Anti-inflammatory effects of Lactobacillus brevis (CD2) on periodontal disease

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OBJECTIVES: To analyze the anti-inflammatory effects of *Lactobacillus brevis* extracts on periodontitis patients and to investigate the involved mechanisms in vitro on activated macrophages.

METHODS: Eight healthy subjects and 21 patients with chronic periodontitis were enrolled to analyze the effect of *L. brevis*-containing lozenges on periodontitis-associated symptoms and signs. Before and after the treatment, the patients received a complete periodontal examination. Saliva samples, collected before and after treatment, were analyzed for metalloproteinase and nitric oxide synthase (NOS) activity, immunoglobulin-A (IgA), prostaglandin E2 (PGE2) and γ-interferon (IFN-γ) levels. Arginine deiminase (AD) and NOS activities were determined through a radiometric assay. Metalloproteinases were assayed by zymogram and Western blotting, whereas IgA, PGE2 and IFN-γ were assayed by enzyme-linked immunosorbent assay tests.

RESULTS: The treatment led to the total disappearance of chronic periodontitis were enrolled to analyze the effect of *L. brevis*-containing lozenges on periodontitis-associated symptoms and signs. Before and after the treatment, the patients received a complete periodontal examination. Saliva samples, collected before and after treatment, were analyzed for metalloproteinase and nitric oxide synthase (NOS) activity, immunoglobulin-A (IgA), prostaglandin E2 (PGE2) and γ-interferon (IFN-γ) levels. Arginine deiminase (AD) and NOS activities were determined through a radiometric assay. Metalloproteinases were assayed by zymogram and Western blotting, whereas IgA, PGE2 and IFN-γ were assayed by enzyme-linked immunosorbent assay tests.

CONCLUSION: Our results suggest that the anti-inflammatory effects of *L. brevis* could be attributed to the presence of AD which prevented nitric oxide generation. We thank the University of L’Aquila for financial support.

Keywords: *L. brevis*; nitric oxide; metalloproteinases; prostaglandin E2; γ-interferon

Introduction

Lactic acid bacteria (LAB) belong to a variety of genera used for milk fermentation. The primary metabolic end product from carbohydrate metabolism is lactic acid, which in turn preserves milk by providing the acidity necessary for a tart flavor and for changes in the structure of casein to achieve syneresis and desired functional characteristics. The extensive knowledge on LAB has opened new possibilities for their application (for reviews see Konings et al., 2000; Çaglar et al., 2005). Reports of many investigators have confirmed that LAB and their products have beneficial effects on the health of animals and humans, i.e., protection against enteric infections, use as an oral adjuvant, immunopotentiation in malnutrition, and prevention of chemically induced tumors (Bodana and Rao, 1990; Roberfroid, 1998; Dugas et al., 1999; Gorbach, 2000). Almost all genera of LAB are able to produce bacteriocins that are primarily lethal to other strains and species of bacteria form short-lived pores in biologic membranes by interactions with different compounds including nisin, lipid II and phospholipids, thereby killing the target bacteria (Konings et al., 2000). In the attempt to better characterize the metabolic features of some LAB strains, our group has recently shown the presence of high levels of arginine deiminase (AD) in *Lactobacillus brevis* (Di Marzio et al., 2001). As bacterial AD may compete with nitric oxide synthase (NOS) by using the same substrate, arginine, we decided to investigate the ability of *L. brevis* extracts to inhibit NOS activity as well other inflammatory parameters [γ-interferon (IFN-γ), prostaglandin E2 (PGE2) and metalloproteinases], known to be associated with NOS induction (Cifone et al., 1999; Goodwin et al., 1999; Di Marzio et al., 2001; Ishii et al., 2003). In particular, the chronic periodontal disease was chosen as the known inflammatory model to test the potential in vivo anti-inflammatory effects of *L. brevis*.

Periodontal disease is a major cause of tooth loss in humans and is one of the most prevalent diseases associated with bone loss. Following bacterial colonization, the gingiva becomes inflamed leading, in some cases,
to the destruction of the alveolar bone. Periodontitis has two distinct but interconnected etiologic components: periodontopathic bacteria adjacent to the periodontal tissues, and host-mediated connective tissue-destructive responses to the causative bacteria and their metabolic products (Golub et al., 1998). Treatment strategies against periodontal diseases have evolved with the aim of eliminating specific pathogens or suppressing destructive host response. Although the pathogenesis of the various forms of this disease is not completely understood, several factors are believed to play an important role, including inflammatory cytokines (McGee et al., 1998; Graves, 1999), PGE2 (Offenbacher, 1996; Tsai et al., 1998; Kornman, 1999), nitric oxide (Matejka et al., 1999; Takeichi et al., 2000) and salivary immunoglobulin-A (IgA) antibodies (Schenk et al., 1993). Moreover, many of the characteristics of periodontal diseases such as inflammation and attachment loss are associated with proteolytic events. Indeed, oral damage can result from the release of an array of proteolytic enzymes by colonizing bacteria as well as host-derived proteases (Henskens et al., 1996; Baron et al., 1999; Korostoff et al., 2000; Travis and Potemba, 2000). Selection of an appropriate delivery system is an important factor and, periodontitis being a 'localized' disease condition, local drug treatment should be preferred.

The aims of this study were firstly to analyze the effects of AD-expressing L. brevis-containing lozenges, both on the clinical signs of chronic periodontitis patients and on the NOS activity levels, matrix metalloproteinase (MMP) levels, eicosanoid generation, IgA levels, IFN-γ levels of the saliva. The mechanisms underlying the anti-inflammatory effect of L. brevis extract were investigated in vitro on rat peritoneal macrophages activated by lipopolysaccharide (LPS).

Materials and methods

Materials

Nitrate reductase, L-lactic dehydrogenase (LDH), pyruvic acid, β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6R)-5,6,7,8-tetrahydrobiopterin (NADPH), flavin adenine dinucleotide (FAD), flavin vic acid, b from Merck (Merck Bioscience GmbH, Schwalbach, Germany). EN^3HANCE spray was obtained from Perkin-Elmer (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA). Methanol, acetic acid, chloroform and ammonium hydroxide were purchased from J.T. Baker (Milan, Italy). Microconcentrators (Centricon 10) from Amicon (Amicon, Millipore Co., Bedford, MA, USA). IFN-γ (Endogen, Inc., Walburn, MA, USA) and PGE2 Elisa kits (Assay Designs Inc., Ann Arbor, MI, USA) were purchased from TEMA ricerca srl (Bologna, Italy). IgAs Elisa kits were obtained from IPR (Catania, Italy).

Preparation of bacterial extracts

Lactobacillus brevis, obtained from VSL Pharmaceuticals (Gaithersburg, MD, USA) in a pure lyophilized form (10⁶ colony-forming units per gram), was routinely grown in MRS broth medium under aerobic conditions.

For the determination of AD activity and for in vitro experiments on macrophages, L. brevis cultures were washed and resuspended in 10 ml phosphate-buffered solution (PBS), sonicated (30 min, alternating 10 s sonication and 10 s pause) with a Vibracell sonicator (Sonic and Materials Inc., Danbury, CT, USA), centrifuged at 7500 g for 20 min at 4°C and the supernatants stored at −20°C until used (bacterial extract). For the in vitro experiments, the bacterial extract was added to cell cultures at 0.5 mg/10⁵ cells/1 ml⁻¹ (final concentration).

For gene expression studies, genomic DNA was isolated from an overnight culture of L. brevis using a WIZARD genomic DNA purification kit (Promega, Milan, Italy), according to the manufacturer’s instructions.

Arginine deiminase activity

Arginine deiminase activity was determined in L. brevis extracts by measuring the conversion of [¹³C]L-arginine to [³H]-L-citrulline. Samples were homogenized by sonication in homogenization ice-cold buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) containing protease inhibitors (0.2 mM PMSF, 10 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ pepstatin A and 10 μg ml⁻¹ leupeptin). The homogenates were centrifuged at 4°C for 20 min at 11 000 g. The supernatants were passed through an AG50W-X-X-8 Dowex resin (Bio-Rad Laboratories, Melville, NY, USA) to remove endogenous arginine. The protein content was determined by the bicinchoninic acid procedure (BCA; Pierce, Rockford, IL, USA) using BSA as standard. Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris-HCl, pH 7.4, containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM L-citrulline (to inhibit the catabolism of [¹³C]L-citrulline), 60 mM L-arginine, 60 mM L-ornithine, 60 mM L-lysine (to inhibit nonspecific arginine activity) and 10 μCi ml⁻¹ of [³H]-L-arginine (Amerham, Bucksinghamshire, UK). The reaction was stopped by the addition of 0.4 ml of stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA). The samples were spotted onto TLC plates and developed in chloroform/methanol/ammonium hydroxide/water (1:4:2:1, by vol) as solvent system. After drying, the radiolabeled spots were visualized by autoradiography, after spraying EN^3HANCE solution (BCA; Pierce, Rockford, IL, USA) using BSA as standard. Enzymatic reactions were conducted at 37°C for 30 min in 50 mM Tris-HCl, pH 7.4, containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM L-citrulline (to inhibit the catabolism of [¹³C]L-citrulline), 60 mM L-arginine, 60 mM L-ornithine, 60 mM L-lysine (to inhibit nonspecific arginine activity) and 10 μCi ml⁻¹ of [³H]-L-arginine (Amerham, Bucksinghamshire, UK). The reaction was stopped by the addition of 0.4 ml of stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA). The samples were spotted onto TLC plates and developed in chloroform/methanol/ammonium hydroxide/water (1:4:2:1, by vol) as solvent system. After drying, the radiolabeled spots were visualized by autoradiography, after spraying EN^3HANCE solution (Perkin-Elmer Life and Analytical Sciences). [³H]-arginine and [³H]-citrulline, identified by co-chromatography with unlabeled standards which were revealed with ninhydrin spray, was scraped, and quantified by liquid scintillation. Where indicated, bacterial extracts were measured by autoradiography, after spraying EN^3HANCE solution (Perkin-Elmer Life and Analytical Sciences). [³H]-arginine and [³H]-citrulline, identified by co-chromatography with unlabeled standards which were revealed with ninhydrin spray, were scraped, and quantified by liquid scintillation. Where indicated, bacterial extracts were measured by autoradiography, after spraying EN^3HANCE solution (Perkin-Elmer Life and Analytical Sciences). [³H]-arginine and [³H]-citrulline, identified by co-chromatography with unlabeled standards which were revealed with ninhydrin spray, were scraped, and quantified by liquid scintillation.
preincubated for 60 min with 20 mM formamidine hydrochloride (Fluka Seelze, Germany), an inhibitor of AD \cite{Weikmann et al., 1978a,b}.

Arginine deiminase gene PCR amplification
The oligonucleotides were designed on the bases of nucleotide sequences of AD genes conserved at EMBL and GeneBank databases (accession number AJ001330). For polymerase chain reaction (PCR) experiments the following primers were used: primer arcA1 frame 5'-ATTTCGACAAACATTACGAGAC and primer arcA2 reverse 5'-GTGAAGACTGTATCTAATG-CAT. Polymerase chain reaction (PCR) to amplify the AD-encoding gene, was performed in a 100 μl volume. The amplification reaction mixture contained 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Life and Analytical Sciences), the reaction buffer provided by the Taq manufacturer (containing 1.5 mM MgCl₂), 200 μM each deoxyribonucleotide triphosphate (dNTPs), 0.5 μM each primer, and 1 μg of genomic DNA as template. Amplification reactions were carried out as follows: 12 min at 95°C for activation of AmpliTaq Gold DNA polymerase; denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, repeated for 30 cycles. A final DNA extension step at 72°C for 7 min was allowed at the end of the termal cycling. Direct sequencing of PCR amplicons was performed on both strands by dideoxy-chain termination method using a dRhodamina Terminator Cycle Sequencing Ready reaction Kit and the ABI PRISM 377 DNA Sequencer (Perkin-Elmer Life and Analytical Sciences). Sequencing was performed on three PCR products derived from three independent reactions.

L. brevis-containing lozenges
Each lozenge (VSL Pharmaceuticals) was composed of the following: lyophilized bacteria (200 mg), fructose (400 mg), mannitol (1020 mg), aspartame (40 mg), t alc (60 mg), aerosil 200 (10 mg), Peg 6000 (50 mg), magnesium stearate (20 mg), tartaric acid (100 mg), and orange flavor (100 mg).

Healthy volunteers, patients, treatment, and sample collection
The study was conducted as a double-blind paired-comparison study, with treatment assignments randomized and balanced to a group of eight healthy volunteers (people of our laboratories; male and female, age 24–47 years, mean age 33.6 ± 7.96), who were clinically free of periodontal disease. Twenty-one patients with chronic periodontitis, 16 men and five women, 30–51 years old (mean age 41.9 ± 7.23) were enrolled to analyze the effect of L. brevis-containing lozenges (4 lozenges/day for 4 days) on periodontitis-associated symptoms and signs, from the clinical point of view. Patients in this study displayed a range of periodontal disease from moderate to severe periodontitis. Pocket depth, measured to the nearest millimeter marking, was > 4 mm. Mean loss of attachment, evaluated by measuring the distance from the cemento-enamel junction to the bottom of the probing pocket, was 4.7 mm. No systemic disease was observed in any patient, and antibiotics were not taken during the previous 4 months. No patient was subjected to oral hygienics. Before and after the treatment with lozenges, the patients received a periodontal examination including plaque index \cite{Silness and Loë, 1964}, gingival index, bleeding on probing, calculus and temperature sensitivity scores. All periodontal disease measurements were performed in four quadrants at six sites per tooth for all teeth, with the exception of plaque index for which four sites were examined. The results were expressed as a mean value accompanied by standard error of the mean. The saliva samples, collected from controls and patients by spitting into an ice-cooled vessel before and after the treatment, were analyzed for MMP and NOS activity (the latter determined as nitrite/nitrate levels in the fluid saliva fraction and as citrulline levels in the cellular saliva fraction), and IgA, PGE₂, IFN-γ. Five patients, four men and one women, 29–48 years old (mean age 40 ± 9.6) manifesting periodontitis were also enrolled in the trial and treated for 4 days with conventional oral antibiotic therapy (spiromycin, 1 × 300 mg day⁻¹) with the aim of comparing the effects of this conventional treatment with those exerted by the bacterial extracts of L. brevis. All participants were carefully informed of the aim of the investigation and were free to withdraw from the study at any time. Written consent was obtained from all patients prior to the collection of samples. The study was approved by the Institute of Odontoiatric Clinics of the La Sapienza University, Rome, and was conducted in according with the principles of the Declaration of Helsinki.

Animals
Wistar rats (5–6 weeks old) were purchased from Charles River Breeding Laboratories (Calco, Como, Italy).

Rat peritoneal macrophages
Rat macrophages were obtained by peritoneal washes with PBS, centrifuged and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM glutamine, 10% heat-inactivated fetal calf serum (FCS) and 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. The cells were counted and their viability, as assessed by Trypan blue dye exclusion, was routinely greater than 98%. Two mililiters of cell suspension (4 × 10⁶cell ml⁻¹) was then plated on a 12-well tissue culture plate (Falcon; Becton Dickinson, Bucinasco, Milan). The following day, cell cultures were washed thrice with medium without FCS and then cultured in the same medium for 18 h with or without LPS (100 ng ml⁻¹) in the presence or absence of bacterial extracts (0.5 mg ml⁻¹). At the end of the treatments, cell pellets were used for NOS activity determination and supernatants, concentrated 10-fold by Centricon C10 (Amicon, Millipore Co.), were used in zymogram experiments to detect metalloproteinase activity.

Nitrite/nitrate levels
In aqueous solution, NO reacts rapidly with O₂ and accumulates in the culture medium as nitrite and nitrate ions. Twenty microliters of saliva samples or superna-
tants from macrophage cultures was used for nitrite measurement by a colorimetric assay based on the Griess reaction. Briefly, 5 μl of HEPES (50 mM, pH 7.4), 5 μl of FAD (5 μM), 10 μl NADPH (0.1 mM), 58 μl of H2O and 2 μl of nitrate reductase (0.2 IU ml−1) were added to 20 μl of samples, which were then incubated for 30 min at 37°C. At the end of the incubation time 10 μl of pyruvic acid (100 mM) and 1 μl of LDH (1500 IU ml−1) were added to the samples. After 10 min of incubation at 37°C, samples were mixed with an equal volume of Griess reagent in a microtiter plate and incubated at room temperature in the dark for 10 min. The OD was measured at 550 nm using a micro-enzyme-linked immunosorbent assay (ELISA) reader (Easy Reader EAR 400; Kontron Analytic, London, UK). KNO3 dissolved in H2O was used as standard and H2O as blank.

Nitric oxide synthase activity
Nitric oxide synthase activity was determined in both macrophage extracts and cell fraction of salivary samples by measuring the conversion of [14C]-L-arginine to [14C]-L-citrulline as above described for AD assay. The assay, performed with the addition to the reaction buffer of tetrahydrobipterin (BH4), 2 μM FAD, 2 μM flavin adenine mononucleotide, CaCl2 (0.6 mM) and calmodulin (CAM) (0.1 μM), measures both the calcium-dependent (constitutive) and calcium-independent (inducible) isoforms of the enzyme. Any activity inhibited by AG or detected in the absence of CaCAM and in the presence of the CAM antagonist W13 (100 μM) (to inhibit endogenous CAM), represented iNOS activity. Where indicated, 20 mM formamidine or 10 mM L-NMMA were added to the assay system to measure AD and/or NOS activity.

Determination of saliva IFN-γ, PGE2, and IgA levels
The IFN-γ (Endogen Inc., Woburn, MA, USA), PGE2 (Assay Designs) and IgA (IPR S.p.A., Catania, Italy) levels were determined in the fluid saliva fraction using ELISA kits, according to the manufacturer’s instructions. Where indicated, PGE2 levels were also assessed in macrophage culture medium.

Substrate gel electrophoresis (zymogram)
SDS-PAGE zymograms containing 0.1% gelatin were performed as described previously (Thorgeirsson and MacKay, 1992) using approximately 2 μg of sample protein from saliva samples or cultured macrophage supernatants to reveal the gelanolytic activity. Gelanolytic activity secreted by cultured macrophages in basal or stimulated conditions was analyzed by electrophoresing gels were rinsed in 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100 followed by 50 mM Tris-HCl (pH 7.4) and incubated overnight for gelatinases, in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM CaCl2, and 1% Triton X-100 at 37°C. Enzyme activity was detected following staining with 0.1% Coomassie brilliant blue in a mixture of acetic acid:methanol:water (1:3:6), destained in the same mixture without dye and dried. Sample collagenase activity was compared with that of partially purified gelatinases (MMP-2 and MMP-9) obtained from the supernatants of HT1080 human fibrosarcoma cell line (ICLC HTL98016, Interlab Cell Line Collection, Genova, Italy) stimulated with TPA (10−7M) for 48 h. All experiments were conducted with or without EDTA, serine or cystein proteinase inhibitors. Bands relative to enzyme activity were quantified by scanning densitometry using Molecular Analyst PC™ software for the Bio-Rad model 670 scanning densitometer.

Western blot
Saliva collected from healthy volunteers and patients were centrifuged at 600 g for 15 min and the supernatants were assayed for protein using the BCA method (Pierce). Twenty-eight microliters of each sample (40 μg of protein) was mixed with 7 μl of nonreducing PAGE buffer 5X [0.5 mol/l Tris-HCl (pH 6.8), 28% glycerol, 4.4% SDS, 0.55% bromphenol blue] and separated on an 8.5% polyacrylamide gel. After electrophoresis, SDS was removed by washing the gel in 50 mM Tris HCl pH 7.4, 2% Triton X-100 for 15 min at 37°C and then with the same buffer lacking Triton X-100 for 15 min at 37°C. Afterwards, the gel was equilibrated in electrotransfer buffer (25 mM Tris-HCl, 192 mM glycine, 10% methanol) for 20 min and transferred to polyvinyl difluoride (PVDF) membrane (Millipore) for 1 h at 4°C at 100V using a Mini Trans-Blot Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding sites were blocked with 10% nonfat dry milk in TBS-T (TBS and 0.05% Tween 20) for 16 h at 4°C. The membrane was then incubated for 1 h at 25°C with a mouse monoclonal antihuman MMP-9 (Calbiochem-Novabiochem, Darmstadt, Germany) diluted 1:400 in 5% nonfat dry milk in TBS-T. After incubation the membrane was incubated for 1 h at 25°C with goat antimouse-horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:3000 in 2.5% non-fat dry milk in TBS-T. Immunoreactivity was assessed by chemiluminescence reaction using ECL Western blotting detection system (Pierce).

Statistical analysis
The differences in the analyzed parameters between the control and patient groups were tested by using the Student’s t-test. The statistical significance of the differences in clinical and laboratory parameters between T0 and T1 was analyzed using the paired sample t-test. The STATPAC program was used to perform statistical analysis and statistical difference set at P < 0.05.

Results
Arginine deaminase activity in L. brevis extracts
Arginine deaminase activity was analyzed in bacterial extracts as described in the Materials and methods section. The enzymatic assay, based on the conversion of L-arginine to L-citrulline in appropriate conditions allowing to exclude NOS and arginase activity, showed that AD activity was present at high levels in L. brevis (Figure 1a). The generation of citrulline by L. brevis
extract could be attributed to AD activity, as it was observed in the absence of NOS cofactors (NADPH, FAD, FMN, and BH₄) and it was not affected either by L-valine, an arginase inhibitor, or by L-NMMA, a NOS inhibitor, and aminoguanidine, a relatively specific inhibitor of the inducible form of NOS. Moreover, the abrogation of citrulline generation observed in the presence of formamidine, a specific inhibitor of AD, definitively established that the conversion of arginine to citrulline was due to the presence of high levels of this enzyme in L. brevis extracts.

The presence of AD in L. brevis was further confirmed through the structural analysis of the gene encoding the enzyme. A couple of primers designed on the basis of consensus sequences known to be an internal region of the AD encoding gene was used to attempt PCR amplification. The PCR, performed on genomic DNA of L. brevis, yielded an amplimer of 665 bp (Figure 1b) which was subjected to direct sequencing. The sequencing of the PCR fragment, named arcA1-2, resulted in a completely identified 478 bp sequence (Figure 1c) encoding a polypeptide of 124 amino acid residues which showed several conserved structural elements typical of AD genes. Comparing amino acids residues of the AD of L. brevis and AD of L. sakei (Figure 1d), a stringent homology (52%) was found. Although the AD gene is scarcely conserved, including the internal regions, our results clearly demonstrated the presence of this gene in L. brevis.

Clinical evaluation
The scores for gingival inflammation, plaque, tartar, temperature sensitivity, and bleeding on probing before and after the treatment with L. brevis lozenges are reported in Table 1. All clinical parameters that were analyzed reached statistically significant differences between patients before treatment (T0) and the same after treatment with L. brevis containing lozenges (T1). On the mean, gingival inflammation was rated as moderate/diffuse by 20 patients at enrollment, and mild by one subject. After treatment, almost all patients showed no gingival inflammation; two individuals complained of a mild inflammation, while just one patient had a still moderate gingival inflammation. The
tolerability was considered as very good by all patients. Overall, the clinical evaluation of both the dentist and the patient overlapped, and the results confirmed recovery in 18 patients and improvement in three individuals. The antibiotic treatment for 4 days did not significantly modify signs and symptoms (data not shown), as expected.

**Effects of L. brevis-containing lozenges on saliva sample NOS activity, PGE₂, IFN-γ, and IgA levels**

The levels of nitrites and nitrates and those of PGE₂, IFN-γ, and IgA, determined in the fluid saliva fraction of healthy controls and periodontitis patients, as described in Materials and methods in an unblinded fashion, are reported in Table 2. Periodontitis patients’ saliva had a significantly higher level of nitrites/nitrates when compared with normal subjects (P < 0.01). In the same Table are also reported the results obtained when periodontitis patients’ saliva was analyzed for nitrite/nitrate levels before (T0) and after (T1) treatment with *L. brevis*-containing lozenges. In all patients, the treatment was associated with a significant reduction in nitrite and nitrate levels. Similar results were obtained when the conversion of radiolabeled arginine to citrulline was analyzed in the cell saliva fraction (data not shown). In addition the PGE₂ and IFN-γ levels were significantly higher in patients with periodontitis than in controls (P < 0.01). Interestingly, treatment with *L. brevis*-containing lozenges was associated with a strong decrease in the levels of these inflammation-associated molecules (P < 0.01). In contrast, IgA levels, which were significantly reduced in patients compared with controls (P < 0.05), were not significantly modified by the treatment.

**Effects of L. brevis-containing lozenges on saliva sample MMP activity and levels**

The measurements of MMP activity in the soluble fraction of saliva samples disclosed that the salivary samples of periodontitis patients contained higher amounts of MMPs than the control samples, thus confirming previous reports. Figure 2 shows gelatinase activity present in five different control saliva samples (panel a) as well as in five different patients’ saliva samples (panel b), representative of all examined cases. Enzymatic activity was compared with that of gelatinases obtained from the supernatants of the HT1080 cell line stimulated with tissue plasminogen activator (TPA) (10⁻⁷ M) for 48 h (the 72 kDa type IV collagenase, gelatinase A, MMP-2, and the 92 kDa type IV collagenase, gelatinase B, MMP-9). The zymogram of healthy control salivas (Figure 2a) shows bands with relative molecular weights of 92 kDa, of about 120 kDa and higher than 200 kDa. Aside from a band of about 37 kDa, little or no enzymatic activity was visible at

<table>
<thead>
<tr>
<th>Samples</th>
<th>Nitrite/nitrate (µM)</th>
<th>PGE₂ (ng ml⁻¹)</th>
<th>IFN-γ (pg ml⁻¹)</th>
<th>IgA (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>4.33 ± 1.31 P &lt; 0.0001</td>
<td>0.32 ± 0.08</td>
<td>10.06 ± 0.84</td>
<td>885.62 ± 118.28</td>
</tr>
<tr>
<td>T0</td>
<td>38.86 ± 6.07* P &lt; 0.0001</td>
<td>1.64 ± 0.1*</td>
<td>28.23 ± 2.11*</td>
<td>309.76 ± 143.06* P &lt; 0.05</td>
</tr>
<tr>
<td>T1</td>
<td>9.39 ± 1.47**</td>
<td>0.46 ± 0.07**</td>
<td>8.82 ± 4.74**1.04</td>
<td>466.51 ± 237.1</td>
</tr>
</tbody>
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HC, healthy controls; T0, before treatment; T1, after treatment. *P = T0 vs HC; **P = T1 vs T0.
molecular weights lower than 92 kDa. The profile of multiple forms of gelatinases found in patients’ saliva is shown in Figure 2b. In addition to the same bands of controls, a strong gelatinolytic activity at molecular weights lower than 92 kDa could be seen in all patients’ salivas. In some cases (i.e., lanes 2 and 3) the high molecular weight activities of gelatinase were almost absent, being prevailing below 92 kDa. These latter species mainly migrated with a connecting smear, being evident as separate bands only at the 37–50 kDa region. The gelatinolytic activity detected by zymogram appeared to be due to the presence of MMPs, as protease activity was completely abrogated by the addition of EDTA, but not by the addition of serine and cysteine proteinase inhibitors to the incubation buffer (data not shown). The treatment with lozenges led to the near disappearance of these MMP bands in all patients’ samples. Figure 3 shows the gelatinolytic activity of five representative periodontitis patients before (T0) and after (T1) the treatment with experimental lozenges. The profile of multiple forms of gelatinases was similar in all patients. The treatment led to the near disappearance of the low molecular weight bands and restored the control profile of gelatinolytic activity.

Western blot experiments demonstrated that the bands revealed by zymograms were due mainly to the enzymatic activity of MMP-9. Figure 4 shows a Western blot of one representative control and three representative patients before and after treatment with experimental lozenges. In the saliva of patients, antibody against MMP-9 revealed some high molecular weight forms (>200 kDa) together with bands at the 180 kDa region and at 120 kDa. Other molecular weights ranging from 92 to 64 kDa and approximately at 54 and 44 kDa were seen in the saliva of patients with chronic periodontitis. After treatment the enzymatic pattern resembled that of healthy controls. Indeed, a relevant reduction in high molecular weight bands was observed and little or no MMP-9 species below 92 kDa were detected. Western blotting with specific antibodies against human MMP-2, MMP-3, MMP-1, and MMP-8 showed no immunoreactivity, suggesting that these MMPs were not detectable in the saliva of either controls or patients (data not shown).

**Effect of L. brevis extracts on macrophage NOS activity**

Rat peritoneal macrophages incubated in vitro in the presence of LPS for 18 h were induced to express iNOS activity (Figure 5a). NOS activity was inhibited by the NOS inhibitors AG and L-NMMA. In the presence of bacterial extracts, NOS activity disappeared and arginine was converted in citrulline through bacterial AD, as demonstrated either by the unsensitivity of this conversion to NOS inhibitor treatment (Figure 5a) or by the disappearance of enzymatic activity in the presence of the AD inhibitor, formamidine (data not shown). Similar results were obtained with *L. fermentum* extracts, but not with *Streptococcus thermophilus*, a LAB in which the AD activity was not detectable (not shown).

These results suggest that bacterial AD was able to totally utilize the arginine present in the medium thus preventing its uptake by macrophages and consequently NOS activity.

**Effect of L. brevis extracts on macrophage PGE2 release**

When rat peritoneal macrophages were cultured for up to 18 h with LPS, a gradual accumulation of PGE2 in the supernatants occurred (fivefold increase over that in control cells) (Figure 5b). This effect was almost totally abrogated in the presence of *L. brevis* extracts as occurred when the cells were previously treated with L-NMMA or aminoguanidine.

**Effect of L. brevis extracts on MMP production by macrophages in the presence of LPS**

To investigate the *L. brevis* effect on MMP production, activated rat peritoneal macrophages were treated with...
bacterial extracts in the presence or absence of LPS and cell culture supernatants were analyzed by zymography for MMP-9 and MMP-2 levels. Compared with macrophages in culture medium alone, LPS increased MMP-9 activity, whereas MMP-2 was not detectable. The presence of \textit{L. brevis} extract in the culture medium led to a relevant decrease in MMP-9 activity, either when added alone or in the presence of stimuli. Similarly, a total inhibition of MMP activity was observed in the presence of iNOS inhibitors. A representative zymography is illustrated in Figure 5c. Enzymatic activity was compared with that of partially purified gelatinases (MMP-2 and MMP-9) obtained from the supernatants of HT1080 cell line stimulated with TPA (10^{-7}M) for 48 h. The bands revealed by zymograms could be attributed to MMPs as they disappear with EDTA but not by the addition of other proteinase inhibitors to the incubation buffer (data not shown).

**Discussion**

Periodontitis is characterized by extensive destruction of the gingival tissues and associated supporting structures of the teeth. In the present work, we first analyzed the potential anti-inflammatory action \textit{in vivo} of \textit{L. brevis}-containing lozenges in 21 adult patients with periodontal disease. Previously, our group reported the presence of arginine-deiminase in \textit{L. brevis}, a strain belonging to LAB (Di Marzio \textit{et al}, 2001). The presence of high levels of this enzyme, which metabolize arginine to citrulline and ammonia, aids \textit{L. brevis} extracts to inhibit NO generation, by competing with NOS for the same substrate, arginine. To confirm the presence of AD in \textit{L. brevis}, previously shown through the assay of enzymatic activity (Di Marzio \textit{et al}, 2001), we checked the presence of the AD gene. The analysis of a fragment obtained by PCR unambiguously revealed the presence of this gene, thus confirming our previous findings.

Nitric oxide is known to be an important inflammatory mediator, and is involved in the pathophysiology of several inflammatory disorders. An increase in NO production has been also demonstrated in periodontitis as its role in the inflammatory response of periodontal tissues has been suggested (Matejka \textit{et al}, 1998, 1999; Lappin \textit{et al}, 2000; Hirose \textit{et al}, 2001; Kendall \textit{et al}, 2001; Lohinai \textit{et al}, 2001; Daghigh \textit{et al}, 2002). These findings are consistent with the hypothesis suggesting that treatment with agents able to block the production of NO or its effects might be therapeutically valuable for periapical lesions (Lohinai \textit{et al}, 1998; Paquette and Williams, 2000; Daghigh \textit{et al}, 2002). Considering the role of NO in inflammatory events in general and in periodontitis in particular, we analyzed the potential beneficial effects of \textit{L. brevis}-based treatment in patients with periodontal disease. Of note, the proposed experimental treatment led to the total disappearance or amelioration of all analyzed clinical parameters (gingival inflammation, plaque, calculus, temperature sensitivity, and bleeding on probing) in almost all 21 patients (recovery in 18 patients and improvement in three individuals). The beneficial effects of the treatment on clinical signs and symptoms were paralleled by a strong and statistically significant decrease in oral NOS activity. Besides its direct role as an inflammatory modulator, NO represents a multifunctional messenger molecule able to modulate the production of inflammatory cytokines (Cifone \textit{et al}, 1999), PGE\textsubscript{2} (Goodwin \textit{et al}, 1999), and MMPs (Maeda \textit{et al}, 1998; Tronc \textit{et al}, 2000;
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Hirai et al, 2001). On the other hand, inflammatory cytokines such as IFN-γ, tumor necrosis factor-α and interleukin-1-α have been shown to be significantly higher in periodontitis patients than in healthy controls (Takeichi et al, 2000; Hirose et al, 2001; Tervahartiala et al, 2001; Ukai et al, 2001). Moreover, the generation of PGE_2, a potent eicosanoid lipid mediator derived from phospholipase-released arachidonic acid that is involved in numerous homeostatic biologic functions and inflammation, has been shown to be strongly incremented in periodontal lesions (Cavanaugh et al, 1998; Leibur et al, 1999; Paquette and Williams, 2000; Tsilingaridis et al, 2003). Furthermore, MMP levels have been previously shown to play a critical role in the inflammatory process leading to alveolar bone and connective tissue loss in periodontal disease (Makela et al, 1994; Ding et al, 1995; Ejeil et al, 2003; Sorsa et al, 2004). Indeed, one of the most salient features of periodontitis and gingivitis is the increase in the levels of bacterial and host-derived proteolytic enzymes in oral inflammatory exudates. Bacterial pathogens involved in periodontal diseases partly trigger and induce host cells to elevate their secretion of MMP. Proteolytic enzymes released by the host cells and/or by pathogen bacteria are associated with tissue destruction and MMP have the primary role in this process as they can degrade most of the extracellular matrix components (Ding et al, 1995).

Altogether, these observations prompted us to analyze the potential effects of L. brevis extracts containing lozenges on IFN-γ, PGE_2, and MMP salivary levels in periodontitis patients. Our findings clearly showed that all these inflammatory-associated factors were drastically reduced in periodontal disease patients after the L. brevis-based treatment. However, no significant effects were observed on salivary IgA levels after the proposed treatment.

In the attempt to define the mechanisms underlying the anti-inflammatory action of L. brevis-based treatment, a set of experiments were performed by using rat peritoneal macrophages activated in vitro with LPS in the presence or absence of bacterial extracts. The results indicated that the LPS-induced iNOS activity, PGE_2 generation, and MMP activation were almost totally abrogated when macrophages were cultured in the presence of bacterial extracts, thus strongly supporting the in vitro observations. The finding that iNOS inhibitors were able to inhibit either PGE_2 release or MMP activity in LPS-activated macrophages, prompted us to hypothesize that NO could be responsible for the latter events, as previously reported in several systems (Maeda et al, 1998; Goodwin et al, 1999; Trone et al, 2000; Hirai et al, 2001). The anti-inflammatory effects of L. brevis extracts could thus be attributed to their ability to prevent iNOS activity and, consequently NO-dependent PGE_2 release and MMP activation.

Considering recent reports showing that Lactobacillus and Bifidobacterium spp. or their components protected the host against certain oral diseases by inhibiting the growth of potential pathogens (Smith et al, 2001; Grudianov et al, 2002; Volozhin et al, 2004), we cannot exclude the possibility that L. brevis extract-based treatment, besides the anti-inflammatory effects reported here, is also able to antagonize the growth of specific periodontopathic pathogens. Taken together, our findings give further insights into the knowledge of the molecular basis of periodontitis and have a potential clinical significance, representing the experimental ground for a new innovative, simple and efficacious therapeutic approach of periodontal disease.

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