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Guiding Periodontal Pocket Recolonization: a Proof of Concept

ABSTRACT

The complexity of the periodontal microbiota resembles that of the gastro-intestinal tract, where infectious diseases are treatable *via* probiotics. In the oro-pharyngeal region, probiotic or replacement therapies have shown some benefit in the prevention of dental caries, otitis media, and pharyngitis, but their effectiveness in the treatment of periodontitis is unknown. Therefore, this study addressed the hypothesis that the application of selected beneficial bacteria, as an adjunct to scaling and root planing, would inhibit the periodontopathogen recolonization of periodontal pockets. Analysis of the data showed, in a beagle dog model, that when beneficial bacteria were applied in periodontal pockets adjunctively after root planing, subgingival recolonization of periodontopathogens was delayed and reduced, as was the degree of inflammation, at a clinically significant level. The study confirmed the hypothesis and provides a proof of concept for a guided pocket recolonization (GPR) approach in the treatment of periodontitis.

KEY WORDS: probiotics, periodontitis, therapy, microbial interference, replacement therapy, treatment.

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INTRODUCTION

Periodontitis painlessly destroys the supporting tissues around teeth, and increases the risk for atherosclerosis (Engebretson *et al.*, 2005) or low-birthweight babies (Lopez *et al.*, 2002). It develops when the equilibrium between microbial load and host defense mechanisms is disturbed in the periodontal tissues (Taubman *et al.*, 2005).

Given the risk of serious side-effects associated with altering the host response (*e.g.*, COX-2 inhibitors), treatment of periodontitis focuses on the reduction of the bacterial threat (Salvi and Lang, 2005). Conventional treatment involves mechanical subgingival debridement, which results in 2- to 3-$\log_{10}$ reductions in the total subgingival microbiota (Maiden *et al.*, 1991; Rhemrev *et al.*, 2006). However, recolonization toward pre-treatment levels, primarily by bacteria less strongly implicated as periodontopathogens, occurs within weeks (Harper and Robinson, 1987), and re-establishment of a more pathogenic microbiota occurs within months (Magnusson *et al.*, 1984). Adjunctive use of local or systemic antibiotics and antiseptics improves the outcome of periodontal therapy only temporarily (Quirynen *et al.*, 2002). Thus, a life-long need for (re)treatment arises, creating a serious socio-economic problem. Additionally, increasing levels of antibiotic-resistant bacteria favor the development of approaches that do not rely on antibiotics.

This study tested the hypothesis that the subgingival application of beneficial bacteria after mechanical debridement would enhance the microbial shift away from periodontopathogens. The beneficial bacteria used were selected because of their ability to prevent colonization of hard tissues and epithelial cells by periodontopathogens (Teughels *et al.*, 2007; Van Hoogmoed *et al.*, 2007), and their ability to prevent other mucosal infections, including pharyngitis (Falck *et al.*, 1999) and acute otitis media (Roos *et al.*, 2001) *in vivo*.

MATERIALS & METHODS

Subjects

Eight male beagle dogs with an average age of 3.08 (± 0.37) yrs were used in this split-mouth, double-blind, randomized trial. The protocol was approved by the University's Ethical Committee for Animal Experimentation. Surgical pocket creation, scaling, and root planing were performed after the animals were anesthetized by an intravenous injection of thiopental sodium (Abbott Laboratories, Leuven, Belgium), and carprofen (Pfizer, Luxembourg, Belgium) was given as a painkiller. All other manipulations were performed with the animals under sedation by medetomidine hydrochloride (Pfizer, Belgium) and ketamine (Eurovet Animal Health, Bladel, The Netherlands). Buprenorfine hydrochloride (Schering-Plough, Luxembourg, Belgium) was administrated as a painkiller. None of the dogs received antibiotics prior to or during the course of the study.

Periodontal Pockets

Bony defects were created surgically 4 mos prior to the experiment, even though the dogs already showed a moderate, naturally occurring periodontitis (localized pockets up to 4 mm). The first premolars were extracted in quadrants 1 and 4. A full-thickness flap was raised, and 5 mm of alveolar bone was removed, with a water-cooled round bur, from the canines, second, third, and fourth premolars. The defects extended from mid-approximal to mid-buccal. Prior to wound closure, the root surfaces were conditioned with heparin (15,000 IU/mL) (Aventis Pharma, Brussels, Belgium) (Wikesjö *et al.*, 1991). We enhanced plaque accumulation by feeding the dogs a soft diet.

Experimental Design

A clinician uninformed as to the study's design randomly assigned the 4 pockets in each quadrant to one of the 4 treatments (n = 2/animals/treatment, 16/treatment):

- a negative control treatment (Nc): no treatment
- a positive control treatment (Rp): subgingival scaling and root planing
- Rp_{single} root planing and a single application of the bacterial mixture at baseline
- Rp_{multi} root planing and repeated applications of the bacterial mixture at baseline and weeks 1, 2, and 4

Bacterial Mixture

Streptococcus sanguinis (ATCC 49297), *Streptococcus salivarius* (TOVE) (Tanzer *et al.*, 1985), and *Streptococcus mitis* (BMS) (Van Hoogmoed *et al.*, 2004) were cultured in 10 mL of Brain-Heart-Infusion broth (Difco Laboratories, Detroit, MI, USA), supplemented with 1 mg/mL Yeast Extract (Difco) at 37°C in 10% H_2, 80% N_2, and 10% CO_2. After 24 hrs, a 200-mL quantity of the respective medium was inoculated with the pre-cultures and grown for 16 hrs under the same conditions. Equal volumes of bacterial pellets harvested by centrifugation [6500 *g*, 10 min, 15°C, *S. sanguinis* - 11.0 ± 0.1 (mean ± standard error), *S. salivarius* - 10.7 ± 0.3, *S. mitis* - 9.6 ± 0.5 $\log_{10}$ CFU/mL] were combined and transferred to a sterile syringe. After undergoing root planing, and at the indicated time-points, the pure, unsuspended, mixed bacterial pellets were locally applied in the designated periodontal pockets by injection with a blunt needle.

Microbiological Parameters

At baseline and 2, 4, 6, 8, and 12 wks later, subgingival plaque samples were taken at each of the above experimental sites. After isolation of the sample sites and thorough supragingival cleaning, subgingival plaque samples were collected with 8 paperpoints (Roeko, Langenau, Germany) *per* site. They were dispersed in Reduced Transport Fluid (Syed and Loesche, 1972), homogenized by vortexing for 30 sec, transferred to the microbiology laboratory, and processed in under 2 hrs.

The samples were cultured under aerobic (3 days) and anaerobic conditions (10% H_2, 80% N_2, and 10% CO_2; 7 days) in non-selective (aerobic and anaerobic counts, black-pigmented bacteria, *Porphyromonas gulae,* and *Prevotella intermedia*) and selective media [Hammond medium for the detection of *Campylobacter rectus* (Hammond and Mallonee, 1988), Trypticase-Soy-Bacitracin-Vancomycin medium for *Aggregatibacter actinomycetemcomitans* (Slots, 1982), and Crystal-Violet-Erythromycin medium for the detection of *Fusobacterium nucleatum* (Walker *et al.*, 1979)]. Specific colony morphologies suggestive for *P. gulae* and *P. intermedia* were pure-cultured and verified *via* a series of biochemical tests (diagnostic tablets for microbial identification, International Medical, Brussels, Belgium). A pilot study on 100 dogs with naturally occurring periodontitis indicated that the species resembling *Porphyromonas gingivalis* in reality (after DNA sequencing) represents *P. gulae* [*i.e.,* the canine form of *P. gingivalis* (Harvey *et al.*, 1995)]. Details concerning the growth conditions, colony selection, and final identification of specific species have been summarized previously (Quirynen *et al.*, 1999). All microbiological evaluations were performed blind.

Periodontal Parameters

The following parameters (in sequential order) were recorded at baseline and at week 12 by one clinician, who was not informed of the treatment strategy:

- Probing pocket depth (PD) and gingival recession/overgrowth were measured from the cemento-enamel junction to the nearest 0.5 mm at 5 buccal sites (mesial, mesio-buccal, mid-buccal, disto-buccal, distal) by means of a Merrit B® probe (Hu-Friedy, Chicago, IL, USA).
- The bleeding tendencies (bleeding on probing, BOP) for the same sites were evaluated 20 sec after probing, with scores being 0 (absent) or 1 (present).

Statistical Analyses

A generalized mixed model was used, where treatment and time were considered as fixed factors, and dog and teeth as random factors. For the selection of the random factor, the model with the lowest Akaike's information criterion was withheld. P-values of the differences between the groups were corrected for simultaneous hypothesis testing according to Tukey's method.

RESULTS

Microbiological Parameters

There were no significant inter-group differences in total anaerobic (6.8 ± 0.1 $\log_{10}$ CFU/mL), black-pigmented bacteria (5.9 ± 0.1 $\log_{10}$ CFU/mL) and aerobic (5.3 ± 0.1 $\log_{10}$ CFU/mL) counts at baseline. After treatment, the shift in anaerobic species ($p < 0.0001$) and black-pigmented bacteria ($p < 0.001$) differed among strategies (Fig., A,B,C). Nc showed negligible changes, whereas all other treatments reduced ($p < 0.001$) the anaerobic species and black-pigmented bacteria. Rp reduced the levels of anaerobic species and black-pigmented bacteria, but a re-emergence reaching baseline levels at week 12 took place. Rp_{single} demonstrated a lesser tendency for re-emergence of anaerobic species when compared with Rp. Rp_{multi} showed the most pronounced reduction in anaerobic species and black-pigmented bacteria, and maintained the reduced levels over the entire study period. The Rp_{multi} strategy was superior to both Rp for anaerobic species and black-pigmented bacteria ($p = 0.002$ and $p < 0.001$) and Rp_{single} ($p = 0.03$ and $p < 0.001$). The changes in the total number of aerobic species (Fig., C), generally regarded as host-compatible species, were minimal in all treatments, although they tended to be elevated at week 12 for Rp_{single} and Rp_{multi}.

At baseline, there were no significant inter-group differences for *P. gulae* (5.8 ± 0.1 $\log_{10}$ CFU/mL), *P. intermedia* (5.4 ± 0.1 $\log_{10}$ CFU/mL), and *C. rectus* (5.2 ± 0.1 $\log_{10}$ CFU/mL). In the Nc sites, the changes in bacterial counts were negligible (Fig., D,E,F), with only a temporary slight reduction in *C. rectus*. Rp resulted in small, temporary

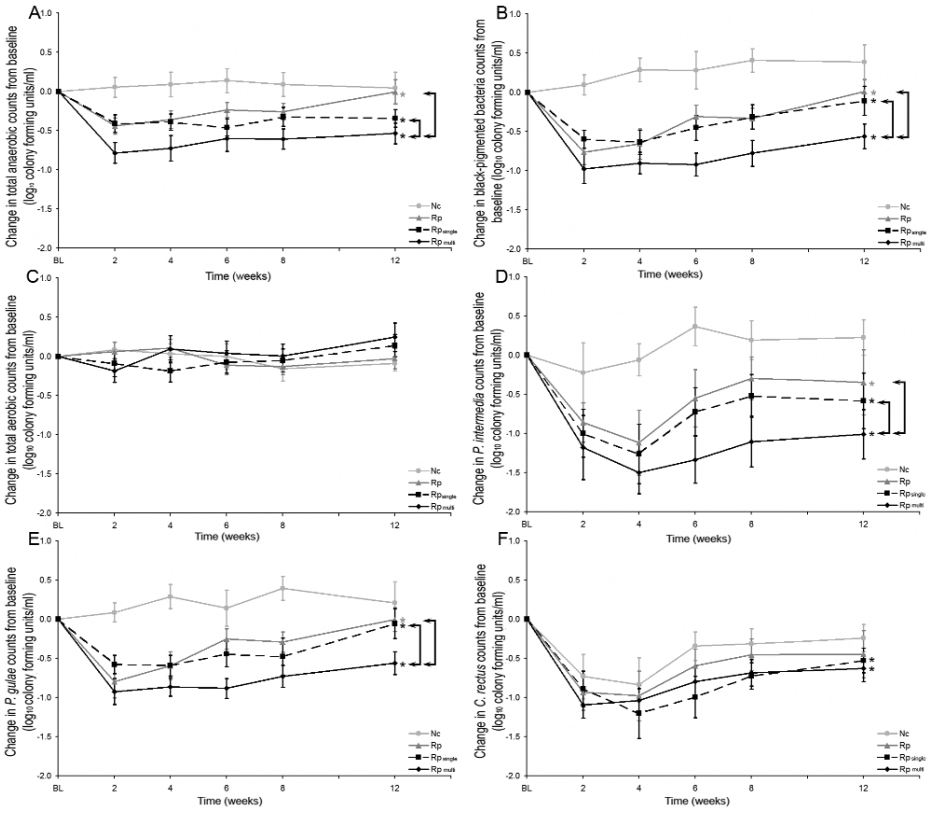

Figure. Mean change (± standard error) in subgingival bacterial load (n = 16/treatment group and time-point) from baseline (BL), and 2, 4, 8, and 12 wks later in each treatment group [Nc = negative control (no treatment), Rp = subgingival debridement *via* root planing, Rp_{single} = root planing + single application of a mixture of beneficial bacteria, Rp_{multi} = root planing + multiple applications of a mixture of beneficial bacteria]. * represents a treatment strategy that is significantly different from Nc ($p < 0.05$). Arrows represent significant differences among treatment strategies ($p < 0.05$). **(a)** Total number of anaerobic species. **(b)** Total number of black-pigmented bacteria. **(c)** Total number of aerobic species. **(d)** Total number of *P. intermedia* species. **(e)** Total number of *P. gulae* species. **(f)** Total number of *C. rectus* species.

reductions of *P. gulae* ($p = 0.001$) and *P. intermedia* ($p = 0.001$), but not for *C. rectus* ($p = 0.17$). Rp_{single} and Rp_{multi} resulted in significant reductions for all 3 species ($p < 0.001$, *P. gulae* and *P. intermedia*; and $p = 0.001$, *C. rectus*). In comparison with Rp, Rp_{single} resulted in minor additional reductions ($p > 0.05$), whereas Rp_{multi} showed clearly reduced levels of *P. gulae* ($p < 0.001$) and *P. intermedia* ($p = 0.02$). Compared with Rp_{single}, Rp_{multi} resulted in additional reductions for *P. gulae* ($p = 0.002$) and *P. intermedia* ($p = 0.05$). *F. nucleatum, A. actinomycetemcomitans,* and *T. forsythia* were never detected.

Periodontal Parameters

Significant reductions in pocket depth, bleeding on probing, and attachment level gain were achieved for all treatments except Nc (Tables 1, 2, 3). The differences among treatments did not reach significance for pocket depth or for gain in attachment, although the improvements in the Rp_{multi} group tended to be superior. In contrast, Rp_{multi} resulted in a more pronounced bleeding on probing reduction, a clinical marker for subgingival inflammation, when compared with Rp ($p = 0.03$). The local application of the bacterial mixture did not result in major adverse effects. During the entire period, only 1 periodontal abscess formed at a Rp_{single} site, and this lesion disappeared spontaneously.

DISCUSSION

'Replacement therapy', also known as 'probiotic therapy', originated over a century ago, but was largely abandoned after the discovery of antibiotics (Wilson, 2005). However, the emerging problem of antibiotic resistance has led to renewed interest in bacterial replacement. The approach is recognized in the management of gastro-intestinal diseases, and encouraging reports have shown possibilities in the oto-oro-pharyngeal area (Falck *et al.*, 1999; Roos *et al.*, 2001). Studies on replacement therapy for caries prevention (for review, see Meurman, 2005) have revealed promising results with the use of a genetically modified *Streptococcus mutans* strain (Hillman, 2002). Our study is the first to test the concept of bacterial replacement therapy in the treatment of plaque-related periodontal disease.

This study assessed quantitative changes in the subgingival microbiota after root planing when beneficial bacteria were applied adjunctively. To challenge the hypothesis of a prolonged beneficial microbial shift after mechanical debridement by deliberate application of beneficial species, we established a reservoir for recolonization (Quirynen *et al.*, 2001), by leaving teeth that were not included in the study unaffected, and by incorporating a negative control. To promote recolonization of periodontopathogens, no oral hygiene was performed (Magnusson *et al.*, 1984). Because ethical considerations imply the performance of oral hygiene after periodontal therapy in humans, a comparison between the obtained microbiological data in this study and the current literature is difficult.

The present findings confirmed a rapid recolonization of debrided periodontal pockets, often reaching pre-treatment levels at week 12 (Magnusson *et al.*, 1984). Although application of beneficial bacteria did not exclude pathogen recolonization, it did delay the recolonization process significantly. Inoculation of beneficial bacteria immediately after root planing, and especially with additional inoculations during the recolonization process, significantly lowered bacterial counts for all monitored pathogens. The differences between Rp and Rp_{multi}, at week 12 for anaerobic, *P. gulae*, and *P. intermedia* species, were identical or exceeded differences reported in similar, human, split-mouth studies where local antiseptics or antibiotics were used as adjuncts to root planing (Quirynen *et al.*, 2002). It is known that the

subgingival microbial profile is related to pocket depth (Socransky and Haffajee, 2005). Therefore, in some of these studies, microbiological differences among treatments might have originated from differences in the space available for recolonization due to differences in pocket depth reduction. The absence of significant differences in pocket depth reduction in our study excludes pocket depth differences as a confounding factor for the interpretation of the microbial changes. This makes the microbiological results even more interesting.

It can be criticized that no placebo control group was included in this study. However, there was, in our opinion, no correct placebo for the pure bacterial pellets used, as long as the exact mode of interference for the species was unknown. Additionally, because sampling the subgingival microbiota implied a disruption of the subgingival ecology (Mousques *et al.*, 1980), it is conceivable that the repeated applications of the bacterial pellets after sample-taking in the Rp_{multi} group did not have a major additional impact on the microbial profiles. Moreover, even in the weeks following the last application (week 4), the microbiota in the Rp_{multi} group remained significantly different from that in the other groups.

We cannot exclude the possibility that the beneficial bacteria applied translocated to control sites, or that their application induced an immunological response that interfered with the recolonization of the control sites. A recent investigation of the effects of gastro-intestinal probiotics has attributed the beneficial gastr-intestinal response to immunomodulation (Madsen, 2006). However, as evidenced by the colonization pattern of *C. rectus* in the Nc group in comparison with the Rp group, the beneficial effect appears to relate to a retarded recolonization.

Clinically, it is well-known that the re-emergence of periodontopathogens is correlated with lack of clinical improvement and the risk for disease relapse (Haffajee *et al.*, 1997). Although not statistically significant, the post-treatment attachment level was slightly lower for the treatments that included the application of beneficial species. The limited clinical improvement can be attributed to the absence of oral hygiene. Interestingly, this improved response also applied to the proportion of sites that exhibited bleeding on probing, a clinical marker for subgingival inflammation. The difference between Rp and Rp_{multi} for this latter variable was statistically significant. This observation supports the concept that the application of beneficial bacteria can lead to a more host-compatible subgingival microbiota. The questions of whether the applied species really colonized the subgingival habitat, and how the prolongation of the microbial shift was induced, remain unsolved. However, re-application of the beneficial bacteria seemed to improve the microbiological outcome of the treatment. In addition, it has been well-established for probiotics in the gastro-intestinal tract that they usually colonize for a short time, or even not at all (for review, see Wilson, 2005). Therefore, extrapolating probiotic colonization behavior on mucosal surfaces, such as in the intestine, to the periodontal pocket and its close association

Table 1. Changes in Probing Pocket Depth

	Probing Pocket Depth (mm) (n = 16/treatment group and time-point)							
	BL[a]			W12[b]			Change BL-W12	
Treatment	Mean	SEM[g]	Range	Mean	SEM	Range	Mean Reduction	SEM
Nc[c]	4.3	0.2	5.2-3.0	4.0	0.1	4.8-3.2	-0.4	0.2
Rp[d]	4.5	0.2	5.8-3.6	3.2[h]	0.1	4.4-2.6	-1.3	0.2
Rp_{single}[e]	4.3	0.2	5.8-3.0	3.2[h]	0.2	4.6-2.4	-1.1	0.2
Rp_{multi}[f]	4.5	0.2	5.8-3.2	3.2[h]	0.2	4.2-2.0	-1.3	0.2

[a] Baseline.
[b] Week 12.
[c] Negative control (no treatment).
[d] Subgingival debridement *via* root planing.
[e] Root planing + single application of a mixture of beneficial bacteria.
[f] Root planing + multiple applications of a mixture of beneficial bacteria.
[g] Standard error of the mean.
[h] Significantly different from BL ($p < 0.05$).

Table 2. Changes in Attachment Level

	Attachment Level (mm) (n = 16/treatment group and time-point)							
	BL[a]			W12[b]			Change BL-W12	
Treatment	Mean	SEM[g]	Range	Mean	SEM	Range	Mean Gain	SEM
Nc[c]	4.6	0.1	6.0-3.8	4.1	0.1	4.8-3.2	0.6	0.2
Rp[d]	5.0	0.2	6.4-4.0	3.7[h]	0.2	5.0-2.8	1.3	0.2
Rp_{single}[e]	4.9	0.2	7.2-3.6	3.8[h]	0.2	5.0-2.8	1.2	0.2
Rp_{multi}[f]	4.9	0.2	6.2-3.4	3.5[h]	0.2	4.6-2.0	1.4	0.2

Legend as for Table 1.

Table 3. Changes in Bleeding on Probing

	Bleeding on Probing (% of sites) (n = 16/treatment group and time-point)							
	BL[a]			W12[b]			Change BL-W12	
Treatment	Mean	SEM[g]	Range	Mean	SEM	Range	Mean Reduction	SEM
Nc[c]	58.8	9.6	100-0	33.8	8.7	100-0	-25.0	10.2
Rp[d]	78.8	6.5	100-0	45.0[h]	7.4	100-0	-33.8	8.1
Rp_{single}[e]	75.0	7.7	100-0	40.0[h]	8.1	100-0	-35.0	7.0
Rp_{multi}[f]	83.8	7.4	100-0	30.0[h,i]	6.6	80-0	-53.8	9.6

[a-h] Legend as in Table 1.
[i] Significantly different from Rp ($p < 0.05$).

with the non-shedding, biofilm-prone root surface might be difficult or even impossible. The mechanisms behind the successful inhibition of periodontopathogen (re-)colonization remain hypothetical. Occupation or a physico-chemical alteration of the subgingival niche (Teughels *et al.*, 2007), competition for essential nutrients (Sanders, 1969), inhibition of the viability or growth of pathogens (Wilson, 2005), and modification of the production or degradation of virulence factors of pathogens or immune responses are possible underlying mechanisms (Yasui *et al.*, 1999).

In summary, these results show that application of beneficial bacteria as an adjunct to root planing is a valid, non-antibiotic treatment approach for periodontitis. Given the emergence of antibiotic resistance and the lack of non-antibiotic treatment options, this Guided Pocket Recolonization approach may provide a valuable addition or alternative to the armamentarium of treatment options for periodontitis.

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