Clin Oral Invest (2012) 16:879–887
DOI 10.1007/s00784-011-0563-6

ORIGINAL ARTICLE

Enterococcus faecalis affects the proliferation and differentiation of ovine osteoblast-like cells

Lamprini Karygianni · Margit Wiedmann-Al-Ahmad · Günter Finkenzeller · Sebastian Sauerbier · Martin Wolkewitz · Elmar Hellwig · Ali Al-Ahmad

Received: 17 November 2010 / Accepted: 26 April 2011 / Published online: 17 May 2011

Abstract *Enterococcus faecalis* (*E. faecalis*) is a Gram-positive bacterium, mostly recovered from root-filled teeth with persistent periapical lesions. Bacterial contamination of root canals inevitably results in interaction between *E. faecalis* and periapical tissues during the dynamic process of periapical inflammation. This study investigated the impact of heat-inactivated endodontic *E. faecalis* on the proliferation and the differentiation of ovine osteoblast-like cells, in an attempt to elucidate its putative enhanced pathogenicity mechanisms. Therefore, two different concentrations of a heat-inactivated endodontic *E. faecalis* isolate (2×10^6 or 2×10^8 CFU/ml) were incubated with ovine osteoblast-like cells for 7 and 14 days, respectively. Cells without antigen served as control. The effects of antigen on cell growth were evaluated by a proliferation assay (EZ4U). Furthermore, the assessment of alkaline phosphatase (ALP) activity, calcium deposition, and osteocalcin (OCN) gene expression through quantitative real-time PCR determined the degree of osteogenic cell differentiation. Scanning electron microscopy (SEM) was also performed to detect alterations in cell morphology. Interestingly, although highly concentrated *E. faecalis* increased cellular reproduction after 14 days, ALP activity and OCN gene expression decreased in an antigen concentration-dependent and incubation time-independent way. SEM images revealed *E. faecalis* adhesion on cells, a fact that might contribute to its virulence. These results suggest that *E. faecalis* stimulated cell multiplication, whereas it likely restrained cell differentiation of ovine osteoblast-like cells. In conclusion, the presence of *E. faecalis* in root canals may negatively affect periapical new bone formation, and thus, the healing of periapical lesions.

Keywords Osteoblasts · Bacteria · Endodontic infection · Cell culture · qPCR

L. Karygianni (✉) · E. Hellwig · A. Al-Ahmad
Department of Operative Dentistry and Periodontology,
Albert-Ludwigs-University,
Hugstetterstrasse 55,
Freiburg 79106, Germany
e-mail: lamprini.karygianni@uniklinik-freiburg.de

M. Wiedmann-Al-Ahmad · S. Sauerbier
Department of Oral and Maxillofacial Surgery,
Albert-Ludwigs-University,
Freiburg, Germany

G. Finkenzeller
Department of Plastic and Hand Surgery,
Albert-Ludwigs-University,
Freiburg, Germany

M. Wolkewitz
Institute of Medical Biometry and Medical Informatics,
Albert-Ludwigs-University,
Freiburg, Germany

Introduction

Up to 90% of the medically demanding enterococcal infections are induced by *Enterococcus faecalis*, a representative member of the *Enterococcus* genus [1]. The Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic *E. faecalis* strains mainly inhabit the alimentary tract of humans and the oral cavity, especially related to teeth with persistent periapical lesions of

endodontic origin [2]. Numerous studies using culture and molecular methods have shown a higher prevalence of *E. faecalis* in obturated root canals with chronic apical periodontitis compared to teeth with primary infections [3–5]. The survival of *E. faecalis* in root canals even under unfavorable environmental conditions may be associated with the presence of an enhanced bacterial pathogenicity.

More specifically, its ability to withstand extreme challenges in root canals can be attributed to resistance to the antimicrobial effects of calcium hydroxide and other intracanal medicaments such as sodium hypochlorite, hydrogen peroxide, and acids [6–8], partly through a functioning proton pump structure, maintaining cytoplasmic pH at favorable levels. *E. faecalis* also shows resistance, intrinsically or via acquisition, to a wide range of antibiotics [9, 10] and can invade in dentinal tubules, where its elimination by root canal disinfectants seems to be a formidable challenge [11]. Finally, *E. faecalis* colonizes the root canal as monocultures, organizes in biofilms without the need for support of other bacteria [12, 13], and possesses a wide array of putative virulence factors that bring about pathological alterations either directly through toxin production or indirectly through the activation of the host immune system. Among these factors belong aggregation substance (AS), surface adhesins (Esp, Efa A, and Ace), sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, cytolysin, and bacteriocins such as AS 48 [14–17].

Up to now, many studies have been conducted concerning the diversity of microorganisms in infected root canals without addressing their impact on periradicular host tissues [18]. The necessity to examine the influence of bacteria on the activity of eukaryotic cells has been recently investigated extensively [19, 20]. Therefore, the aim of this study is to focus on the effect of a heat-inactivated endodontic *E. faecalis* isolate originating from an endodontic patient on the proliferation and the differentiation of ovine osteoblast-like cells, in order to illuminate the virulence potential of *E. faecalis* towards the major cellular component of periapical tissues, the osteoblasts.

Materials and methods

Antigen preparation

The endodontic *E. faecalis* FRs 112 isolate was acquired from a patient's periapically inflamed root canal [21]. The activated stock-culture microorganisms firstly reached their exponential growth phase when incubated in Caso-Bouillon broth (Merck, KGaA, Darmstadt, Germany) overnight at 37°C. For its production, 15 g of the medium was suspended in 0.5-l distilled water. They were mixed thoroughly and heated gradually until the medium was completely dissolved. It was then poured into suitable containers and subjected to a 15-min-long sterilization at 121°C in an autoclave. The bacterial strains of *E. faecalis* were then centrifuged twice in 0.9% NaCl at 4.000×*g* for 20 min (Hettich Zentrifugen Universal 32, Tuttlingen, Germany), heated at 90°C for 1 h (Thermomixer Comfort, Eppendorf GmbH, Hamburg, Germany), and stored at −80°C until use, according to a modified protocol [22]. The suspensions were adjusted to a concentration of 2×10^8 CFU/ml by using a SmartSpec plus spectrophotometer (Bio-Rad, Life Science Group, Hercules, USA). The bacterial suspensions remained sterile, and the complete inactivation of *E. faecalis* was certified after recultivation of the heat-inactivated strains on CBA plates.

Cell culture

For the cultivation of ovine osteoblast-like cells, the pieces of pelvic bone (3×3 mm), from seven ($n=7$) sheep, 9–12 months old, were crumbled with the aid of the explant technique. Cells in primary and secondary culture were cultivated in opti-minimal essential medium (Opti-MEM, Gibco Laboratories Life Technologies Inc, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin/streptomycin (Penicillin/Streptomycin liquid, PAA Laboratories GmbH, Pasching, Austria), at 37°C/5% CO_2. The first passage of cells, detached by 0.5% trypsin from the culture flask, was diluted to a final cell concentration of 1×10^5 cells/ml. Cells were then seeded with two different *E. faecalis* concentrations onto 12- and 24-well culture plates. The first group (0) lacked antigen and served as control. The second (1) and third groups (2) were cultivated with *E. faecalis* antigen concentrations I (2×10^6 CFU/ml) and II (2×10^8 CFU/ml), respectively. After inoculation with antigen, the ovine osteoblast-like cell cultures were incubated over a 14-day period in a humidified atmosphere of 5% CO_2 at 37°C. The culture medium and *E. faecalis* suspensions were replaced every 2 days, and the experimental procedures were conducted twice, after 7 and 14 days, respectively.

Cell proliferation assay

A colorimetric, nonradioactive, and nontoxic assay kit (EZ4U, Biozol diagnostica GmbH, Eching, Germany) enabled the assessment of cell proliferation of three

different samples for each individual. Intact mitochondria turned lightly yellow-colored tetrazolium salts into intense red-colored formazan derivatives, indicative of the total amount of living cells in the samples [23]. In this assay, 125 μl of solubilized ready-to-use dye substrate was added in every well, and 24-well plates were stored at 37°C and 5% CO_2 for 3 h, as specified by the manufacturer. Two hundred microliters of each supernatant was then dispensed into a 96-well microplate in triplicate for each sample. Wells filled only with pure culture medium served as negative control (blind value). The exact amount of the active osteoblast-like cells was photometrically measured by a microtiterplate reader (Infinite M200, Tecan Austria GmbH, Groedig, Austria) at a wavelength of 450 nm (reference wavelength 620 nm) and was further demonstrated as an optical density (OD value). The EZ4U assay was conducted twice, after 7 and 14 days, respectively.

Cell differentiation

Alkaline phosphatase activity and protein assay

An assay, based on the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricycle [3.3.1.1^{3,7}] decan}-4-yl) phenylphosphate (CSPD,0,4 mM Ready to use with Sapphire-II Enhancer, Tropix Applied BioSystems, Foster City, CA, USA) determined the alkaline phosphatase (ALP) activity in the cell lysate after 7 and 14 days, as previously described [24]. The measurement was held in triplicate for each of the three different samples per individual with luminescence by an ELISA reader after 5, 15, and 30 min, respectively. The ALP detection signal was recorded in relative luminescence units (RLU), and the graphic demonstration of the ALP activity was logarithmic. BCA Protein assay (Thermo Fisher Scientific Inc, Rockford, USA) normalized the cellular ALP activity to total protein concentration utilizing bovine serum albumin to derive a standard curve.

Calcium deposition assay

von Kossa silver nitrate staining manifests extracellularly positioned insoluble calcium phosphate salts [25]. After fixation in 4% (*w*/*v*) paraformaldehyde in phosphate-buffered saline for 10 min, the cells were rinsed with distilled water and incubated with 10 mg/ml silver nitrate solution under UV light for 1 h. Following rinsing in distilled water, 50 mg/ml sodium thiosulphate solution was added to the cultures for 2 min and then they were washed again. Phosphate deposits stained black.

PCR assay

RT-PCR

Total RNA extraction was accomplished with the aid of RNeasy mini kit (Qiagen, Hilden, Germany), while iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) assisted complementary DNA (cDNA) synthesis, specified by the manufacturer.

Primer design and direct sequencing

Utilizing the Blast nucleotide algorithm (NCBI, http://www.ncbi.nlm.nih.gov/) and Beacon Designer Software (Premier Biosoft International, Palo Alto, CA, USA), signature primers that are most exclusive to ovine osteoblast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and osteocalcin (OCN) genes were designed and further constructed (Thermo Fisher Scientific GmbH, Ulm, Germany). Direct sequencing of the PCR products was performed by GATC Biotech (Konstanz, Germany).

Quantitative real-time PCR

Real-time PCR cycles were performed in the presence of iQ Sybr Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on iCycler (Bio-Rad Laboratories GmbH, Munich, Germany). For PCR assay, 1 μl of cDNA was amplified in a 25-μl-reaction mixture consisting of specific primer pairs to detect transcripts of OCN and GAPDH gene. PCR primer sequences and PCR product sizes are demonstrated in Table 1. The amplifications were conducted in triplicate for each of the three different samples per individual, while H_2O was used as negative control. The following temperature profile was optimized and adopted during thermocycling: 45 cycles were run, where each cycle includes denaturation (94°C for 30 s), annealing (54°C for 30 s) and synthesis (72°C for 30 s). The gene expression of the housekeeping gene was utilized to normalize the content of cDNA samples. The increased fluorescence of GAPDH-reference gene and OCN-target gene PCR amplicons was monitored in the form of dissociation curves, and the average threshold cycle (Ct) was calculated with a specified software (Bio-Rad Laboratories GmbH, Munich, Germany). The delta/delta calculation method was used for quantification [26].

Agarose gel electrophoresis

The electrophoresis of PCR products (100 V, 400 mA, 1 h) in 1% agarose gel, mediated by ethidium bromide, certified the progress of the reaction, and visualized by means of a

Table 1 Forward (F) and Reverse (R) PCR primers used for determination of OCN and GAPDH gene expression

Genes	Primers	Primer sequence	Length
GAPDH	GAPDH-F	5′-ACA TCA TCC CTG CTT CTA CTG-3′	181 bp
	GAPDH-R	5′-GCC TGC TTC ACC ACC TTC-3′	
OCN	OCN-F	5′-CAG CGA GGT GGT GAA GAG-3′	145 bp
	OCN-R	5′-CTG GAA GCC GAT GTG GTC-3′	

UV lightbox (Universal Hood II, Bio-Rad Laboratories, Milan, Italy), the specificity of the GAPDH and OCN primers on DNA templates.

Scanning electron microscopy

After fixation in 8% formaldehyde, specimens were dehydrated in an ascending ethanol series (50–100%), sputter-coated with gold/palladium, and examined by an environmental scanning electron microscope (Electroscan ESEM 2020, Wilmington, MS, USA) at 10 kV.

Statistical analysis

A linear mixed model was fitted, where each continuous response variable (proliferation rate, ALP activity, and OCN gene expression) was modeled as a linear function of time and antigen concentration. The interaction between time and antigen concentration was utilized as an explanatory variable [27]. Least-square means with 95% confidence interval were calculated and subsequently graphically displayed.

Results

Effects of *E. faecalis* on cell proliferation

The predicted means determining OD of ovine osteoblast-like cell reproduction in the absence of *E. faecalis* were 0.74 after 7 days and 1.06 after 14 days. Accordingly, the corresponding OD values for ovine osteoblast-like cells seeded with *E. faecalis* concentration I (1) were 0.71 and 1.07 after 7 and 14 days, respectively, whereas with *E. faecalis* concentration II (2) 0.76 after 7 days and 1.39 after 14 days as shown in Fig. 1a. When considering each osteoblast-like cell group with a specific antigen concentration measured at two time points (7 and 14 days) as a single group, no significant alterations in the calculated proliferation rates were observed ($p \geq 0.2$) among the different cell-antigen combinations over the first week. On the contrary, the differences between the predicted means were statistically significant after a 2-week time period. Osteoblast-like cells with *E. faecalis* concentration II (2) proliferated in greater proportions than the control group (0; $p=0.0053$) and the cell group with *E. faecalis* concentration I (1; $p=0.007$). A significant time-antigen concentration interaction was only detected for the cell group with *E. faecalis* antigen concentration II (2; $p \leq 0.05$), indicating that there was a time effect only among antigen concentration II cell groups (2) and not among other cell groups.

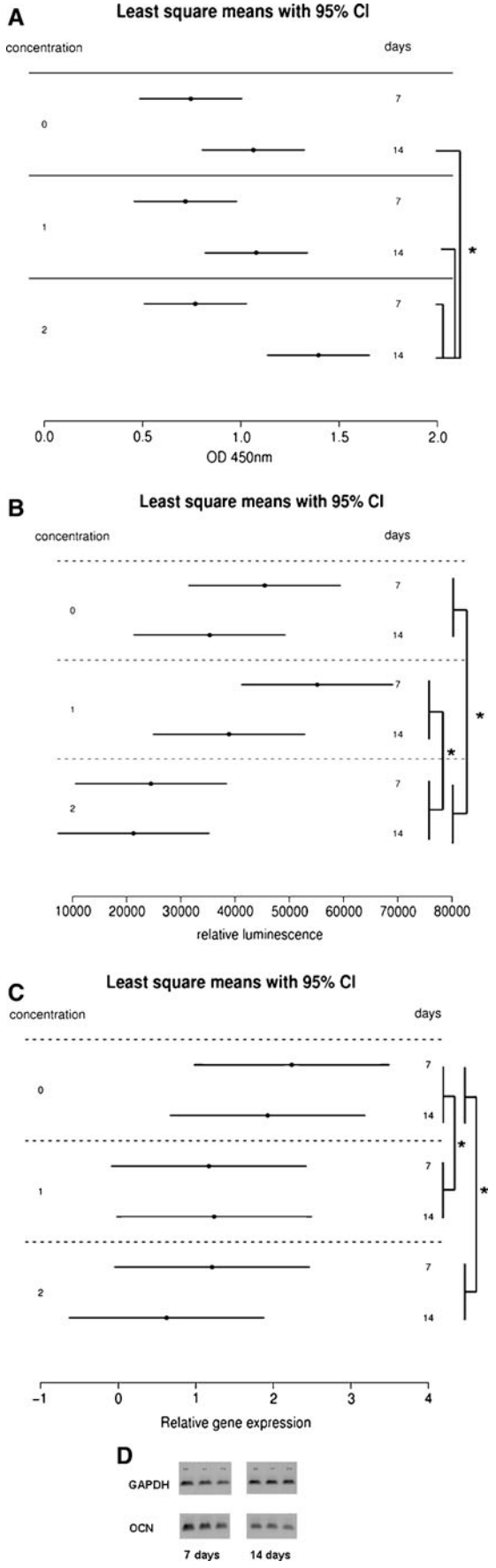
Effects of *Enterococcus faecalis* on cell differentiation

Alkaline phosphatase activity

When *E. faecalis* antigen is absent (control group), the predicted means for ALP activity showed RLU of 45,400 after 7 days and 35,293 after 14 days. Accordingly, the corresponding values for ovine osteoblast-like cells cultivated with *E. faecalis* concentration I (1) were 55,159 and 38,886 after 7 and 14 days, respectively; while for osteoblast-like cells seeded with *E. faecalis* concentration II (2), the values were 24,463 after 7 days and 21,260 after 14 days as shown in Fig. 1b. When considering each osteoblast-like cell group with a specific antigen concentration measured at two time points (7 and 14 days) as a single group, the significant changes in the estimated ALP activity values were detected. There was less ALP activity in the cell group with *E. faecalis* concentration II (2) than in the control group (0; $p=0.003$) and the cell group with *E. faecalis* concentration I (1; $p=0.0001$). There was a significant influence of time among the same cell groups assessed at different time points.

Osteocalcin gene expression

The procedure of quantitative real-time PCR (qPCR) facilitated the evaluation of osteodifferentiation-indicative OCN gene expression twice, after 7 and 14 days, respectively. The predicted means for the relative OCN gene expression of the control group (absence of *E. faecalis*) were 2.2384 units and 1.9268 units after 7 and 14 days, respectively; whereas, the equivalent values for the cell group with *E. faecalis* concentration I (1) were 1.1690 units after 7 days and 1.2364 units after 14 days. The cell group with *E. faecalis* concentration II (2) showed mean values of 1.2097 units after 7 days and 0.6219 units after 14 days (Fig. 1c, d). The statistical evaluation of the results did not show a meaningful effect of time among the same cell groups. When each osteoblast-like cell group with a



◀ **Fig. 1** The proliferation rate (**a**), endogenous cellular ALP activity (**b**), and OCN gene expression (**c**) of a total of seven (n=7) ovine individuals were statistically evaluated as a linear function of the measurement time points (7 and 14 days) and the osteoblast-like cell groups with variant antigen concentrations (*0* absence of antigen/control group, *1* low *Enterococcus faecalis* concentration I (2×10^6 CFU/ml), *2* high *E. faecalis* concentration II (2×10^8 CFU/ml)). **a**–**c** Graphical displays of the anticipated estimates with 95% CI for all cell groups with different *E. faecalis* concentrations and all times of assessment ($p\leq0.05$). The *asterisks on the right side* display the statistically significant differences between the predicted means. **d** Exhibited data represent mRNA levels of GAPDH and OCN documented by agarose gel electrophoresis of qPCR products from osteoblast-like cells (n=7) cultivated with different antigen concentrations (*0* absence of antigen/control group, *1* low *E. faecalis* concentration I (2×10^6 CFU/ml), *2* high *E. faecalis* concentration II (2×10^8 CFU/ml)). The OCN-target gene mRNA expressions normalized to those of GAPDH were estimated twice, after 7 and 14 days, respectively

specific antigen concentration assessed at a particular time point (7 and 14 days) is regarded as a single group, significant changes were observed between the control group (0), where OCN gene was expressed more intensely, compared to the cell group with *E. faecalis* concentration I (1; p=0.0131) and the cell group with *E. faecalis* concentration II (2; p=0.0016).

Matrix mineralization

The *E. faecalis*-inoculated osteoblast-like cell groups (1 and 2) demonstrated very little von Kossa-positive mineralized nodules, while calcium precipitation was exhibited more intensely in the control group (0) by multiple von Kossa-positive areas (Fig. 2).

Scanning electron microscopy

General view and more detailed SEM exposures of ovine osteoblast-like cells seeded with *E. faecalis* are shown in Fig. 3. Ovine osteoblast-like cells (magnification, ×2,000) tended to form a highly packed network when cultivated without *E. faecalis*. Spindle- or cuboidal-shaped cells with long cytoplasmic filaments oriented in parallel. The interconnection and therefore the communication between the osteoblast-like cells were distinctly achieved through numerous filopodia and long lamellipodia (Fig. 3a). Osteoblast-like cells seeded with *E. faecalis* concentration I (2×10^6 CFU/ml) constitute confluent cell monolayers (magnification, ×5,000), which demonstrate flat, mainly stretched or polygonal morphology of closely packed cells with numerous filopodia. SEM analysis of osteoblast-like cells cultivated with *E. faecalis* concentration II (2×10^8 CFU/ml) revealed a layer of regularly spread cells (magnification, ×2,000) with cytoplasmic processes that mediate cell-to-cell communication. The attachment of *E. faecalis* to cell surfaces, through capillaceous extensions of

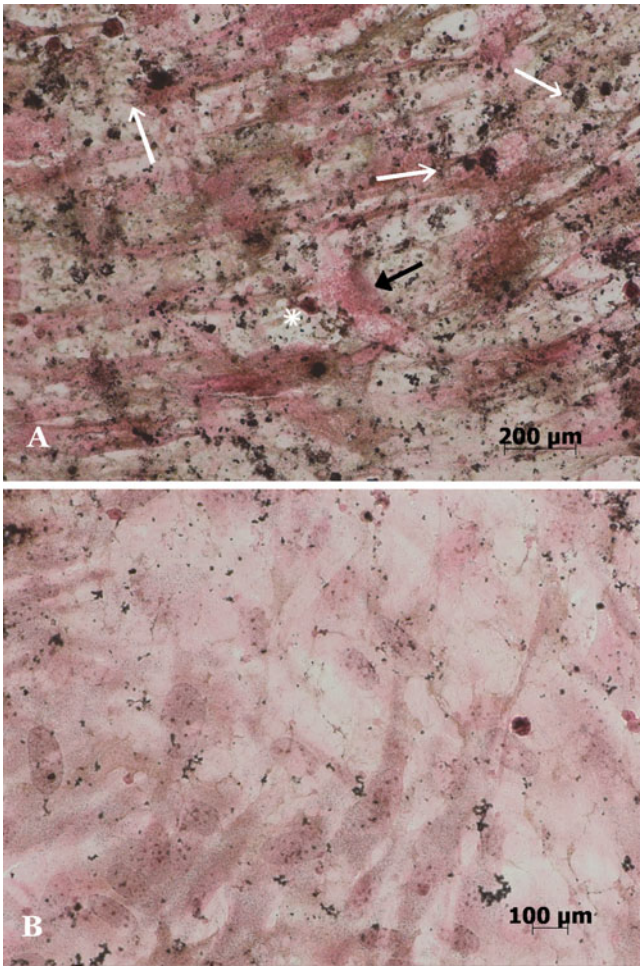


Fig. 2 Light microscopy of *Enterococcus faecalis*-inoculated ovine osteoblast-like cell cultures following von Kossa staining on day 14. **a** In the absence of *E. faecalis* (control group), multiple black-stained insoluble calcium phosphate salts (magnification, ×200), indicative of intense matrix calcification, are demonstrated in the extracellular spaces (*white arrows*). Pink-tinged cytoplasm (*black arrow*) and red-dyed nuclei (*asterisk*) reveal the osteoblast-like cellular presence. **b** Ovine osteoblast-like cells seeded with *E. faecalis* concentration II (2×10^8 CFU/ml) exhibit very little black-stained von Kossa-positive areas (magnification, ×200), due to a markedly decreased mineral nodule formation

proteinaceous nature presumably, was also demonstrated (Fig. 3c). SEM observation showed no detectable morphological alterations of osteoblast-like cells, resulting from the bacterial influence of *E. faecalis*.

Discussion

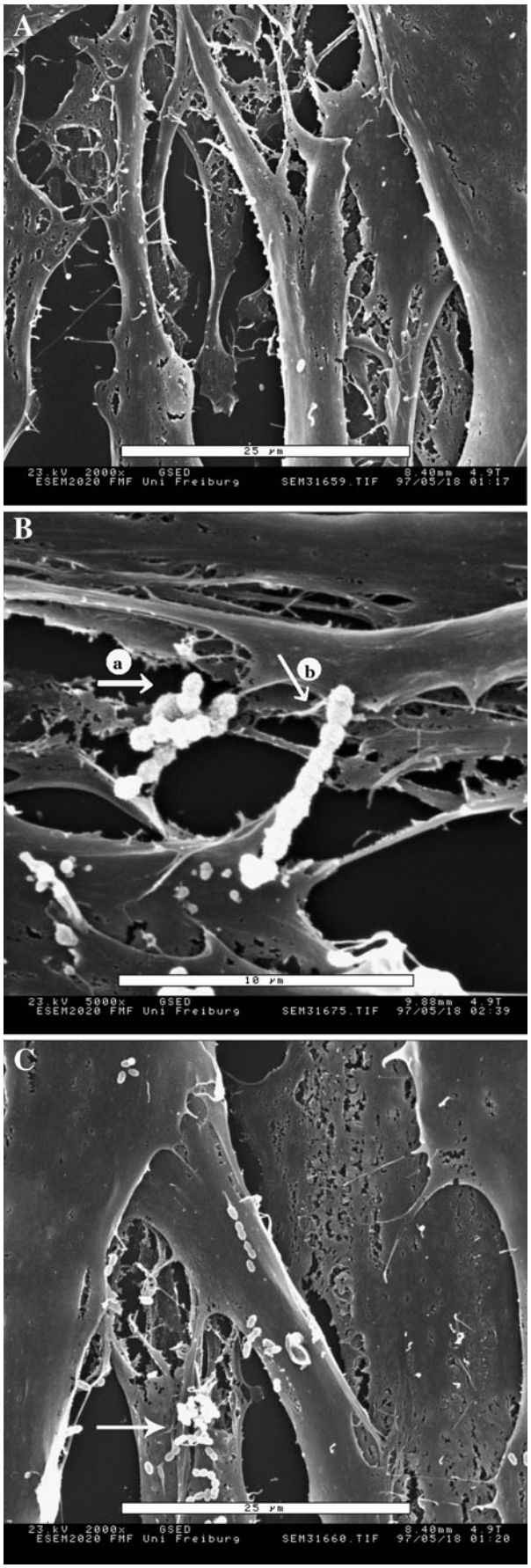
Analyzing experimental design and methodological parameters, the use of osteoblast-like cells derived from ovine pelvic bone provided a highly representative culture model for the study of the influence of *E. faecalis* on eukaryotic

Fig. 3 Scanning electron microscopic (SEM) exposures of ovine osteoblast-like cells seeded with different *Enterococcus faecalis* concentrations, after the first 7 days of culture. **a** Ovine osteoblast-like cells (magnification, ×2,000) cultivated without *E. faecalis*. **b** Osteoblast-like cells seeded with *E. faecalis* concentration I (2×10^6 CFU/ml). Spherical or ovoid *E. faecalis* cocci (**a**) tended to form chains that adhered via hair-like structures (**b**) protruding from the bacteria to the cells. **c** Osteoblast-like cells cultivated with *E. faecalis* concentration II (2×10^8 CFU/ml). *E. faecalis* attached in the form of cocci or chains, indicated with a *white arrow*, to the cells

cells. Past studies have indicated that cancellous bone of iliac crest, compared with other osseous sites, has a high metabolic turnover rate and can represent a large potential reservoir of osteoprogenitor cells [28]. In our study, ovine osteoblast-like cells were used because previous studies showed that ovine osteoblast-like cells are sensitive to substrate composition and topography [29, 30]. Consequently, it was also interesting to examine the affect of microorganisms like *E. faecalis* on proliferation and differentiation on the already established cells. Moreover, in these in vitro examinations, ovine osteoblasts were investigated for potential later in vivo studies (sheep model to examine proliferation, differentiation, and healing process) because spectroscopical analysis shows similarities between sheep and human bones regarding the micromorphological structure [30]. The ovine osteoblasts were characterized as previously described by Wagner et al. [30]. The characterization of ovine and human osteoblast-like cells depends on the osteocalcin amount. For the quantification of osteocalcin in the cell culture supernatant of ovine and human osteoblast-like cells, the MetraTM osteocalcin EIA-Kit (Quidel, San Diego, USA) was performed.

Monolayer culture was the most appropriate method to raise the amount of osteoblast-like cells needed [31]. Moreover, heat inactivation of *E. faecalis* aimed at preparation of experimental inoculum without loss of virulence or number of bacteria and at impeding the overexpansion that a live bacterial population normally develops. Indeed, bacterial adherence to cells does not seem to be inhibited by heat inactivation [32]. Although heat killing at 90°C would be expected to denaturate all or most of the protein virulence factors, other components such as LTA do not seem to be affected. Therefore, SEM images revealed hair-like structures that have been previously identified as an AS, an adhesin which enables binding of *E. faecalis* to eukaryotic cells, and therefore, it might contribute to its virulence [33].

As far as the cell reproduction-related outcomes are concerned, literature research did not provide any information about *E. faecalis*-driven stimulation of cell proliferation. However, a similar in vitro study showed that the treatment of basal urothelial cells with lipoteichoic acid



(LT-2) stimulated a high proliferative subpopulation of quiescent cells, possibly through NO-mediated mechanisms [34]. In contrary, viability and proliferation assays did not disclose any cell number changes when human dental pulp fibroblasts were seeded with whole heat-inactivated *E. faecalis* [35]. The results of the present study contribute to the above conflicting published data. These contradictions may reflect different biological responses related to the source and progenitor status of cells analyzed. The source and the preparation of the pathogenic agent should also be considered with regards to the diversity of the above published conclusions, due to the multiple putative virulent factors involved in the use of whole heat-inactivated *E. faecalis*.

The assessment of ALP activity revealed an antigen concentration-dependent enzyme activity reduction. However, the duration of cell cultivation proved to have no influence on ALP activity. The interpretation of the results necessitates the consideration of the in vivo regulation of cell differentiation by inflammation-associated cytokines. It is already described that a series of putative virulence factors of *E. faecalis*, e.g., LTA, could trigger the release of inflammation mediators (TNF-α, IL-1β, IL-6, IL-8, PGE2, lysosomal enzymes, superoxide anions). Depending on cell differentiation stage, the prolongation of culture period and cytokine concentration, both IL-1α and IL-1β, has been shown to stimulate osteoblast proliferation and to inhibit bone formation through reduced ALP activity [36]. Although host response cannot be estimated in our in vitro study, the suppression of ALP activity under concentration-dependent influence of *E. faecalis* through still unknown mechanisms is in agreement with the above statements. These cell differentiation-related outcomes are supported by several research projects studying different bacterial effects on osteoblast cell differentiation [37, 38].

Additionally, qPCR disclosed the most bone-specific OCN gene expression downregulation in an *E. faecalis* concentration-related manner. It was previously reported that a prostaglandin-independent inhibitory effect of Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* on OCN biosynthesis was observed after 24 h of treatment with human osteoblastic cells [39], while OCN levels significantly decreased in *S. aureus*-infected mice [40]. Moreover, CCAAT/enhancer binding protein beta is known to play an important role of development- and homeostasis-associated gene expression and it was proved to increase proliferation and suppress osteogenic differentiation [41]. Putative intracellular signaling mechanisms responsible for the negative effect of *E. faecalis* on differentiation could involve LTA-mediated upregulation of Toll-like receptor 2 (TLR2) that recognizes Gram-positive cell wall structures [42]. Upon TLR2 stimulation, osteoblast-like cells may go through a functional alteration

that results in the decrease of matrix synthesis and mineralization [43].

Finally, the data of this study exhibited the ability of *E. faecalis* to affect cell differentiation, implying that bone loss in *E. faecalis*-induced inflammatory processes may not be caused only by enhanced bone resorption, but also by suppressed bone formation.

Acknowledgments Gabi Braun, Ute Hübner, and Heike Jahnke are acknowledged for the skilful technical laboratory assistance. We also thank Dr. Yi Thomann for the interpretation of the SEM images. This study was supported in part by the German Research Foundation (DFG proposal AL 1179/1-1).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Jett BD, Huycke MM, Gilmore MS (1994) Virulence of enterococci. Clin Microbiol Rev 7:462–478
2. Portenier I, Waltimo TMT, Haapasalo M (2004) *Enterococcus faecalis*—the root canal survivor and 'star' in post-treatment disease. Endod Topics 6:135–159
3. Pirani C, Bertacci A, Cavrini F, Foschi F, Acquaviva GL, Prati C, Sambri V (2008) Recovery of *Enterococcus faecalis* in root canal lumen of patients with primary and secondary endodontic lesions. New Microbiol 31:235–240
4. Gomes BP, Pinheiro ET, Jacinto RC, Zaia AA, Ferraz CC, Souza-Filho FJ (2008) Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction. J Endod 34:537–540
5. Ozbek SM, Ozbek A, Erdorgan AS (2009) Analysis of *Enterococcus faecalis* in samples from Turkish patients with primary endodontic infections and failed endodontic treatment by real-time PCR SYBR green method. J Appl Oral Sci 17:370–374
6. Evans M, Davies JK, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 35:221–228
7. Radcliffe CE, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H, Drucker DB (2004) Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms *Actinomyces israelii*, *A. naeslundii*, *Candida albicans* and *Enterococcus faecalis*. Int Endod J 37:438–446
8. Delgado RJ, Gasparoto TH, Sipert CR, Pinheiro CR, Moraes IG, Garcia RB, Bramante CM, Campanelli AP, Bernardineli N (2010) Antimicrobial effects of calcium hydroxide and chlorhexidine on *Enterococcus faecalis*. J Endod 36:1389–1393
9. Dahlen G, Samuelsson W, Molander A, Reit C (2000) Identification and antimicrobial susceptibility of enterococci isolated from the root canal. Oral Microbiol Immunol 15:309–312
10. Sun J, Song X, Kristiansen BE, Kjaereng A, Willems RJ, Eriksen HM, Sundsfjord A, Sollid JE (2009) Occurrence, population structure, and antimicrobial resistance of enterococci in marginal and apical periodontitis. J Clin Microbiol 47:2218–2225
11. Chivatxaranukul P, Dashper SG, Messer HH (2008) Dentinal tubule invasion and adherence by *Enterococcus faecalis*. Int Endod J 41:873–882
12. Distel JW, Hatton JF, Gillespie MJ (2002) Biofilm formation in medicated root canals. J Endod 28:689–693
13. Takemura N, Noiri Y, Ehara A, Kawahara T, Noguchi N, Ebisu S (2004) Single species biofilm-forming ability of root canal isolates on gutta-percha points. Eur J Oral Sci 112:523–529
14. Signoretto C, Lleo MM, Tafi MC, Canepari P (2000) Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. Appl Environ Microbiol 66:1953–1959
15. Rozdzinski E, Marre R, Susa M, Wirth R, Muscholl-Silberhorn A (2001) Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilized extracellular matrix proteins. Microb Pathog 30:211–220
16. Low YL, Jakubovics NS, Flatman JC, Jenkinson HF, Smith AW (2003) Manganese-dependent regulation of the endocarditis-associated virulence factor EfaA of *Enterococcus faecalis*. J Med Microbiol 52:113–119
17. Kayaoglu G, Orstavik D (2004) Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. Crit Rev Oral Biol Med 15:308–320
18. Sundqvist G, Figdor D (2003) Life as an endodontic pathogen. Endod Topics 6:3–28
19. Siqueira JF Jr, Rocas IN (2008) Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod 34:1291–1301, e1293
20. Siqueira JF Jr, Rocas IN (2009) Diversity of endodontic microbiota revisited. J Dent Res 88:969–981
21. Schirrmeister JF, Liebenow AL, Braun G, Wittmer A, Hellwig E, Al-Ahmad A (2007) Detection and eradication of microorganisms in root-filled teeth associated with periradicular lesions: an in vivo study. J Endod 33:536–540
22. Noel RF Jr, Sato TT, Mendez C, Johnson MC, Pohlman TH (1995) Activation of human endothelial cells by viable or heat-killed gram-negative bacteria requires soluble CD14. Infect Immun 63:4046–4053
23. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. Cancer Res 47:943–946
24. Blum JS, Li RH, Mikos AG, Barry MA (2001) An optimized method for the chemiluminescent detection of alkaline phosphatase levels during osteodifferentiation by bone morphogenetic protein 2. J Cell Biochem 80:532–537
25. Schenk RK, Olah AJ, Hermann W (1984) Preparation of calcified tissue for light microscopy. In: Dickson GR (ed) Methods of calcified tissue preparation. Elsevier Science, New York, pp 26–32
26. Winer J, Jung C, Shackel I, Williams P (1999) Development and validation of real-time quantitative reverse transcriptasepolymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270:41–49
27. Brown H, Prescott R (1999) In: Barnett V (ed) Applied mixed models in medicine. John Wiley and Sons, Hoboken, pp 33–83
28. Defino HL, da Silva Herrero CF, Crippa GE, Bellesini LS, Beloti MM, Rosa AL (2009) In vitro proliferation and osteoblastic phenotype expression of cells derived from human vertebral lamina and iliac crest. Spine (Phila Pa 1976) 34:1549–1553
29. Schmitt SC, Wiedmann-Al-Ahmad M, Kuschnierz J, Al-Ahmad A, Huebner U, Schmelzeisen R, Gutwald R (2008) Comparative in vitro study of the proliferation and growth of ovine osteoblast-like cells on various alloplastic biomaterials manufactured for augmentation and reconstruction of tissue or bone defects. J Mater Sci Mater Med 19:1441–1450
30. Wagner M, Kiapur N, Wiedmann-Al-Ahmad M, Hübner U, Al-Ahmad A, Schön R, Schmelzeisen R, Mülhaupt R, Gellrich NC (2007) Comparative in vitro study of the cell proliferation of ovine and human osteoblast-like cells on conventionally an rapid prototyping produced scaffolds tailored for application as potential replacement material. J Biomed Mater Res A 83:1154–1164

31. Wiedmann-Al-Ahmad M, Gutwald R, Lauer G, Hubner U, Schmelzeisen R (2002) How to optimize seeding and culturing of human osteoblast-like cells on various biomaterials. Biomaterials 23:3319–3328
32. Ostad SN, Salarian AA, Ghahramani MH, Fazeli MR, Samadi N, Jamalifar H (2009) Live and heat-inactivated lactobacilli from feces inhibit *Salmonella typhi* and *Escherichia coli* adherence to Caco-2 cells. Folia Microbiol (Praha) 54:157–160
33. Al-Ahmad A, Muller N, Wiedmann-Al-Ahmad M, Sava I, Hubner J, Follo M, Schirrmeister J, Hellwig E (2009) Endodontic and salivary isolates of *Enterococcus faecalis* integrate into biofilm from human salivary bacteria cultivated in vitro. J Endod 35:986–991
34. Elgavish A (2000) NF-kappaB activation mediates the response of a subpopulation of basal uroepithelial cells to a cell wall component of *Enterococcus faecalis*. J Cell Physiol 182:232–238
35. Sipert CR, Moraes IG, Bernardinelli N, Garcia RB, Bramante CM, Gasparoto TH, Figueira EA, Dionisio TJ, Campanelli AP, Oliveira SH, Cunha FQ, Santos CF (2010) Heat-killed *Enterococcus faecalis* alters nitric oxide and CXCL12 production but not CXCL8 and CCL3 production by cultured human dental pulp fibroblasts. J Endod 36:91–94
36. Tanabe N, Ito-Kato E, Suzuki N, Nakayama A, Ogiso B, Maeno M, Ito K (2004) IL-1alpha affects mineralized nodule formation by rat osteoblasts. Life Sci 75:2317–2327
37. Kadono H, Kido J, Kataoka M, Yamauchi N, Nagata T (1999) Inhibition of osteoblastic cell differentiation by lipopolysaccharide extract from *Porphyromonas gingivalis*. Infect Immun 67:2841–2846
38. Roberts HC, Moseley R, Sloan AJ, Youde SJ, Waddington RJ (2008) Lipopolysaccharide alters decorin and biglycan synthesis in rat alveolar bone osteoblasts: consequences for bone repair during periodontal disease. Eur J Oral Sci 116:207–216
39. Lerner UH, Sundqvist G, Ohlin A, Rosenquist JB (1998) Bacteria inhibit biosynthesis of bone matrix proteins in human osteoblasts. Clin Orthop Relat Res 346:244–254
40. Verdrengh M, Carlsten H, Ohlsson C, Tarkowski A (2006) Rapid systemic bone resorption during the course of *Staphylococcus aureus*-induced arthritis. J Infect Dis 194:1597–1600
41. Iyer VV, Kadakia TB, McCabe LR, Schwartz RC (2004) CCAAT/enhancer-binding protein-beta has a role in osteoblast proliferation and differentiation. Exp Cell Res 295:128–137
42. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S (1999) Differential roles of TLR2 and 51TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443–451
43. Durand SH, Flacher V, Roméas A, Carrouel F, Colomb E, Vincent C, Magloire H, Couble ML, Bleicher F, Staquet MJ, Lebecque S, Farges JC (2006) Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts. J Immunol 176:2880–2887