ACTA
STOMATOLOGICA
CROATICA
www.ascro.hr

Acta stomatol Croat. 2014;48(2):116-122.

IZVORNI ZNANSTVENI RAD
ORIGINAL SCIENTIFIC PAPER

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Detekcija bakterija koje reduciraju sulfate i ostalih uzgojivih fakultativnih bakterija u zubnim tkivima

Detection of Sulphate-Reducing Bacteria and Others Cultivable Facultative Bacteria in Dental Tissues

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Sažetak

Svrha: Željelo se detektirati bakterije koje reduciraju sulfate (BRS) i procijeniti moguću povezanost između BRS-a i fakultativnih bakterija na različitim mjestima u usnoj šupljini s različitim stanjem parodonta. **Metode:** Za istraživanje je od osmero pacijenata uzeto devet uzoraka (od jednog pacijenta uzeta su dva) s različitih mjesta u usnoj šupljini. Skupljeni su s pomoću modificiranog medija Postgate E namijenjenog uzgoju, rastu i izolaciji BRS-a. Osim toga upotrijebljena je i reducirana otopina za anaerobne bakterije kao otopina za transport fakultativnih bakterija identificiranih s pomoću lančane reakcije polimeraze te sekvencije gena 16S rRNK. **Rezultati:** BRS je pronađen u trima uzorcima – u fragmentu korijena, u fragmentu korijena i u zdravom zubu s vertikalnim gubitkom kosti i pomičnošću treće razine, te u zdravom zubu izvađenom zbog ortodontskih razloga. Kod posljednjeg pacijenta fakultativna bakterija identificirana je kao *Lactobacillus casei*. Ostale bakterije bile su *Kurthia gibsonii* (pacijent 5) i *Pseudomonas aeruginosa* (pacijent 7). **Zaključak:** Otkrivanje BRS-a na različitim zubnim tkivima s izrazitim parodontnim problemima upozorilo je na to da su potrebna nova istraživanja kako bi se otkrila prava uloga BRS-a u oralnoj mikroflori. Također smo, osim BRS-a, u istom uzorku mogli potvrditi i prisutnost *Lactobacillus casei*.

Zaprimljen: 16. svibnja 2013.
Prihvaćen: 30. siječnja 2014.

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Ključne riječi
parodontitis; sumpor-reducirajuće bakterije; Desufovibrio

Uvod

Bakterije koje reduciraju sulfate (BRS) isključivo su anaerobne s optimalnom temperaturom između 25 i 44° C i rasponom pH između 5,5 i 9,0. Danas se zna za dvadesetak podvrsta BRS-a, primjerice za *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfolobus, Desulfobacter, Desulfococus, Desulfosarcina* (1). Te izbirljive mikroorganizme možemo pronaći u okolišu – u slatkoj vodi i slanim močvarama ili u ljudskom tijelu, ponajprije u crijevnoj mikroflori gdje se najčešće nalazi *Desulfovibrio desulfuricans* (2, 3).

Sefer i Cálinescu (4), (1969.), u jednom od prvih radova o toj temi, otkrili su BRS, uz *Streptococcus* sp., na sedam izvađenih zuba zbog propadanja tvrdoga zubnog tkiva. Dokazali su *Desulfovibrio* sp. i u strugotinama karioznog dentina. Istraživanja BRS-a nastavljena su tek tri desetljeća poslije kada je u jednoj studiji opisana njegova pojava na bukalnoj sluznici, jeziku, u slini te u sub- i supragingivnom biofilmu (5-9).

Introduction

Sulphate-reducing bacteria (SRB) are strict anaerobes, with an optimal temperature range of between 25 and 44°C and a pH between 5.5 and 9.0. There are currently over 20 well know generas such as *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfolobus, Desulfobacter, Desulfococus, Desulfosarcina,* among others (1). These fastidious microorganisms can be found in environments such as freshwater and salt marshes or in the human body, mainly in the intestinal microbiota, where the species *Desulfovibrio desulfuricans* is frequently detected (2,3).

In a pioneering work, Sefer and Cálinescu (4),(1969) reported the presence of SRB associated with *Streptococcus* sp. in seven teeth extracted due to extensive tooth decay. Furthermore they reported the presence of *Desulfovibrio* sp. in carious dentine scrapings. Only after almost three decades, studies on the presence of SRB in the oral cavity resumed, with a study describing the occurrence of SRB in the buccal

Boopathy i suradnici. (6) otkrili su BRS u uzorku tkiva uzetom iz parodontnih džepova 9 od 17 pacijenata te su između ostalih bakterija izolirali i *Desulfovibrio spp*. U ostalim istraživanjima upozoreno je da je BRS brojniji u subgingivnom plaku negoli na stražnjem i prednjem dijelu jezika, u bukalnoj i vestibularnoj sluznici te supragingivnom plaku (7 – 9).

U nekoliko istraživanja ističe se povezanost između parodontnog statusa i prisutnosti BRS-a u oralnoj mikroflori u kojoj su *Desulfovibrio fairfieldensis* i *Desulfovibrio desulfuricans* izolirani kao uzročnici parodontitisa, iako se nije znao točan utjecaj tih bakterija na gubitak kosti (7, 10 – 13). Te iste bakterije stvaraju agresivnije komplekse s drugim parodontnim patogenima kao što su *T. denticola*, *T. forsythus*, *P. gingivalis* i metalogene bakterije (9 – 14).

Svrha ovog istraživanja bila je otkriti prisutnost BRS-a i procijeniti njegovu moguću povezanost s fakultativnim bakterijama nađenima na raznim mjestima u oralnoj šupljini s različitim stanjem parodonta.

Materijali i metode

Istraživanje je obavljeno na devet uzoraka uzetih od osam pacijenata (od pacijenta broj 4 uzeta su dva uzorka s različitih lokacija) (tablica 1.) Pritom smo se koristili modificiranim medijem Postgate E koji je namijenjen rastu i izolaciji BRS-a (1), a sadržava (g/L destilirane vode): KH_2PO_4 (0,5); NH_4Cl (1,0); Na_2SO_4 (,10); $CaCl_2$ $2H_2O$ (0,67); $MgCl_2$

mucosa, tongue, saliva, subgingivial and supragingivial biofilms (5-9).

As well as tissue samples from periodontal pockets, Boopathy et al. (6), identified the presence of SRB in 9 to 17 patients with periodontitis, identifying *Desulfovibrio spp*. among the samples tested. Other studies indicate a greater number of SRB in subgingivial biofilms when compared to the posterior and anterior tongue, the buccal mucosa, vestibular mucosa and the supragingivial biofilm (7-9).

Several studies indicate a strong association between periodontal status and the presence of SRB in the oral microbiota, with *Desulfovibrio fairfieldensis* and *Desulfovibrio desulfuricans* species being reported as causative agents of periodontitis, without a true understanding of their role in bone loss (7,10-13). Such bacterial species can form a more aggressive complex in periodontal diseases when associated with other periodontal pathogens such as *T. denticola*, *T. forsythus*, *P. gingivalis* and metallogenic bacteria (9-14).

The objective of this study was to detect for the presence of SRB and evaluate the possible association between SRB and cultivable facultative bacteria of oral sites with different periodontal conditions.

Material and Methods

The study was carried out on 9 samples from different oral sites in 8 patients (in the patient number 4, two samples were collected from two different oral sites) (Table 1). Material was collected using a modified Postgate E culture medium, indicated for the growth and isolation of SRB (1), composed of the following (g/Litre of distilled water): KH_2PO_4

Tablica 1. Demografski podatci, sistemske bolesti i opisi mjesta uzimanja uzoraka
Table 1 Demographic data, systemic conditions and description of the sites collected from eight patients

Pacijent • Patient	Spol • Sex	Dob • Age	Pušač • Smoker	Sistemska bolest • Systemic disease	Prikupljeni materijal • Material collected	Opis mjesta • Site description
1	M	21	N	N	Strugotine karijesa • Caries scraping	Zub 36 s dubokim karijesom saniranim amalgamom • Tooth 36 with deep caries restored with amalgam
2	Ž • F	59		Hipotiroidizam • Hypothyroidism	Fragment korijena • Root fragment	Zub 15 s ranijim apscesima, predviđen za ekstrakciju • Tooth 15, indicated for extraction, with a history of abscesses
3	M	50	N	N	Fragment korijena • Root fragment	Zub 35 s ranijim apscesima, predviđen za ekstrakciju • Tooth 35, indicated for extraction, with a history of abscesses
4	M	46	N	N	Fragment korijena • Root fragment	Zub 25 s ranijim apscesima, predviđen za ekstrakciju • Tooth 25, indicated for extraction, with a history of abscesses
					Zdravi zub • Healthy tooth	Zub 22 3. stupnja pomičnosti (gubitak alveoralne kosti) • Tooth 22 with dental mobility degree of 3 (advanced bone loss)
5	Ž • F	83	N	Hipertenzija i osteoporoza • Hypertension and osteoporosis	Zub nosač s mostom • Abutment tooth with fixed bridge	Zub 16 3. stupnja pomičnosti • Tooth 16 with dental mobility degree of 3
6	Ž • F	46	N	N	Bioptat • Biopsy	Bioptat endodontske lezije zuba 11 • Endodontic lesion biopsy by paraendodontic surgery of tooth 11
7	Ž • F	19	N	N	Papirnati štapić • Paper point	Sterilni papirnati štapić koji je 3 sekunde bio u kanalu korijena tijekom druge sekcije pod potpunom izolacijom • Sterile paper cone, inserted for 3 seconds to the root canal of a tooth, during the second section under absolute isolation
8	M	22	N	N	Zdravi zub • Healthy tooth	Zub 48 (bez gubitka kosti) predviđen za ekstrakciju • Tooth 48 (without bone loss) indicated for extraction because of orthodontic treatment

N = Ne • No; M = Muško • Masculine; Ž • F = Žensko • Feminine

$6H_2O$ (,.68); natrijev laktat (7,0), ekstrakt kvasca (1,0), askorbinsku kiselinu (0,1); agar-agar (1,9), NaCl (5,0), rezasurin (4,0 mL) i $FeSO_4$ $7H_2O$ (0,5). Uz to, korištena je reducirajuća otopina za anaerobne bakterije kao transportni medij za fakultativne bakterije koja je sadržavala (g/L destilirane vode) natrijev tioglikolat (0,124); askorbinsku kiselinu (0,1); NaCl (5,0) i rezasurin (4,0ml). pH medija iznosio je 7,6 s NaOH-om.

Identificiranje BRS-a i izoliranje fakultativnih bakterija

Čim su uzorci skupljeni svaki je odmah stavljen u posebnu epruvetu s reducirajućom otopinom za anaerobne bakterije. Nakon što ih se protreslo desetak sekundi, 1,0 ml otopine inokuliran je u modificirani medij Postgate E.

Zatim su uzorci u reduciranoj otopini od po 1 mL preneseni u hranjivi medij te prebrojeni. Nakon toga su kolonije izolirane te inkubirane od 24 do 48 sati i ponovno su inokulirane u hranjivom mediju za naknadnu biomolekularnu identifikaciju vrsta bakterija.

Uzorci inokulirani u posebnim epruvetama koje su sadržavale modificirani medij Postgate E bile su inkubirane 28 dana na temperaturi od 30° C. Medij je pregledan mikroskopski te je uočena diskoloracija zbog stvaranja crnog taloga raspršenog u mediju, što je upućivalo na prisutnost BRS-a. Nakon 28 dana medij je potpuno pocrnio, a to je značilo da sadržava BRS. Talog je nastao nakon vezanja iona željeza prisutnih u modificiranom mediju Postgate E zato što su bakterije reducirale sulfat pa se željezo vezalo za sulfide (1). Uzorci bez BRS-a bili su ružičasti ili prozirno bijeli, što je upućivalo na rast ostalih anaerobnih bakterija koje ne reduciraju sulfate.

Biomolekularna identifikacija fakultativnih bakterija

Fakultativne bakterije mogle su se izolirati jedino iz uzoraka pacijenata 5, 7 i 8 kod kojih su provedeni protokoli za njihovo otkrivanje. Pritom je korišten set za ekstrakciju DNK MOBio UltraClean Microbial DNA®. Ekstrahirani DNK proveden je kroz elektroforezu na 1-postotnom agargelu obojenom SYBR Safeom.® Nakon ekstrakcije je gen 16S RNK (1500bp) pojačan PCR-om s univerzalnim primerima za bakterije SAdir (5'-AGAGTTTGATCATGGCTCAGA-3') i S17rev (5'-GTTACCTTGTTACGACTT-3'). Amplifikacija je obavljena sustavom GeneAmp PCR System 9700® (Applied Biosystems) te je uključen inicijalni ciklus denaturacije (94° C tijekom 5 minuta), 30 srednjih ciklusa denaturacije (94° C tijekom 30 sekundi), prekaljivanje (55° C tijekom 30 sekundi) i dva finalna ciklusa (72° C tijekom 30 sekundi te 72° C tijekom 5 minuta).

Ishod svake reakcije PCR-a analiziran je s pomoću elektroforeze na agarozi (1 %), obojen bojilom SYBR Safe i nakon toga pročišćen UltraCleanom® PCR Clean-up® (MOBio). Nakon toga je kvantiteta i čistoća rezultata PCR-a određena optičkom spektrofotometrijom (NanoDrop® ND-1000 UV-Vis – Thermo Scientific).

(0.5); NH_4Cl (1.0); Na_2SO_4 (1.0); $CaCl_2$ $2H_2O$ (0.67); $MgCl_2$ $6H_2O$ (1.68); sodium lactate (7.0); yeast extract (1.0); ascorbic acid (0.1); agar-agar (1.9); NaCl (5.0); rezasurina (4.0 mL); $FeSO_4$ $7H_2O$ (0.5). In addition, a reducing solution for anaerobic bacteria was used as a transport solution for facultative bacteria, consisting of the following (g/Litre of distilled water): sodium thioglycolate (0.124); ascorbic acid (0.1); NaCl (5.0); and rezasurina (4.0 ml). The pH of the medium was adjusted to 7.6 with NaOH.

Identifying SRB and isolating facultative bacteria

Immediately after collection, each sample was placed in separate tubes containing reducing solution for anaerobic bacteria, shaken for 10 seconds and 1.0 ml of this solution was inoculated in modified Postgate E medium.

Subsequently, samples in reducing solution were transferred, in 1.0 ml aliquots, to nutrient broth culture medium, and the striation method using plate count medium was performed. After verifying the isolation of colonies, incubated from 24 to 48 hrs, they were again inoculated in nutrient broth medium for subsequent biomolecular identification of the species present.

Samples inoculated in separate tubes containing modified Postgate E medium were incubated for 28 days at 30°C. The media was macroscopically inspected, observing discoloration due to the formation of black precipitates dispersed throughout the culture medium when positive for the presence of SRB. Then, after 28 days, the media with black color confirmed the presence of SRB. The precipitates were representative of the binding of the iron ion present in modified Postgate E culture medium with the reduction of sulphate by SRB to form iron sulphide (1). Samples negative for the presence of SRB developed a pink or white-transparent coloration, indicating the growth of other anaerobic bacteria that do not reduce sulphate.

Biomolecular identification of facultative bacteria

The isolation of facultative bacteria was possible only for samples from patients numbers 5, 7 and 8, where the protocol was carried out to identify these microorganisms. The MOBio UltraClean Microbial DNA® kit was used to extract DNA from the sample. The extracted DNA was visualized by electrophoresis of a 1% agarose gel stained by SYBR Safe®. After the extraction step, the 16S RNA gene (1500bp) was amplified by PCR using the universal primers for bacteria: SAdir (5'-AGAGTTTGATCATGGCTCAGA-3') and S17rev (5'-GTTACCTTGTTACGACTT-3'). Amplification was performed in a GeneAmp PCR System 9700® thermocycler (Applied Biosystems) and included an inital denaturation cycle (94°C for 5 minutes); 30 intermediate denaturation cycles (94°C for 30 seconds), annealment (55°C for 30 seconds) and an extension cycle (72°C for 30 seconds) and one final extension cycle (72°C for 5 minutes).

All PCR products were analysed by agarose gel (1%) electrophoresis, stained by SYBR Safe and then purified with the UltraClean® PCR Clean-up® kit (MOBio). After this step, the quantity and purity of the PCR products was determined

Sekvencioniranje rezultata PRC-a obavljeno je automatskim uređajem MEGABACE 1000, odnosno s pomoću Sector of DNA Sequncinga. Dobiveni rezultati analizirani su u programu Chromas Lite, version 2,01 (McCarthy, 1996, www.tecnelysium.com.au) te su sekvencije DNK uspoređene s već poznatim sekvencijama iz baze podataka Genbank (www.ncbi.nlm.nih.gov).

Uspoređivanje podjednakosti u sekvencijama uzoraka 16S rRNK provedeno je BLAST-om (Basic Alignment Search Tool, http://genome.eerie.fr/bin/blast-guess.cgi.) koji je otkrio izolirane bakterijske vrste.

Rezultati

Od osam pacijenata uključenih u ovo istraživanje, samo su tri bila pozitivna na BRS (tablica 2.). Od prvog pacijenta (broj 2) uzorak je uzet s fragmenta korijena, od drugog pacijenta (broj 4) izdvojen je s fragmenta korijena i sa zdravog zuba s vertikalnim gubitkom kosti i trećim stupnjem pomičnosti, a od trećeg pacijenta (broj 8) uzet je sa zdravog zuba ekstrahiranog u ortodontske svrhe. Kod posljednjeg pozitivnog pacijenta izolirana je fakultativna bakterija *Lactobacillus casei.* Ostale fakultativne bakterije pronađene su kod pacijenta broj 5 (*Kurthia gibsonii*) i broj 7 (*Pseudomonas aeruginosa*) (tablica 2.).

by optical density spectrophotometry (NanoDrop® ND-1000 UV-Vis - Thermo Scientific).

Sequencing of the PCR products was carried out by the Sector of DNA Sequencing, in a MEGABACE 1000 automatic sequencer. The resulting sequence electropherograms were analysed by the program Chromas Lite, version 2.01 (McCarthy, 1996, www.tecnelysium.com.au). The DNA sequences obtained were compared to those already in Genbank (www.ncbi.nlm.nih.gov).

The search for sequence similarity of the 16S ribosomal RNA gene of the isolated samples was carried out using BLAST (Basic Alignment Search Tool, http://genome.eerie.fr/bin/blast-guess.cgi.) revealing the bacterial species isolated.

Results

Of the eight patients included in this study, only three were positive for the presence of SRB (Table 2)**.** In the first patient (number 2), the sample was isolated from a root fragment; in the second (number 4), from a root fragment and a healthy tooth with vertical bone loss and with a mobility degree of 3; and in the third (number 8), a healthy tooth extracted for orthodontic purposes. In the final patient sample, *Lactobacillus casei,* a cultivable facultative bacterial species was identified. Other facultative bacterial species were identified in patient 5 (*Kurthia Gibsonii*) and patient 7 (*Pseudomonas aeruginosa*) (Table 2).

Tablica 2. Usporedba mjesta uzorka i mikrobioloških rezultata
Table 2 Relating the collected sites and the microbiology results

Pacijent • Patient	Uzorak • Sample	SRB	FAB
1	Strugotine karijesa • Caries scapings	-	
2	Fragment ekstrahiranog korijena • Extracted root fragment	+	
3	Fragment ekstrahiranog korijena • Extracted root fragment	-	
4	Fragment ekstrahiranog korijena • Extracted root fragment	+	
	Zdravi zub • Healthy tooth	+	
5	Zub nosač s mostom • Abutment tooth with fixed bridge	-	*Kurthia gibsonii*
6	Bioptat • Biopsy	-	
7	Papirnati štapić • Paper point	-	*Pseudomonas aeruginosa*
8	Zdravi zub • Healthy tooth	+	*Lactobacillus casei*

(-) Negativno • Negative; (+) Pozitivno • Positive; FAB = Fakultativni anaerobi • Facultative Anaerobic Bacteria

Rasprava

Bisson-Boutelliez i suradnici (11) otkrili su da invazivni kapacitet bakterije *Desulfovibrio spp.* potiče u epitelnim stanicama izlučivanje citokina IL-6 i IL-8. Prema njihovu mišljenju, takvi dokazi potvrđuju činjenicu o nastanku i napredovanju parodontne bolesti. Ti su istraživači također povezali nakupljanje sulfata kao konačnog metaboličkog proizvoda BRS-a u parodontnim džepovima i smrt parodontnih stanica (7). Tu tvrdnju podupire činjenica da je BRS potvrđen u deset uzoraka subgingivnog plaka uzetoga od različitih pacijenata s parodontitisom te da su te iste bakterije pronađene u barem jednom džepu kod 64 posto pacijenata s parodontitisom (7,8).

Discussion

Bisson-Boutelliez et al. (11), (2010) reported that the invasive capacity of *Desulfovibrio* spp. in oral epithelial cells induced the secretion of cytokines, IL-6 and IL-8 by these cells. According to the author, such evidence contributes to the initialization and perpetuation of periodontal disease. In addition, the author also related the accumulation of sulphate, as a final metabolic product of SRB in periodontal pockets, as responsible for causing cellular damage to cells (7). This is demonstrated by the isolation of sulphate-reducing bacteria in 10 subgingivial plaque samples from various patients with periodontitis and no less than one periodontal pocket in 64% of patients with periodontitis (7,8).

U ovom istraživanju četiri od devet uzoraka bilo je pozitivno na BRS (66, 67 %), što upućuje na to da su ove bakterije možda dio normalne oralne mikroflore s potencijalom da uzrokuju parodontnu bolest. Takav slučaj otkriven je kod jednog pacijenta koji je imao BRS na dva različita mjesta – na zubu 35 i na zubu 31. Na zubu 31 radilo se o velikom razaranju parodonta i o vertikalnom gubitku kosti, što upućuje na to da je BRS možda utjecao na parogenezu parodontne bolesti. To se moglo vidjeti i kod pacijenata 2 i 4 kod kojih su uzorci bili uzeti sa zuba s različitim stupnjevima parodontne bolesti. Ovi rezultati u skladu su s rezultatima Langendijka i suradnika. (7) koji su uočili veću količinu BRS-a kod pacijenata s parodontnim džepovima i krvarenjem tijekom sondiranja, s raznim koštanim defektima, otvorenim furkacijama i endodontskim komplikacijama. To ih je navelo na zaključak da postoji izravna povezanost između dubine džepova i prisutnosti BRS-a. Willis i suradnici (5) otkrili su veću učestalost BRS-a u slini nego u sub- i supragingivnom plaku, prednjem i stražnjem dijelu jezika te na oralnoj sluznici.

Na mjestima koja nisu u izravnom doticaju s oralnim okružjem nije izoliran BRS kao što je, primjerice, bioptat iz endodontske lezije ili korijenskog kanala. To upućuje na činjenicu da je potrebno izlaganje oralnom okružju kako bi se kolonizirao BRS.

Jedno od važnih svojstava BRS-a je prianjanje na različite podloge na kojima stvara plak s ostalim bakterijama (15, 16). Treba istaknuti da, kad se nalazi u oralnom mediju, BRS može kolonizirati različita mjesta u usnoj šupljini što je i vidljivo kod pacijenta 4 kod kojega su bakterije kolonizirale različita udaljena mjesta (zub 35 i 31).

Bakterija *Lactobacillus* spp. usko je povezana s nastankom karijesa i nalazi se u oralnoj mikroflori, plaku i na površinama zuba te mijenja razinu mliječne kiseline u mikrookolišu (17). *Lactobacillus casei* može se pronaći u supragingivnom aproksimalnom plaku (17) i biofilmu (18). Smanjenje pH vrijednosti koje uzrokuje *Lactobacillus* spp. potiče metaboličku inhibiciju te povećava broj bakterija koje preživljavaju u novonastalim uvjetima (19). Unatoč svemu ovo je istraživanje potvrdilo BRS i *Lactobacillus casei* u istom uzorku, što može upućivati na simbiotsku aktivnost tih mikroorganizama. Aerobne bakterije pogoduju BRS-u jer svojim metabolizmom stvaraju anaerobne uvjete za metabolizam i rast BRS-a (16). *Lactobacillus casei* fakultativna je bakterija koja proizvodi mliječnu kiselinu (18) kojom se BRS koristi kao izvorom organskoga ugljičnog laktata (1), što je zapravo sol mliječne kiseline. Langendijk i suradnici (8) istraživali su oksidaciju laktata u prisutnosti sulfata u deset podvrsta BRS-a izoliranih iz oralne mikroflore.

Mnogo crijevnih bakterija proizvodi laktate, što stimulira BRS na proizvodnju sulfata u debelom crijevu (20). Newton i suradnici (21) procijenili su utjecaj *Desulfovibrio desulfuricans* na populaciju trajnih crijevnih bakterija kao što je *Lactobacillus acidophilus*. U ovom istraživanju zabilježena je velika redukcija laktata, koji služi kao važan donor elektrona potrebnih bakteriji *Desulfovibrio spp.,* te velika redukcija u populaciji bakterije *Lactobacillus acidophilus.*

In the current study, of the nine samples, four were positive for the presence of SRB (66.67%), indicating that this bacterial group may be a part of the normal oral microbiota while also potentially being associated with periodontal disease. This was observed in one patient where SRB was found in two distinct sites, tooth 35 and 31. However the sample from tooth 31 had severe periodontal destruction, with vertical bone loss, presenting the possibility that SRB has a role in the pathogenesis of periodontal disease. The same was similar for two other samples analyzed from root fragments (patients numbers 2 and 4), where clinically these teeth presented different degrees of periodontal involvement. These findings are in accordance with Langendijk et al. (7), who reported a higher prevalence of SRB in patients with periodontal pockets and bleeding upon probing and in patients with various angular bone defects, furcation and endodontic complications, observing a direct relationship between the pocket depth and the presence of SRB. Willis et al. (5), identified a greater prevalence of SRB in saliva when compared with sub- and supragingivial biofilms, the anterior and posterior tongue and the oral mucosa.

In sites without direct contact with the oral environment, SRB was not isolated from the endodontic lesion biopsy or the root canal paper cone, which highlights the need for oral microbiota exposure for potential colonisation by SRB.

One of the important characteristics of SRB is its ability to associate with different types of surfaces where it constructs a cooperative consortium with other types of bacteria to form a biofilm (15,16). It may be suggested that once present in the oral microbiota, different oral sites can be colonized by SRB, as demonstrated by patient 4, with the colonization of SRB in distant sites (tooth 35 and 31).

The *Lactobacillus* spp., which is strongly associated with dental caries is present in the oral microbiota, dental biofilm and the dentine surface and changes the level of lactic acid in these microenvironments (17), while the species *Lactobacillus casei* can be found in supragingivial interproximal plaque (17) and dental biofilms (18). The reduction of pH in the microenvironment caused by *Lactobacillus* spp. results in the metabolic inhibition and increase of bacteria present in the oral microbiota of this microenvironment (19). However, the current study identified SRB and *Lactobacillus casei* in the same sample, which may indicate a possibility symbiotic relationship between these microorganisms. In association with aerobic bacteria, SRB benefits the formation of an anaerobic microenvironment in the biofilm caused by the metabolism of the aerobic bacteria present, where oxygen consumption will ensure a reduction in the necessary conditions for the growth of SRB, leading to maximum metabolic activity (16). *Lactobacillus casei* is a facultative bacteria that produces lactic acid (18) which can be used by the SRB as a source of organic carbon lactate (1), which is a salt of lactic acid. Langendijk et al. (8), reported the oxidation of lactate in the presence of sulphate in ten SRB strains isolated from the oral microbiota.

Many intestinal bacteria produce lactate which stimulates the production of sulphate by the SRB present in the colon (20). Newton et al. (21), evaluated the effect of *De-*

Rezultati ovog istraživanja moraju se oprezno interpretirati zbog malog uzorka. Upravo zato se i ne mogu pronaći poveznice između BRS-a i ostalih bakterija.

Također je bilo moguće dokazati veću pojavnost BRS-a u uzorcima sline kod pacijenata s gastritisom i parodontitisom (22). Metodologija korištena u ovom istraživanju bila je usmjerena više na detekciju mjesta na kojima djeluje BRS. Prisutnost BRS-a u različitim oralnim tkivima s različitim parodontnim statusom upućuje na to da su potrebna nova istraživanja kako bi se ustanovila njegova prava uloga u oralnoj mikroflori. Osim toga bilo je moguće u istom uzorku potvrditi prisutnost *Lactobacillus casei* i BRS-a.

Zahvala

Autori zahvaljuju svim pacijentima koji su sudjelovali u istraživanju. Rad su poduprli Zaklada za postdoktorsko obrazovanje (CAPES) i Nacionalni institut za tehnologiju (LABIO i DCOR).

Sukob interesa

Svi autori potvrdili su da nisu u sukobu interesa.

sulfovibrio desulfuricans on intestinal bacterial populations in continuous cultures, among these, *Lactobacillus acidophilus*. This study noted a great reduction of lactate, which is an important electron donor for *Desulfovibrio* sp., as well as a significant reduction in the population of *Lactobacillus acidophilus* species.

The findings here need to be considered with caution because the small number of samples. In this way, it could not be able to establish associations of SRB with other cultivable bacteria. Therefore, it is necessary to increase the number of samples for future effective conclusions.

In the current study it was possible to demonstrate a higher prevalence of SRB in saliva samples from patients with gastritis and periodontal diseases (22). The methodology applied was more selective in the detection of the site of action of SRB. The presence of SRB in different dental tissues with distinct periodontal features demonstrates that new studies need to be developed in order to determine the true role of SRB in the oral microbiota. In addition, it was possible to verify the presence of *Lactobacillus casei* together with SRB in one sample.

Acknowledgements

The authors gratefully acknowledge the patients for their participation in this study. This work was supported by the Foundation for Post-Graduate Education (CAPES), and the National Institute of Technology (LABIO and DCOR).

Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Aim: To detect for the presence of sulphate-reducing bacteria (SRB) and evaluate the possible association between SRB and cultivable facultative bacterial of oral sites with different periodontal conditions. **Methods:** The study was carried out on 9 samples from different oral sites in 8 patients (two samples were collected from the same patient). Material was collected using modified Postgate E culture medium, indicated for the growth and isolation of SRB. In addition, a reducing solution for anaerobic bacteria was used as a transport solution for facultative bacteria and identified by polymerase chain reaction amplification (PCR) and sequencing of the 16S rRNA gene. **Results:** SRB was found in 3 patient samples: the first in a root fragment, the second in a root fragment and a healthy tooth with vertical bone loss and a mobility degree of 3; and the third in a healthy tooth extracted for orthodontic treatment. In the final patient, the cultivable facultative species *Lactobacillus casei* was identified. Other facultative bacterial species were identified in patient 5 (*Kurthia Gibsonii*) and patient 7 (*Pseudomonas aeruginosa*). Conclusions: The detection of SRB in different dental tissues with distinct periodontal features demonstrated that new studies need to be developed in order to determine the true role of SRB in the oral microbiota. In addition, it was possible to verify the presence of *Lactobacillus casei* together with SRB in one sample.

Received: May 16, 2013
Accepted: January 30, 2014

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Key words
Periodontitis; Sulphur-Reducing Bacteria, Desulfovibrio.

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