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Abstract: Objective. To evaluate the clinical effect of a probiotic mouthwash in reducing generalized marginal chronic gingivitis using positive and negative control groups. Methodology. Four-week study conducted in San Luis Potosí, Mexico, from January to March 2017. Participants were healthy, non-smokers with generalized marginal chronic gingivitis; age range 18-45 years. Subjects were randomized and divided into three groups: Group A: mouthwash based on 0.05% cetylpyridinium chloride (CPC) (positive control); Group B: mouthwash based on probiotics (experimental); Group C: placebo mouthwash (negative control). No oral hygiene practices or routines were modified; subjects were followed for 4 weeks. The primary outcome variable of interest was the Löe and Silness gingival index, and the secondary one, the Quigley Hein plaque index modified by Turesky. Results. Of the 45 patients included, 19 (42.2%) were men and 26 (57.7%) women, mean age was 22.8±2.07. Each group consisted of 15 subjects; all subjects completed the study. There was no statistically significant reduction in gingival inflammation when comparing the 3 treatment groups (p=0.540) with respect to the gingival index. A comparison was made before and after the treatment and in the 3 groups there was no reduction of the gingival inflammation. Plaque reduction was not statistically significant when comparing the 3 groups (p=0.278). However, when doing intra-group comparison, it was found that the patients in group A had a reduction in plaque index (p<0.005), which was not observed in groups B (p=0.1103) and C (p=0.1508). Conclusions. The use of a probiotic mouthwash did not reduce gingival inflammation or the accumulation of dentobacterial plaque in a period of 4 weeks. There were no statistically significant differences between the study groups.

Keywords: dental plaque; gingivitis; probiotics; periodontal diseases; mouthwashes.

INTRODUCTION.

Gingivitis is the initial stage of periodontal disease. It is related to a dysbiotic state of the biofilm bacteria accumulated on the dental surface near the gingival sulcus.1 Its main clinical manifestation is inflammation,2,3 which is related to an increase in the number of pathogenic bacteria such as Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythia, although these are also found, in lower numbers, in healthy individuals. Given its characteristics, treatment based on basic cleaning and on an improvement in oral hygiene tend to reverse the development and progression of gingivitis before it causes irreversible damage.4 However, it has been reported that tooth
brushing alone is not completely effective to significantly remove the dental biofilm. Consequently the use of various antimicrobials in the form of mouthwashes, gels or pastes has been proposed, to complement tooth brushing. In this sense, cetylpyridinium chloride (CPC), a quaternary ammonium compound, has been clinically proven to be safe and effective in concentrations 0.045 to 0.1%, it has a prolonged effect on the oral cavity as it remains attached to the glycoproteins that cover teeth and the oral mucosa. Some authors have shown that it has activity against gram positive bacteria, fungi and yeasts at 0.05%. However, eliminating both pathogenic and commensal bacteria with the use of antimicrobials perpetuates the imbalance of the oral microbiota.

New preventive and therapeutic approaches based on restoring the balance of the dental biofilm seem to be a better option. Shimauchi et al. found that the administration of the probiotic strain Lactobacillus salivarius WB21 to smokers with periodontal disease significantly reduced gingival sulcus depth and plaque index compared to a placebo group. Probiotic bacteria administered in sufficient quantity contribute to periodontal health through different mechanisms: 1) they interact directly with the pathogenic microbial agents neutralizing them, 2) they occupy niches, preventing colonization by pathogens, and 3) they modulate the inflammatory response efficiently. On the other hand, Alkaya et al., evaluated a mixture of the three probiotic strains Bacillus subtilis, Bacillus megaterium, and Bacillus pumulus in patients with generalized gingivitis and found that there was no reduction in the clinical parameters under study after 8 weeks of treatment.

The effectiveness of probiotics is determined by the bacterial strain, as well as the dose and administration route; for this reason, results reported in the literature are inconclusive. Multispecies probiotics can be a good alternative to recover the balance of the dental biofilm.

The aim of this pilot study was to determine the clinical effect on generalized marginal chronic gingivitis of ProBiseis™, a mixture of six probiotic strains (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium infantis and Streptococcus thermophilus) as a mouthwash.

MATERIALS AND METHODS.

Study design and subjects
Randomized pilot study based on the CONSORT 2010 guidelines extended to pilot studies. A population of 18-45 year-old subjects with a diagnosis of generalized marginal chronic gingivitis were included in the study. Subjects agreed to participate by signing an informed consent. They had available time, good general health status, no history of allergies to the components used in this study, were non-smokers, with 1.0 in Löe and Silness gingival index and Quingley Heinn plaque index modified by Turesky. The study was carried out for 4 weeks at the Periodontics Clinic of the School of Stomatology at Universidad Autónoma de San Luis Potosí (UASLP), San Luis Potosí, México. Forty-five subjects were included for convenience from January to March 2017.

Intervention
The three study groups were distributed by simple randomization as follows: Group A, mouthwash based on 0.05% CPC as a positive control (Plax™, Colgate Palmolive); Group B, mouthwash based on probiotics as experimental group (ProBiseis™, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium infantis, and Streptococcus thermophilus, Solanum laboratories); Group C, placebo mouthwash (saline solution with flavor). Each subject was instructed verbally and in writing on how to use the mouthwash. They were asked to use the product daily (Group A and C: two times a day, keeping it for 1 minute in the mouth before spitting, after teeth brushing; Group B, twice a day after teeth brushing, by dissolving the content in 50ml of water and keeping the suspension for 2 minutes in mouth before spitting). Solutions and probiotics were provided for 15 days only, without displaying trademarks, in order to keep subjects blind to the components. No additional instructions were given on tooth brushing techniques or the use of toothpaste, nor were there restrictions on diet during the study.

Measurements
The primary and secondary output variables recorded were: Löe and Silness gingival index and Quingley Heinn plaque index modified by Turesky. The evaluation of clinical parameters was performed by a
dentist specialized in periodontics and blinded to each treatment group. Calibration of the evaluator was carried out before the study to verify the intra- and inter-examiner reproducibility; a correlation of 0.98 was obtained. Each subject completed three visits: the first one corresponded to day 0, informed consent was obtained, evaluation of the soft and hard structures of the oral cavity was carried out, basal measurements were made and indications were given. Second visit, at day 15, the content of the mouthwash bottles was verified to validate the use of the product, indications of use were repeated, any doubts regarding its use were resolved, and the product was given again for another 15 days. Third visit, at day 30, the final measurements of the gingival and plaque indices was performed. Patients were selected consecutively and for convenience in the period January-March of 2017.

Statistical analysis
Descriptive statistics calculations, using measures of central tendency for continuous variables and percentages for nominal variables, were performed. Normality of the data was checked by the D’Agostino-Pearson test and, according to the results, a t-paired test was used to assess the efficiency of the mouthwashes before and after the treatment. The Kruskal-Wallis test was used to evaluate differences between the groups; \( p<0.05 \) was considered statistically significant.

Figure 1. Study design and sample selection.

**Figure 2.** Comparison of the gingival index after 4 weeks of intervention according to the treatment.

**Figure 3.** Comparison of the posterior plaque index after 4 weeks of intervention according to the treatment.

Table 1. Demographic characteristics of the participants according to group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive Control</th>
<th>Experimental Probiotic</th>
<th>Negative Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cetylpyridinium (n=15)</td>
<td>Group (n=15)</td>
<td>Placebo (n=15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N%</td>
<td>N%</td>
<td>N%</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>7(15.5)</td>
<td>6(13.3)</td>
<td>6(13.3)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8(17.7)</td>
<td>9(20.0)</td>
<td>9(20.0)</td>
</tr>
<tr>
<td></td>
<td>Med ± SD</td>
<td>22.6 ±2.5</td>
<td>22.8 ±1.6</td>
<td>23.07 ±2.08</td>
</tr>
</tbody>
</table>

**Table 2.** Comparison of the initial and final gingival index in the three groups evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IGI* (n=15)</th>
<th>FGI* (n=15)</th>
<th>p&lt;0.05*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (positive control)</td>
<td>1.18 ± 0.20</td>
<td>0.98 ± 0.40</td>
<td>p=0.111</td>
</tr>
<tr>
<td>Group B (experimental group)</td>
<td>1.24 ± 0.30</td>
<td>1.14 ± 0.03</td>
<td>p=0.38</td>
</tr>
<tr>
<td>Group C (negative control)</td>
<td>1.2 ± 0.20</td>
<td>1.08 ± 0.40</td>
<td>p=0.70</td>
</tr>
</tbody>
</table>

*Initial gingival index. & Final gingival index. + t-paired.

**Table 3.** Comparison of the initial and final plaque index in the three groups evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IPI* (n=15)</th>
<th>FPI* (n=15)</th>
<th>p&lt;0.05*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (positive control)</td>
<td>1.38 ± 0.25</td>
<td>1.05 ± 0.38</td>
<td>p=0.009</td>
</tr>
<tr>
<td>Group B (experimental group)</td>
<td>1.5 ± 0.35</td>
<td>1.3 ± 0.42</td>
<td>p=0.380</td>
</tr>
<tr>
<td>Group C (negative control)</td>
<td>1.5 ± 0.60</td>
<td>1.3 ± 0.60</td>
<td>p=0.700</td>
</tr>
</tbody>
</table>

*Initial plaque index. & Final plaque index. + t-student test ^ U-Mann-Whitney test

**RESULTS.**

The total population evaluated consisted of 45 patients, randomization and treatment received by each group is shown in Figure 1. The demographic characteristics of the population are shown in Table 1. The baseline measurement of the gingival and plaque indices in the three groups were homogeneous; there were no statistically significant differences (p=0.1153, p=0.6480 respectively). None of the patients reported adverse effects and, likewise, no adverse effects were identified by the researcher at the end of the 4th
week of treatment.

Regarding the primary outcome variable, gingival inflammation, Table 2 shows the intra-group comparison, before and after the intervention with the different treatments. There was no statistically significant difference in any of the groups in the reduction of the gingival index. However, in Group A, a gingival index reduction of 1.18 to 0.98 was observed ($p=0.112$), progressing from slight inflammation to absence of inflammation. Figure 2 shows the inter-group comparison, in which group A is identified as having a greater reduction in the gingival index; however, this difference is not statistically significant with respect to the other treatment groups.

The intra-group comparison regarding plaque index, the secondary output variable, is shown in Table 3, where it can be observed that in Group A there was a statistically significant reduction in the plaque index, but not in Groups B and C. Figure 3 shows the inter-group comparison after 4 weeks of treatment. There was no statistically significant difference with respect to the reduction of the plaque index; however, in Group A, a greater reduction is observed in comparison with Groups B and C.

The treatments had a greater effect on plaque reduction than on reducing gingival inflammation.

**DISCUSSION.**

The effectiveness of probiotics use in the treatment of gingivitis is still debatable. Some authors support its effectiveness, while others have not reported any conclusive effects. This study evaluated the effectiveness of a mouthwash, ProBiseisTM, composed of six bacterial strains (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium infantis and Streptococcus thermophilus) as an adjuvant treatment to the routine teeth brushing in patients with generalized marginal chronic gingivitis. The mouthwash was kept in the subjects’ mouths for only two minutes. For the evaluation, two widely validated indices were used, Löe and Silness gingival index and Quingley Heinn plaque index modified by Turesky. These allowed for the degree of gingival inflammation and the amount of dental biofilm accumulated per subject to be determined, by accurately measuring its reduction. Regarding the primary output variable, no inter-group and intra-group differences were found after 4 weeks of using the mouthwashes.

As only patients with mild gingivitis were evaluated in this study, reductions in the indices are discrete; however, they are statistically significant for CPC regarding reduction of plaque, but not gingival inflammation. The latter may be due to the design of the study, since the participants’ brushing technique was not modified, to avoid changing their daily hygiene habits. On the other hand, the period of time the mouthwash was kept in mouth may not have been sufficient to achieve an adequate colonization. Likewise the length of the intervention may have not been enough (4 weeks), since reduction in gingival inflammation has been reported after using probiotics for 8 weeks. To remedy these limitations, there are plans to conduct this study for a longer period of time. Another factor that may have influenced results was the use of mouthwashes. Subjects were instructed about their administration at the beginning of the study.

Then it was corroborated that the containers were empty when they came back for the checkup. Then the product was restocked, however, it is difficult to ascertain if the subjects followed the instructions correctly. The probiotic mouthwash used in this study did not reduce mild chronic marginal gingivitis or the associated dental biofilm. However, it is important to consider other studies where its efficacy has been demonstrated in the treatment of severe gingivitis. The effectiveness of probiotic strains could be due to the prevention of pathogenic bacteria from adhering to dental and gingival surfaces. In addition, these strains modify the composition of salivary proteins, balance the oral pH and improve the expression of helper T cells. The expected outcome was not reached due to the following factors: a) the probiotic strain(s) used; b) the dose or concentration of these, c) the administration route and form of application, and d) the length of administration.

Although the use of probiotics has increased in recent years and several lines of research have been generated, in Mexico there are not enough commercially available options, either in the form of chewable tablets, mouthwashes or gels. In addition, there are no studies evaluating the effect of specific species or bacterial
mixtures in the Mexican population. Therefore it was considered that it would be a contribution to evaluate the mixture of probiotic strains present in ProBiseis™, dissolved in running water and used as mouthwash twice a day and kept in the mouth for 2 minutes in a similar way to commercial antimicrobial mouthwashes. Few authors have conducted similar studies so literature regarding this protocol is scarce.¹⁸

It is important to evaluate in clinical trials new and better probiotic strains isolated from the oral cavity, different means and alternatives to achieve appropriate contact time so they become active in saliva and on the structures of the mouth. A product containing oral probiotic strains may have better results in maintaining or restoring the balance of the dental biofilm, since the effect of probiotics is niche- and species-specific as demonstrated by Shimauchi et al., these authors studied patients with moderate to severe gingivitis, who were given tablets of Lactobacillus salivarius WB21 for 8 weeks, 3 times a day. After the trial, patients had achieved a reduction in periodontal pocket depth, clinical insertion level and in dental biofilm.¹⁶ Krasse et al., also reported a reduction in the level of inflammation and dental biofilm using L. reuteri (LR-1 or LR-2) in patients with moderate to severe gingivitis.¹⁵

In 2011 Montero et al., reported the use of probiotics in dentistry stating that, despite the limitations of existing studies, clinical results seem to be more limited than laboratory microbiological results.¹⁷ Based on the above, it is possible to state that probiotics may be potentially beneficial as adjuvant therapy to periodontal treatment; however, the information resulting from clinical trials so far is limited to support this. On the other hand, some authors, such as Inesta et al., reported not having observed clinical changes in patients or in bacteriological samples between groups, using Lactobacillus reuteri and Lactobacillus salivarius against a placebo during a period of 8 weeks, partially agreeing with the results obtained in this study.²²

Finally, a reduction in gingival inflammation and biofilm formation in the placebo group was not observed, which indicates that the tooth brushing technique used by the subjects was not adequate, as it did not remove dental biofilm nor reduce gingival inflammation.

For the secondary output variable intra-group differences were evident in Group A, and based on the results obtained, the 0.05% CPC mouthwash used as a positive control significantly reduced dental biofilm, which agrees with the findings of Kozak et al., They reported a reduction in plaque accumulation of 55% when using a 0.05% CPC mouthwash + routine brushing compared to tooth brushing alone.¹⁹ Costa et al., on the other hand, found that CPC mouthwash at 0.05% and 0.07% have been shown to be safe and effective, with virtually no adverse effects, raising the possibility of using these routinely, making it the ideal product to use as a positive control.⁶,²⁰

One of the limitations of this study is the fact that it is not possible to make an analysis with greater precision since previous clinical studies evaluating multispecies probiotic mixtures have not been reported in the literature.

For that reason, this was considered a pilot study, with the aim of determining its feasibility and being able to later conduct an RCT. However, to demonstrate the effectiveness of this probiotic mixture based on observations that have been made in previous studies, 253 subjects per group are required to find statistically significant differences.¹⁸ Other possible limitations were that patients with mild gingivitis were included, the length of time that the mouthwash was used (2 minutes), as well as the duration of the intervention. For these reasons the evaluation of the same mixture of probiotics is planned, and measuring its impact in the periodontal treatment of patients with moderate to severe gingivitis, increasing the length of exposure to the probiotic in the mouth using, for example, a chewable pill or candy lollipop for 8 weeks.

**CONCLUSION.**

A mouthwash containing the probiotic strains: Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium infantis and Streptococcus thermophilus, did not reduce gingival inflammation or the accumulation of dental plaque in a period of 4 weeks. There were no statistically significant differences between the groups under study.
REFERENCES.