. mutans*, can trigger inflammatory processes not only through membrane-bound interactions but also through their release into the environment (Kang et al., 2016; Klein et al., 2015). In order to reproduce this complex interplay and to study both cell–cell and cell-bacteria interactions, the aim of this study was to establish an *in vitro* coculture.

As initial carious lesions are predominantly colonized by streptococci, lactobacilli and actinomyces (Love & Jenkinson, 2002), *S. mutans* is a suitable model organism for pathogenic Gram-positive bacteria (Lemos et al., 2013). However, it is also known that anaerobic Gram-negative bacteria can be found in deeper cavities (Hoshino, 1985). It should be noted that cocultures technically require selection of microorganisms that can be cultured under cell biological conditions (37°C, neutral pH, 5% CO_2, 21% O_2).

Limitations of the model in terms of translating the *in vitro* situation to the clinical scenario of a deep carious lesion are of course present and should not be discounted. As indicated above, the microbial diversity and biofilm organization present in a clinical cavity cannot be reproduced *in vitro*. Culture conditions are well defined, but do not take into account environmental factors, such as dietary nutrients, which can impact on bacterial activity and function, for example. In addition, bacterial metabolites, which are known to diffuse through the dentinal tubules, trigger the initial immune response in the pulp by interacting with odontoblast processes (Galler et al., 2021). When the bacteria finally get in contact with the pulp cells, as simulated in the model, the first immunological processes are probably already in progress *in vivo* (Seltzer et al., 1963). Similarly, the concentration of endotoxins and bacteria increases as the caries progresses into the pulp, whereas the *in vitro* load is determined by growth and is certainly high from the outset. However, despite the limitations, this approach offers a specific and well-controlled model situation that can provide important insights into the interaction between Gram-positive bacteria and pulp cells and allow for further modifications in the future.

Bacterial growth in different media and impact on medium pH

The first objective was to assess whether *S. mutans* (ATCC25175) tolerates cell culture conditions. Typically, bacterial growth *in vitro* can be divided into four distinct phases, including lag phase, log phase, stationary phase and death phase (Maier & Pepper, 2015). *S. mutans* in TSB exhibited these phases, with the exception of the lag phase, which may be too short to be correctly represented. In contrast, cultivation in MEM α resulted in a growth curve with a lower peak, but a longer stationary phase with higher counts. A longer stationary phase, where cell division and death are equal, might contribute to a stable production of signalling molecules. Obviously, differences in media composition had an effect on *S. mutans*, but aerobic cell culture conditions were consistent with growth patterns reported in the literature (Kawada-Matsuo et al., 2012).

The metabolism of *S. mutans* depends primarily on the metabolism of cariogenic carbohydrates, which are internalized by the bacteria and fermented by glycolysis to provide energy in the form of adenosine triphosphate (ATP). In the intracellular environment, glycolysis mainly leads to the production of lactic acid, which is excreted and accumulates in the biofilm matrix *in vivo* (Dashper & Reynolds, 2000; Lemos et al., 2019). Although high levels of lactic acid can cause a decrease in blood or tissue pH (Kraut & Madias, 2014), the pH in the coculture medium appeared to be stable due to the sodium bicarbonate buffering system of the culture medium. Accordingly, changes in pH did not appear to affect the results described below.

Proliferation and viability of cells and bacteria

While both DPCs and *S. mutans* showed constant growth, the proliferation and viability of *S. mutans* was not affected by either DPCs or eDMP. Although antimicrobial effects of dentine matrix components have been reported (Smith et al., 2012), no impact on *S. mutans* was observed at the concentration used in this study.

In contrast, proliferation and viability of DPCs were affected by *S. mutans* and eDMP. While eDMP only affected proliferation, exposure to *S. mutans* also reduced cell viability. The literature on the effects of microorganisms on cells is highly controversial and suggests that bacteria have no effect on cells or even increase their viability and number, but only over a short period of 24 h (Shayegan et al., 2021). Hattar et al. reported that LTA from *Staphylococcus aureus* induced proliferation of

TLR-2-positive cells in a concentration-dependent manner (Hattar et al., 2017). These observations could be attributed to the activation of TLR-2, which targets genes involved in cell proliferation and survival, and induces cytokine expression (Taylor et al., 2010). On the other hand, Zhang et al. (2011) reported reduced proliferation of human lung fibroblasts following activation of TLR-4 by lipopolysaccharide and attributed these effects to cytokines. Thus, the antiproliferative effect of *S. mutans* on DPCs observed in this study may be due to the production of the cytokines IL-6 and IL-8. Similarly, antiproliferative effects have been described for eDMP, probably due to cell cycle regulation by TGF-β1 (Kubiczkova et al., 2012; Widbiller, Eidt, Lindner, et al., 2018). The inhibition of proliferation could also indicate a progression of differentiation by dentine matrix compounds, which has been described *in vitro* and *in vivo* (Tziafas et al., 1992; Widbiller, Eidt, Wölflick, et al., 2018).

As carbohydrates can be a limiting metabolite for both bacteria and cells (Püschel et al., 2020), glucose starvation may also play a role in cell proliferation in the coculture environment. It was therefore important to ensure that no organism was subjected to prolonged starvation, which could affect the functionality of the organisms, as discussed in more detail below.

Glucose degradation and lactate formation

Glucose is a key metabolite for *S. mutans*, as catabolism to lactic acid provides the basis for its pathogenicity. While glycolysis is primarily used for energy production, lactate serves as a major virulence factor that initiates demineralisation of enamel and dentin, leading to cavitation (Harper et al., 2021; Tanzer et al., 1974). Interestingly, previous studies have shown that strains deficient in lactate dehydrogenase are less cariogenic (Banas, 2004; Fitzgerald et al., 1989). Furthermore, carbohydrates are also involved in the biosynthesis of extracellular polysaccharides and cell wall components such as LTA (Kawada-Matsuo et al., 2016). It was therefore of great interest to analyse the glucose metabolism of *S. mutans* in order to assess its pathogenic qualities in the coculture environment.

Of course, not only bacteria but also cells use glucose to maintain their metabolism. While the DPCs consumed the glucose in the culture medium over 72 h, the fast-growing *S. mutans* consumed the supply in only 30 h. Thus, glucose consumption in the coculture is cumulative, resulting in a rapid reduction in the glucose concentration in the medium. Despite the short culture time, this observation raises the question of whether the observed effects are caused by glucose starvation, lactate or cell-bacteria interactions.

Apparently, the bacteria were not affected by the limited supply of glucose over 72 h and reached almost the same numbers as in TSB. Interestingly, a study by Tong et al. (2011) found that when *S. mutans* was cultured in glucose-free conditions such as water or PBS, bacterial cell numbers decreased by one log level within 6 h, but the same conditions increased the bacteria's resistance to stress from lethal acid and anti-caries agents. Similarly, in the present study, the maximum bacterial growth in coculture was not affected by the absence of glucose after 30 h.

In contrast, DPCs appeared to have reduced growth and viability in the presence of bacteria; however, it was not possible to distinguish whether this was due to glucose starvation or interaction with bacteria and their metabolites. Previous studies have shown that starvation leads to cell adaptation, production of cytokines such as IL-6 and IL-8, and ultimately cell death (Püschel et al., 2020). In this study, the effect of starvation did not appear to be the dominant factor, as no cell death was observed after 30 h, but growth stagnated, similar to culture with eDMP. Furthermore, the profiles of growth factors and cytokines produced did not show a consistent pattern suggesting glucose starvation of DPCs causative, which will be discussed later.

Medium glucose was completely converted to L-lactate by DPCs and *S. mutans*, which is consistent with previous findings (Carr et al., 2002; Ewaschuk et al., 2005). The excretion of L-lactate, which is impermeable to the cell membrane, requires no metabolic energy once a chemical potential has been established by F_1F_0-ATPases (Dashper & Reynolds, 1996). However, relatively little is known about the cellular effects of lactate. It has been reported, particularly in the context of inflammatory diseases such as rheumatoid arthritis, that free lactate in the extracellular space can be taken up by immune cells such as $CD4^+$ T cells, macrophages, dendritic cells, and osteoclasts. The uptake of lactate can induce the differentiation and activation of these cells, affecting their function and leading to disease progression (Yi et al., 2022).

Release of inflammatory cytokines

To further investigate the functional response of DPCs to *S. mutans* in the coculture system, the production of characteristic signalling molecules was examined. These included the cytokines IL-6 and IL-8, and the growth factors VEGF and TGF-β1.

IL-6 is a pleiotropic interleukin produced by both immune and nonimmune cells to modulate inflammation and recruit leukocytes and mesenchymal stem cells following TLR-2/6 activation (Hunter & Jones, 2015). As a Gram-positive bacterium, *S. mutans* most likely enhances

IL-6 production through LTA, an amphiphilic molecule that is localized in the cell wall (Kawada-Matsuo et al., 2016) and can be excreted, e.g. during proliferation or under antibiotic therapy (Schröder et al., 2003). LTA is reported to induce immune responses leading to the activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)- and the mitogen-activated protein kinase (MAPK)-pathway, resulting in the production of chemokines and cytokines to control inflammation and restore tissue homeostasis (Carrouel et al., 2013).

Interestingly, IL-6 production was significantly upregulated in DPCs exposed to *S. mutans* with a continuous increase. However, as the glucose in the medium appeared to be depleted after about 30 h in coculture, an effect of starvation could not be ruled out initially. Therefore, in a side experiment (data not shown), DPCs were cultured for 120 h without any change in medium and IL-6 levels were monitored and showed no increase. Consequently, it seems legitimate to attribute the main effect to the interaction with the bacteria.

IL-8, a pro-inflammatory cytokine, has chemotactic activity and mainly leads to neutrophil recruitment and degranulation (Hébert & Baker, 1993) mediated by G-protein coupled chemokine receptors (Bernhard et al., 2021). Similar to IL-6, IL-8 is released upon activation of TLR-2 by LTA (Keller et al., 2010). IL-8 was produced by DPCs exposed to *S. mutans*, however, to a lesser extent and without statistical difference. Previous studies also reported increased IL-8 levels in DPCs, THP-1 and PDL 4 h after incubation with *S. mutans* (Engels-Deutsch et al., 2003). Clinically, high IL-8 levels have been described in inflamed pulps (Huang et al., 1999) and in primary pulp cell cultures from carious teeth (Arora et al., 2022).

Another signalling molecule that modulates inflammation is TGF-β1 (Cooper et al., 2010), which can be released from dentine by decalcification through organic acids in caries or dental materials (Binanzan & Alsalleeh, 2022; Sloan et al., 1999) and act locally on cells (Dung et al., 1995; Tomson et al., 2007, 2017). It is a homodimer that binds to specific membrane receptors with multiple regulatory properties such as cell proliferation, apoptosis or differentiation (Conery et al., 2004; Gray & Mason, 1990; Kubiczkova et al., 2012; Roberts et al., 1985). When evaluating TGF-β1 in the coculture environment, it is important to consider that it is not only produced by DPCs but also present in the groups initially supplemented with eDMP (Widbiller et al., 2019; Widbiller, Eidt, Wölflick, et al., 2018). Therefore, TGF-β1 was detected in these respective groups at baseline, and its ongoing cellular uptake was monitored. The stability of TGF-β1 from eDMP in the culture medium was observed throughout the designated culture period, which has been reported previously (Galler et al., 2014). Dental pulp cells actively internalized TGF-β1 over 72 h probably through TGF-β1 receptors activation with the aim of initiating regenerative goals (Bizet et al., 2012; Kubiczkova et al., 2012). Surprisingly, fewer TGF-β1 was taken up by DPCs exposed to *S. mutans*, which could be attributed to factors such as metabolic stress. From a clinical perspective, the reduced utilization of TGF-β1 by DPCs due to microbial stimulation could indicate a diminished regenerative capacity.

In addition, the homodimeric glycoprotein VEGF plays an important role in tissue regeneration by promoting the migration, proliferation and differentiation of DPCs (D'Alimonte et al., 2011; Muller et al., 1997; Sun et al., 2019). It exhibits pro-angiogenic properties, facilitating endothelial cell activation and vasculogenesis to restore proper blood supply (Gong et al., 2019). Although VEGF is also found in the dentine matrix, the amount of supplementation was negligible compared to TGF-β1 (Widbiller, Eidt, Wölflick, et al., 2018). Interestingly, DPCs consistently produced VEGF throughout the culture period in all groups. However, it appeared that *S. mutans* only induced VEGF production when combined with eDMP, whereas eDMP alone limited VEGF production and *S. mutans* alone had no significant effect. Several inducers of VEGF are known, including growth factors, such as TGF-β1, hypoxia (Shweiki et al., 1992), oxidative stress (Schäfer et al., 2003) or LTA (Telles et al., 2003). Thus, the increased production of VEGF in the coculture may be due to a combinatorial effect of TGF-β1 from eDMP and LTA from *S. mutans*.

CONCLUSION

In this study, a coculture system was established to allow the simultaneous cultivation of DPCs and *S. mutans*. The proliferation and viability of DPCs were affected by both *S. mutans* and eDMP, whereas *S. mutans* was not affected by either cells or eDMP. Glucose catabolism, lactate, cytokine and growth factor production demonstrated the functionality of the organisms in the coculture system.

Given the clinical challenges of vital pulp therapy and recent advances in treatments aimed at preserving the pulp, this approach holds great promise for investigating the molecular interactions between bacteria and pulp cells. It also has the potential to elucidate the essential mechanisms involved in pulp regeneration and repair and may contribute to the development of pharmacological strategies to regulate these processes.

AUTHOR CONTRIBUTIONS

Tobias Akamp: Data curation; formal analysis; investigation; methodology; writing – original draft preparation. Andreas Rosendahl: Data curation; investigation; methodology. Kerstin M. Galler: Writing – review & editing.

Melanie Wölflick: Investigation. Wolfgang Buchalla: Resources; writing – review & editing. Matthias Widbiller: Conceptualization; formal analysis; funding acquisition; methodology; project administration; visualization; writing – original draft preparation.

CONFLICT OF INTEREST STATEMENT
The authors state that there is no conflict of interests related to this study.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Regensburg (16-101-0022).

PATIENT CONSENT STATEMENT
Informed consent was obtained from all subjects involved in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.