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Adjunctive efficacy of probiotics in the treatment of experimental peri-implant-mucositis with mechanical and photodynamic therapy: a randomized, cross-over clinical trial

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ABSTRACT

Aim: to evaluate the adjunctive clinical efficacy of probiotics in the treatment of peri-implant mucositis (p-iM) with professionally administered plaque removal (PAPR) and photodynamic therapy (PDT).

Materials and methods: Following p-iM induction, patients underwent PAPR+PDT and were randomly assigned to receive the professional and home-based administration of probiotics (*Lactobacillus plantarum* and *Lactobacillus brevis*) (test treatment) or placebo preparation (control treatment) according to a cross-over design. Clinical parameters were assessed at 6 sites for each implant before as well as at 2 and 6 weeks after professional treatment administration.

Results: Twenty patients contributing 1 dental implant each were included. Immediately before treatment and at 6 weeks, the median number of sites with bleeding on probing (BoP+) sites per implant unit was 4 (3-6) and 2 (0-2) ($p< 0.001$), respectively, for test treatment, and 3.5 (2-4) and 2 (0-3) ($p= 0.03$), respectively, for control treatment. No significant difference in clinical outcomes was observed between treatment groups.

Conclusion: The combination of PAPR and PDT either alone or associated with probiotics determined a significant reduction of the number of BoP+ sites at 2 and 6 weeks around implants with p-iM. The adjunctive use of probiotics did not significantly enhance the clinical outcomes of PAPR + PDT.

CLINICAL RELEVANCE

Scientific background: Peri-implant mucositis (p-iM) is a frequent finding in patients rehabilitated with dental implants. Residual bleeding is commonly reported following treatment of p-iM.

Principal findings: The combination of professionally administered plaque removal (PAPR) and photodynamic therapy (PDT) either alone or associated with probiotics determined a significant reduction of the number of BoP+ sites at 2 and 6 weeks around implants with p-iM. The adjunctive use of probiotics did not significantly enhance the clinical outcomes of PAPR + PDT.

Practical implications: The adjunctive use of probiotics seems not justified when treating p-iM with PAPR+PDT.

INTRODUCTION

The plaque-induced inflammation of the peri-implant, supracrestal soft tissues, named as peri-implant mucositis (p-iM), is a highly frequent clinical finding in patients rehabilitated with dental implants, with a weighted prevalence of 43% as reported in a recent systematic review (Derks & Tomasi 2015). p-iM is fully reversible following treatment (Pontoriero et al. 1994, Salvi et al. 2012), but may progress into peri-implantitis if untreated (Costa et al. 2012), with the irreversible loss of implant-supporting structures. Since the treatment of peri-implantitis may require surgical intervention and no universally accepted treatment protocols have been established for the management of such disease, which remains a challenge for the clinician (Lindhe & Meyle 2008, Froum et al., 2016), the prevention and treatment of p-iM acquired growing importance (Heitz-Mayfield & Mombelli 2014, Salvi & Zitzmann 2014, Jepsen et al., 2015, Clark & Levin 2016).

Professionally administered plaque removal (PAPR) is currently recognized as the indispensable, basal procedure in the treatment of p-iM (Figuero et al. 2014, Schwarz et al. 2015). The complete resolution of p-iM following PAPR, however, remains an unfrequent event, with residual bleeding on probing (BoP) ranging from 14.3% to 47.5% as reported in a recent systematic review (Schwarz et al. 2015). In the attempt to enhance the clinical outcomes of PAPR, several adjunctive treatments (including antiseptics, local and systemic antibiotics, and air abrasive devices) were proposed. On the basis of the existing evidence, however, none of them was shown to have a superior efficacy to PAPR alone in reducing the level of inflammation of the peri-implant mucosa on the short-term (Faggion et al. 2014, Schwarz et al. 2015).

When used for the treatment of periodontal and peri-implant diseases, the effect of photodynamic therapy (PDT) is mainly due to the bactericidal properties displayed when free reactive oxygen specimens are produced by the light activation of a dye within a pocket (Konopka & Goslinski 2007, Takasaki et al. 2009). Positive microbiological and clinical outcomes were reported for PDT

in the treatment of periodontitis (Sgolastra et al. 2013, Talebi et al. 2016), with scaling and root planing plus PDT being superior to SRP alone on the short-term (Sgolastra et al. 2013). From the results of previous *in vitro* and *in vivo* studies, PDT can safely and effectively reduce the prevalence of pathogens on the implant surface (Takasaki et al. 2009). Clinically, PDT was shown to be a promising treatment for the non-surgical management of mild peri-implantitis, with results comparable to those observed for locally applied antibiotics (Schär et al. 2013, Bassetti et al. 2014). On average, however, residual bleeding was reported at either 6 months (Schär et al. 2013) or 12 months (Bassetti et al. 2014) following treatment.

Probiotics were defined by the World Health Organization as "*living micro-organisms which, when administered in adequate amounts, confer a health benefit to the host by suppressing endogenous and exogenous pathogens and by promoting beneficial host response*" (http:// www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf). In addition to their anti-microbial effects, they can act on a variety of cells to modulate the immune system towards anti-inflammatory action (Teughels et al. 2011). To date, a limited number of studies evaluated the adjunctive efficacy of probiotics in the treatment of p-iM with conventional scaling and root planing, with contrasting results. While Flichy-Fernandes et al. (2015) reported superiority of systemically administered probiotics compared to placebo, Hällstrom et al. (2016) failed to find significant differences in the effect of probiotic supplements and placebo lozenges.

The aim of the present study was to evaluate the adjunctive clinical efficacy of probiotics in the treatment of experimentally-induced p-iM with PAPR and PDT.

MATERIALS & METHODS

Ethical aspects

The experimental protocol was approved by the Ethical Committee of the University of Rome, La Sapienza (date of approval: 27.11.14; protocol number: 3339). The clinical procedures were performed in accordance with the Declaration of Helsinki and the Good Clinical Practice (GCPs) guidelines. Each patient provided a written informed consent before participation.

Experimental design

The study was a 14-day, experimental p-iM trial with a randomized, cross-over, placebo-controlled, double blind design, and was conducted at the University of Rome La Sapienza. The investigated treatments consisted of PAPR and PDT with adjunctive, local and systemic administration of probiotics (test treatment) or placebo preparation (control treatment) (see "*Clinical procedures*" for details). The study was extended from July 2014 to June 2015 including a first phase of recruitment and a second part from the first patient starting the induction phase to the last one completing the study.

The phases of the experimental period for each patient are illustrated in Table 1 and described in details in the section "*Clinical procedures*". Briefly, at the screening visit each patient underwent professional oral hygiene and received oral hygiene instructions. This preparation phase terminated after 4 weeks. Then, patients wore an acrylic stent during self-performed oral hygiene procedures for 14 days (induction phase). After 2 weeks of experimentally-induced plaque accumulation at implant sites, the treatment phase was initiated with the professional administration of PAPR, PDT, and test or control treatment (as determined by the randomization list). The treatment phase was then continued for 2 weeks with the consumption of test or control tablets. After 6 weeks of tablet administration, professional oral hygiene was administered after experimental assessments

according to individual needs. After a wash-out period of 4 weeks, the induction and treatment phases were repeated for the evaluation of the remaining (test or control) treatment.

Treatment allocation and allocation concealment

According to the cross-over design, each patient underwent both investigated treatments. For each patient, the sequence of treatment administration was randomly determined through coin toss by an investigator not involved in the clinical procedures. For test treatment, probiotics were professionally delivered to the peri-implant sulcus in a 1:1 saline dilution with blunted tip mounted on a 5 ml syringe, while professional administration of placebo consisted of a saline irrigation with an identical syringe. Test and placebo tablets were prepared with identical tablet shape, size, color, product taste and packaging. The examiner and the patient were blinded as to the allocation sequence. The allocation sequence was disclosed at the completion of the experimental period for the last subject enrolled in the trial.

Study population

In general, screening aimed at identifying systemically and periodontally healthy patients rehabilitated with a single, implant-supported unit. Patient selection was conducted according to the following criteria.

Patient-related criteria

- age ≥ 18 years;
- healthy systemic conditions (i.e. no past or current diabetes, HIV, hepatitis or other infectious diseases, radiation in head and neck area, kidney disorders, immunological disorders, hematologic diseases);
- no current or chronic (more than 2 weeks) consumption of drugs with a documented influence on the gingival inflammatory response to dental plaque (e.g., antibiotics, non

steroidal anti-inflammatory drugs, phenytoin, calcium channel blockers, cyclosporin) within 3 months prior to study initiation;

- no use of any mouthwash, chewing gum or foods/drinks containing probiotics within 1 month from participation in the study;
- never smoked, former smoker, or smoking less than 5 cigarettes/day;
- no history of periodontitis or no residual sites with probing depth ≥ 5 mm following active treatment of periodontitis;
- full-mouth plaque score (FMPS) ≤ 15% at the screening visit;
- full-mouth bleeding score (FMBS) ≤ 15% at the screening visit;
- no drug or alcohol abuse;
- no pregnant or lactating.

Implant-specific criteria

In eligible patients, the presence of a dental implant with the following characteristics was verified:

- located in the molar or premolar region;
- loaded with a single-unit crown not interfering with the assessment of clinical parameters;
- in function for at least 1 year;
- distance between the peri-implant bone crest and the implant shoulder (as assessed on a peri-apical radiograph) < 2 mm;
- peri-implant probing depth ≤ 4 mm;
- keratinized tissue width ≥ 2 mm.
- negative to BoP.

Clinical procedures

The procedures by visit are outlined in Table 1 and reported in detail in the following paragraphs. Clinical procedures differed between treatment groups only for the administration of probiotics or placebo.

Screening visit (day -30)

In order to standardize baseline conditions, all eligible patients were instructed to apply a standardized regimen for self-performed oral hygiene, including a powered toothbrush (Oral B Professional Care, P&G, Rome, Italy) and toothpaste (AZ total care, P&G, Rome, Italy), and interdental floss/brush when indicated. Patients were supplied with the oral hygiene devices and received oral hygiene instructions. If their hygiene regimen was judged sufficient, only few individual instructions were given on how to improve their performance. Also, patients were instructed not to use any mouthwash, chewing gum or foods/drinks containing probiotics during the study. An individual acrylic stent was prepared as previously described by Trombelli et al. (2004). The stent included the dental implant selected for experimental assessments and, whenever possible, was extended at least two teeth (or implants) mesially and distally.

Induction phases (day 0 - +14; and day +56 - +70)

If one or more sites were BoP+ at the beginning of an induction phase (day 0 and day +56), polishing was performed with a rubber cup, oral hygiene instructions were reinforced, and the patient was asked to return in 1 week. After one week, the induction phase was initiated. Patients were instructed to use the stent before brushing and then remove it afterwards for the following 14 days.

Treatment phases (day +14 - +28; and day +70 - +84)

At the beginning of each treatment phase (day +14 and day +70), plaque was professionally removed with soft bristle brushes and low abrasive polishing paste (Nupro®; Dentsply, New York,

USA). Interdental spaces were cleaned with Super Floss® (Oral B, Procter & Gamble©, Cincinnati, Ohio). The experimental implants were isolated with cotton rolls, and toluidine blue O gel of moderate density (CMS Dental, Copenhagen, Denmark) was applied in the peri-implant sulcus with a blunted tip syringe and left in place for 1 minute. Afterwards, the gel was removed and submucosal irrigations with saline solution were performed to remove gel remnants. The implant surface was then dried, and a cold red light of 630 nm was applied with a LED FotoSan lamp (CMS Dental, Copenhagen, Denmark) using a blunted tip. At 6 sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual) for each implant, the lamp was activated for 10 seconds. Then, the implant was isolated again with cotton rolls. For the administration of test treatment, a probiotic mixture prepared with saline and probiotic powder (CMS Dental, Copenhagen, Denmark) in 1:1 ratio was delivered to the peri-implant sulcus with a blunted tip mounted on a 5 ml syringe. For the administration of control treatment, saline was delivered to the peri-implant sulcus according to the same procedure.

After the professional administration of test treatment, patients were supplied with probiotic tablets containing *Lactobacillus plantarum* and *Lactobacillus brevis* (AB-Dentis®, CMS Dental, Copenhagen, Denmark) and were instructed to consume one tablet per day (before breakfast) for 14 days. Similarly, patients undergoing professional administration of control treatment were supplied with placebo tablets to be consumed with the same dosage. In order to evaluate the level of patient compliance with probiotics (or placebo) intake, each patient was asked to return empty lozenges at the end of the two treatment phases. The level of compliance was considered sufficient if the patient had consumed ± 2 probiotics (or placebo) tablets with respect to the dose prescribed at the beginning of the treatment phase.

Wash-out (day +28 - day +56)

At the end of the first recovery phase (day +28), oral hygiene instructions were reinforced. In the period between the end of the first treatment phase (day +28) and the beginning of the second induction phase (day +56), patients continued their self-performed oral hygiene regimen.

Study termination (day +112)

At 4 weeks following the termination of the second treatment phase, patients were recalled for the assessment of experimental parameters. Then, a full-mouth session of mechanical prophylaxis was performed, and the patients exited the study.

Experimental assessments

Clinical parameters were measured by a single, trained operator not involved in clinical procedures for the administration of experimental treatments. At the beginning and completion of each induction phase as well as 2 and 6 weeks following the professional administration of test and control treatment, experimental parameters were assessed at 6 peri-implant sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual) according to the following sequence: Modified Plaque Index (mPlI) (Mombelli et al., 1987) and Bleeding on Probing (BoP) (Mühlemann & Son 1971). BoP was assessed using a manual pressure sensitive periodontal probe (UNC15; Hu Friedy, Milan, Italy) with a force of about 0.2 N.

STATISTICAL ANALYSIS

Data were recorded in a Microsoft Excel file and analyzed with a statistical software (STATA version 13.1; StataCorp LP; College Station, TX). Each patient contributed with one implant to the study, therefore the patient was regarded as the statistical unit. Values were expressed as median and interquartile range.

The Shapiro-Wilk tests and eyeball assessment of the empirical distributions were used to assess the evidence in rejecting the normality distribution resemblance of each parameter. The Kruskal-Wallis test was applied to evaluate the influence of time on each parameter, and two sample Wilcoxon rank sum test was used to evaluate the difference in each parameter between two treatment protocols at each observation interval. The level of statistical significance was fixed at 5%.

Due to the lack of previous controlled studies investigating the efficacy of probiotics in the treatment of p-iM, no sample size calculation could be performed *a priori*. Therefore, a sample size of 20 patients was arbitrarily determined. A *post-hoc* power calculation showed that the study had a power of 97.2% in detecting a difference in mean BoP of 0.8 between treatments with a standard deviation of 1.7.

RESULTS

Study population

Twenty patients (9 males; mean age: 57 ± 11 years, range: 39-78; 11 smokers) participated in the study, each contributing one implant in function for an average of 5 years (range: 1-13 years). Seven patients had a history of treated periodontitis. All participants fully complied with the study protocol, except for 1 drop-out (due to travelling abroad) at day +112. Based on the number of empty lozenges returned at the end of the treatment phases, all participants were compliant with probiotics and placebo intake.

Plaque and bleeding levels at the beginning and completion of the induction phase

At the beginning of the induction phase, similarly low levels of plaque accumulation were observed at implant units undergoing test treatment (mPlI: 0; 0-0) and control treatment (mPlI: 0; 0-0.17) (Table 2). At the completion of the induction phase, plaque accumulation and mucosal bleeding around dental implants as observed before the administration of test treatment (mPlI: 1.2; 0.92-1.59;

number of BoP+ sites per implant unit: 4; 3-6) were not significantly different from those observed before the administration of control treatment (mPlI: 1.42; 0.92-1.75; number of BoP+ sites per implant unit: 3.5; 2-4) (p= 0.6 and p= 0.3, respectively) (Table 2).

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Plaque and bleeding levels for test and control treatment are reported in Table 3. Both treatments were significantly effective in reducing plaque levels compared to first day of the treatment phase. At 2 and 6 weeks, mPlI was reduced to 0.17 (0-0.33) ($p<0.001$) and 0 (0-0.17) ($p<0.001$), respectively, following test treatment, and to 0 (0-0.33) ($p<0.001$) and 0.17 (0-0.33) ($p<0.001$), respectively, following control treatment. The number of BoP+ sites per implant unit was reduced to 3 (1.5-5) and 2 (1-5) at 2 weeks for test and control treatment, respectively. At 6 weeks, a further reduction was observed for both treatments. No statistically significant difference in mPlI and number of BoP+ peri-implant sites was observed between test and control treatment at each observation interval (Table 3). No significant inter-group differences in implant distribution according to the number of BoP+ sites were observed at each observation interval, as well (Table 4).

For each investigated treatment, no significant difference in the mean number of BoP+ sites per implant unit was observed between first and second treatment phase at each observation interval (i.e., end of induction phase, 2-week and 6-week visit of the treatment phase), thus indicating that the treatment phase (first or second) had no significant effect on clinical outcomes.

DISCUSSION

The present randomized, cross-over, placebo-controlled trial, was performed to evaluate the adjunctive clinical efficacy of probiotics in the treatment of experimentally-induced p-iM with

PAPR and PDT. Twenty patients, contributing 1 dental implant each, participated in the study and received PAPR and PDT in combination with the professional and home-based administration of either probiotics (*Lactobacillus plantarum* and *Lactobacillus brevis*) or placebo preparation according to a cross-over design. Clinical parameters were assessed immediately before as well as at 2 and 6 weeks after treatment administration.

In the present study, the efficacy of probiotics was investigated in an experimental p-iM study. To date, the existing evidence does not indicate how well this experimental disease model mimics naturally-occurring p-iM. Based on information from previous experimental gingivitis studies (Trombelli et al. 2010, Farina et al. 2012), which reported higher levels of plaque accumulation and gingival inflammation as well as qualitative differences in the microbiological profile in the experimentally-induced compared to the naturally occurring condition, it could be hypothesized that differences may exist also between experimental p-iM and its natural-occurring counterpart. Future studies, however, are needed to clarify this issue.

The experimental design of the present study was based on a p-iM induction phase of 2 weeks and a follow-up period of 6 weeks. Differently, other p-iM trials adopted a 3-week duration of the induction phase (Pontoriero et al. 1994, Zitzmann et al. 2001, Salvi et al. 2012). The choice to use a 2-week induction phase was based on some considerations. First, the severity of peri-implant inflammation (as obtained only with oral hygiene abstention) was shown to be not significantly different after 2 and 3 weeks of plaque accumulation (Salvi et al. 2012). Second, previous experimental gingivitis trials have shown that the 2-week use of a stent may lead to substantial accumulation of plaque deposits, with limited, although significant, differences in plaque deposits between 2- and 3-week of plaque accumulation (Trombelli et al. 2004). Third, a shorter duration of the induction phase is expected to improve the tolerability of the model as well as the patient adherence to study procedures. Data reported in Table 1 seem to suggest that a 2-week p-iM

induction phase may be sufficient to obtain levels of plaque accumulation and mucosal bleeding adequate to perform an intervention, experimental p-iM study. The duration of the resolution period was set at 6 weeks, based on the findings from Salvi et al. (2012) that suggested that the complete resolution of p-iM may require periods longer than 3 weeks.

BoP was used as the primary outcome variable. Due to its correlation with mucosal inflammation as assessed at the histological level (Lang et al. 1994), BoP is currently recognized as a key parameter to distinguish between peri-implant health and disease (Jepsen et al. 2015). Moreover, the available evidence seems to indicate that peri-implant BoP has a prognostic value, its presence (or absence) being associated with the deterioration (or stability) of peri-implant conditions overtime (Luterbacher et al. 2000). For this reason, BoP is also commonly used as a treatment outcome in the management of peri-implant diseases, in general, and p-iM, in particular (Schwarz et al. 2015).

To the best of our knowledge, this is the first study evaluating the clinical effectiveness of the combination of mechanical and photodynamic therapy in the treatment of p-iM. In our material, such combination resulted in a significant reduction of the number of BoP+ sites per dental unit at either 2 or 6 weeks following treatment administration. Despite these clinical improvements, only 6 out of 20 (30%) implant units were free of BoP+ sites at 6 weeks (Table 4). Consistently with our findings, a previous study evaluating the effect of PAPR+PDT in the treatment of mild peri-implantitis lesions showed a reduction of BoP+ sites by 44% after 3 months and 63% after a second application (Schär et al. 2012). Similarly, Bassetti et al. (2014) reported significant reductions of BoP+ sites at 12 months following administration of PAPR+PDT every 3 months. While indicating that PAPR+PDT seems not to predictably allow for a complete resolution of p-iM, these findings must also be considered in relation to some limitations of BoP assessments around implants. In particular, residual BoP could be partly due to mechanical trauma during the probing maneuver (Lang et al. 1994, Abrahamsson & Soldini 2006, Gerber et al. 2009, Salvi et al., 2012).

The adjunctive professional and home-based administration of probiotics did not improve the clinical performance of PAPR+PDT in the treatment of experimentally-induced p-iM. This finding is consistent with the results of a placebo-controlled study evaluating the topical use of an oil containing probiotics (two strains of *Lactobacillus reuteri*) as adjunct to PAPR followed by the administration of probiotics lozenges for 3 months in the treatment of p-iM (Hällstrom et al. 2016). At the end of probiotic administration, the clinical, microbiological and GCF levels of inflammatory mediators showed significant improvements and were maintained at 3 months after treatment administration, without significant inter-group difference (Hällstrom et al. 2016). Since residual BoP observed in PAPR+PDT group could suggest the difficulty of this treatment strategy in disrupting the biofilm on implant surface, a possible explanation for the lack of adjunctive effective of probiotics may reside in the partial effectiveness of mechanical + photodynamic therapy to control the implant biofilm. In this respect, Teughels et al. (2013) pointed out that probiotics might not be effective when applied on undisrupted biofilms. Similarly, a limited clinical efficacy of probiotics (*Lactobacillus reuteri*) in the prevention and treatment of gingivitis was observed when treatment was administered without self-performed or professional oral hygiene (Iniesta et al. 2012, Hällstrom et al. 2013). Furthermore, it may also be hypothesized that the effect of probiotics may become manifest at time intervals longer than those used in the present study.

In the present study, a wash-out period of 4 weeks was adopted in order to allow for complete recovery following p-iM therapy as well as to avoid the carry-over effect of probiotics for patients who had randomly assigned to test treatment during the first treatment phase. To date, limited data is available on the persistence of probiotic bacteria in the subgingival environment in relation to the time elapsed from their administration. The heterogeneity in the duration of wash-out periods as adopted in previous studies on probiotics (Del Piano et al., 2010, Hallström et al. 2013, Ashwin et al., 2015, Jørgensen et al. 2016) seems to reflect the lack of this information. Our choice to adopt a

4-week wash-out period was therefore based on the fact that at least 2 weeks following the suspension of probiotics administration were needed for pro and anti-inflammatory cytokines to return to baseline levels (Twetman et al. 2009).

In conclusion, the results of the study indicated that (i) the combination of PAPR and PDT either alone or associated with probiotics determined a significant reduction of the number of BoP+ sites at 2 and 6 weeks at implant units with experimentally-induced p-iM; and (ii) the adjunctive use of probiotics did not significantly enhance the clinical outcomes of PAPR + PDT.

CONFLICT OF INTERESTS

Authors declare no conflict of interest with the funder or producers and distributor of any of products and instruments used for this trial.

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TABLES

Table 1. Clinical procedures for each study phase. OHI, oral hygiene instructions; PAPR, professionally administered plaque removal; PDT, photodynamic therapy

Table 2. Modified Plaque Index (mPlI) and number of BoP+ sites per implant unit as assessed at the beginning and completion of each induction phase. Values are expressed as median and interquartile range. Two-sample Wilcoxon rank-sum test was performed with a significance level set at $p < 0.05$.

Table 3. Modified Plaque Index (mPlI) and number of BoP+ sites per implant unit as assessed at the beginning of each treatment phase as well as 2 weeks and at 6 weeks following the professional treatment administration. Values are expressed as median and interquartile range. Two-sample Wilcoxon rank-sum test was performed with a significance level set at $p < 0.05$.

Table 4. Distribution of implant units according to the number of BoP+ sites per implant unit as observed at the beginning of each treatment phase as well as 2 weeks and at 6 weeks following the professional treatment administration.

Table 1. Clinical procedures for each study phase. OHI, oral hygiene instructions; PAPR, professionally administered plaque removal; PDT, photodynamic therapy

	Day	Inclusion/ exclusion criteria	Stent	Clinical measurements	PAPR	Treatment
Screening visit	**-30**	+	preparation	+		
First induction phase	**0-14**		use	+	+	
First treatment phase	**14- 28**			+	+	administered
Wash-out	**28-56**					
Second induction phase	**56-70**		use	+	+	
Second treatment phase	**70-84**			+	+	administered
Study termination	**112**			+	+	

Table 2. Modified Plaque Index (mPlI) and number of BoP+ sites per implant unit as assessed at the beginning and completion of each induction phase. Values are expressed as median and interquartile range. Two-sample Wilcoxon rank-sum test was performed with a significance level set at $p < 0.05$.

	Test (probiotics)			**Control (placebo)**		
	Beginning of the induction phase	Completion of the induction phase	p value	Beginning of the induction phase	Completion of the induction phase	p value
PlI	0 (0.00-0.00)	1.25 (0.92-1.59)	< 0.001	0 (0.00-0.17)	1.42 (0.92-1.75)	< 0.001
n° of BoP+ sites per implant unit	2 (1-4)	4 (3-6)	0.04	2 (1-3)	3.5 (2-4)	0.03

Table 3. Modified Plaque Index (mPlI) and number of BoP+ sites per implant unit as assessed at the beginning of each treatment phase as well as 2 weeks and at 6 weeks following the professional treatment administration. Values are expressed as median and interquartile range. Two-sample Wilcoxon rank-sum test was performed with a significance level set at $p < 0.05$.

		Beginning of the treatment phase	2 weeks	p value	6 weeks	p value
PlI	**Test (Probiotics)**	1.2 (0.92-1.59)	0.17 (0.00-0.33)	p<0.001	0 (0.00-0.17)	p<0.001
	Control (Placebo)	1.42 (0.92-1.75)	0 (0.00-0.33)	p<0.001	0.17 (0.00-0.33)	p<0.001
	p value	0.64	0.23		0.17	
n° of BoP+ sites per implant unit	**Test (Probiotics)**	4 (3-6)	3 (1-5)	0.2	2 (0-2)	0.001
	Control (Placebo)	3.5 (2-4)	2 (1-5)	0.1	2 (0-3)	0.03
	p value	0.30	0.23		0.53	

Table 4. Distribution of implant units according to the number of BoP+ sites per implant unit as observed at the beginning of each treatment phase as well as 2 weeks and at 6 weeks following the professional treatment administration.

	Number of implant units					
	Beginning of the treatment phase		2 weeks		6 weeks	
Number of BoP+ sites	Test (Probiotics)	Control (Placebo)	Test (Probiotics)	Control (Placebo)	Test (Probiotics)	Control (Placebo)
0	1	2	2	3	6	6
1	3	1	3	6	3	3
2	0	3	3	5	7	1
3	3	4	3	0	2	6
4	5	5	2	0	1	1
5	2	2	5	4	0	1
6	6	3	2	2	1	1

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6-7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	7- 8
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6-7, 8-10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	11
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	NA
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	7

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	11
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	NA
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	11
	13b	For each group, losses and exclusions after randomisation, together with reasons	11
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	NA
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	NA
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	12
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	12
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	12
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	NA
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	15
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	14
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14
Other information			
Registration	23	Registration number and name of trial registry	NA
Protocol	24	Where the full trial protocol can be accessed, if available	NA
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Adjunctive efficacy of probiotics in the treatment of experimental peri-implant-mucositis with mechanical and photodynamic therapy: a randomized, cross-over clinical trial

(ref. CPE-10-16-6621)

AUTHORS' RESPONSE TO THE REVIEWERS' COMMENTS

Associate Editor

Please carefully address the minor issues raised by the referees.

ALL THE MINOR ISSUES RAISED BY REFEREE 1-3 HAVE BEEN ADDRESSED. BELOW PLEASE FIND A STRUCTURED REPONSE TO EACH COMMENT.

Section Discussion should also consider some thoughts on how well experimental peri-implant mucositis lesions mimic their naturally occurring counterpart.

TO ADDRESS THE EDITOR'S ISSUE, THE DISCUSSION HAS BEEN IMPLEMENTED WITH THE FOLLOING PARAGRAPH: "*In the present study, the efficacy of probiotics was investigated in an experimental p-iM study. To date, the existing evidence does not indicate how well this experimental disease model mimics naturally-occurring p-iM. Based on information from previous experimental gingivitis studies (Trombelli et al. 2010, Farina et al. 2012), which reported higher levels of plaque accumulation and gingival inflammation as well as qualitative differences in the microbiological profile in the experimentally-induced compared to the naturally occurring condition, it could be hypothesized that differences may exist also between experimental p-iM and its natural-occurring counterpart. Future studies, however, are needed to clarify this issue.*".

P14 L12-16: please double check this sentence - "experimental p-iM trial" appears to be redundant.

THE REDUNDANT PART OF THE SENTENCE HAS BEEN ELIMINATED.

Referee: 1

1. Sample size: How was the sample size determined? Was a power calculation performed?

THE STATISTICAL ANALYSIS SECTION WAS CHANGED AS FOLLOWS, INCLUDING THE REQUESTED WITH INFORMATION ON SAMPLE SIZE CALCULATION: "*Data were recorded in a Microsoft Excel file and analyzed with a statistical software (STATA version 13.1; StataCorp LP; College Station, TX). Each patient contributed with one implant to the study, therefore the patient was regarded as the statistical unit. Values were expressed as median and interquartile range.*

The Shapiro-Wilk tests and eyeball assessment of the empirical distributions were used to assess the evidence in rejecting the normality distribution resemblance of each parameter. The Kruskal-Wallis test was applied to evaluate the influence of time on each parameter, and two sample Wilcoxon rank sum test was used to evaluate the difference in each parameter between two treatment protocols at each observation interval.

Due to the lack of previous controlled studies investigating the efficacy of probiotics in the treatment of p-iM, no sample size calculation could be performed a priori. Therefore, a sample size of 20 patients was arbitrarily determined. A post-hoc power calculation showed that the study had a power of 97.2% in detecting a difference in mean BoP of 0.8 between treatments with a standard deviation of 1.7.".

2. Spelling/wording: Schär (p6 l16), of any mouthwash (p9 l7), located in (p9 l34), in detail (p10 l2), contributed with one implant to the study (p12 l38), in the study (p13 l3), 6 out of 20 (p15 l32).
ALL THESE MINOR ERRORS HAVE BEEN AMENDED.

3. Missing reference: The last sentence of the second paragraph on page 15 (ls14-18) requires a reference.
THE SENTENCE IS NOW SUPPORTED WITH PERTINENT REFERENCES: "*...Also, a recent systematic review on the efficacy of PAPR with or without adjunctive measures for the treatment of p-iM showed that the majority of the included studies reported treatment outcome in terms of BoP reduction (Schwarz et al. 2015).*"

Referee: 2
This is a cross over study with two treatments two periods with a wash out period between. The subjects were randomized to the sequence of treatments. In a cross over study it is of interest to determine if there is a period effect (was the treatment more effective in the first or second period). The analysis presented by the authors did not test for this type of effect.
AS REQUESTED BY THE REFEREE, WE HAVE PERFORMED AN ADDITIONAL ANALYSIS AIMED AT EVALUATING THE DIFFERENCES IN CLINICAL OUTCOMES BETWEEN 1ST AND 2ND TREATMENT PHASE WITHIN EACH TREATMENT. FOR EITHER TEST OR CONTROL TREATMENT, THE RESULTS DID NOT SHOW A DIFFERENCE IN THE MEAN NUMBER OF BOP+ SITES PER IMPLANT UNIT AT BASELINE, 2- AND 6-WEEK VISIT BETWEEN 1ST AND 2ND TREATMENT PHASE, THUS INDICATING THAT TREATMENT PHASE (1ST VS 2ND) HAD NO SIGNIFICANT EFFECT ON CLINICAL OUTCOMES. THIS FINDING HAS BEEN ADDED TO THE RESULTS SECTION OF OUR REVISED MANUSCRIPT: "*For each investigated treatment, no significant difference in the mean number of BoP+ sites per implant unit was observed between first and second treatment phase at each observation interval (i.e., end of induction phase, 2-week and 6-week visit of the treatment phase), thus indicating that the treatment phase (first or second) had no significant effect on clinical outcomes.*".

The authors found no difference between the control and experimental groups. The question is: are two applications of the accompanying therapy more effective than one and was the wash out time adequate?
DATA STEMMING FROM OUR ADDITIONAL ANALYSIS (SEE OUR RESPONSE TO THE PREVIOUS COMMENT) SHOWED THAT NO CUMULATIVE EFFECT OF TREATMENT WAS OBSERVED FOR EITHER TEST OR CONTROL TREATMENT.
THE 4-WEEK DURATION OF THE WASH-OUT PERIOD IS NOW DISCUSSED IN A DEDICATED PARAGRAPH OF THE DISCUSSION: "*In the present study, a wash-out period of 4 weeks was adopted in order to allow for complete recovery following p-iM therapy as well as to avoid the carry-over effect of probiotics for patients who had randomly assigned to test treatment during the first treatment phase. To date, limited data is available on the persistence of probiotic bacteria in the subgingival environment in relation to the time elapsed from their administration. The heterogeneity in the duration of wash-out periods as adopted in previous studies on probiotics (Del Piano et al., 2010, Hallström et al. 2013, Ashwin et al., 2015, Jørgensen et al. 2016) seems to reflect the lack of this information. Our choice to adopt a 4-week wash-out period was therefore based on the fact that at least 2 weeks following the suspension of probiotics administration were needed for pro and anti-inflammatory cytokines to return to baseline levels (Twetman et al. 2009).*"

Referee: 3
1.How was compliance with intake of probiotics tablets or placebo tablets at home checked?
THE MATERIALS & METHODS HAVE BEEN IMPLEMENTED WITH INFORMATION ON THE METHOD USED TO EVALUATE THE LEVEL OF PATIENT COMPLIANCE: "*In order to evaluate the level of patient compliance with probiotics (or placebo) intake, each patient was asked to return empty lozenges at the end of the two treatment phases. The level of compliance was considered sufficient if the patient had consumed ± 2*

probiotics (or placebo) tablets with respect to the dose prescribed at the beginning of the treatment phase.". IN THE RESULTS, WE HAVE REPORTED INFORMATION ON THE LEVEL OF PATIENT COMPLIANCE: "*Based on the number of empty lozenges returned at the end of the treatment phases, all patients were compliant with probiotics and placebo intake.*".

2. Why was the BoP parameter and not the modified Gingival Index (Mombelli et al. 1987) used to assess inflammation of the marginal mucosa? Are the authors confident that BoP reflects the changes in inflammation of the marginal mucosa following delivery of therapy?

WE AGREE WITH THE REFEREE THAT THE CHOICE OF BOP AS TREATMENT OUTCOME MUST BE SUPPORTED WITH ADDITIONAL CONSIDERATIONS. IN ORDER TO ADDRESS THE REFEREE'S ISSUE, WE HAVE IMPLEMENTED THE DISCUSSION WITH AN ADDITIONAL PARAGRAPH: "*BoP was used as the primary outcome variable. Due to its correlation with mucosal inflammation as assessed at the histological level (Lang et al. 1994), BoP is currently recognized as a key parameter to distinguish between peri-implant health and disease (Jepsen et al. 2015). Moreover, the available evidence seems to indicate that peri-implant BoP has a prognostic value, its presence (or absence) being associated with the deterioration (or stability) of peri-implant conditions overtime (Luterbacher et al. 2000). For this reason, BoP is also commonly used as a treatment outcome in the management of peri-implant diseases, in general, and p-iM, in particular (Schwarz et al. 2015).*".