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Inhibitory effect of the methanolic fraction of Oxapampa propolis on different isolated genotypes of *Streptococcus mutans* in children with caries*

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ABSTRACT

Objective: To determine the inhibitory effect of the methanolic fraction of propolis from Oxapampa, Peru, on different genotypes of *Streptococcus mutans* in children diagnosed with caries. **Materials and methods:** 150 oral swab samples were collected, seeded for isolation of *S. mutans* and then identified by biochemical tests of carbohydrate fermentation, Api 20 Strep (bioMérieux) and conventional PCR. The 138 strains isolated and identified from patients and the *S. mutans* ATCC® 25175™ control were confronted with the methanolic fraction by the disk diffusion method, having as positive control 0.12% chlorhexidine and negative control Milli-Q water and DMSO (1:1). **Results:** It was found that the inhibition diameter of the strains extracted from children diagnosed with caries against the methanolic fraction showed greater diameter (14.13 mm) in relation to the strain of *S. mutans* ATCC® 25175™ (10.16 mm) with statistically significant differences. In the genotyping of the 138 strains with the different specific primers (c, e, f, and k), it was found that 63.77% belonged to genotype c, 21.73% to genotypes c and e, and 14.50% to genotypes c and f. **Conclusions:** The methanolic fraction of Oxapampa propolis presents inhibitory activity on *S. mutans* strains isolated and genotyped in children diagnosed with caries. In addition, it presents greater inhibitory activity in strains obtained from children compared to *S. mutans* ATCC® 25175™.

Keywords: *Streptococcus mutans*; propolis extract; dental caries; bacterial inhibition.

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INTRODUCTION

Dental caries is a bacterial etiology disease that constitutes a public health problem and is characterized by the destruction of dental tissues caused by acids produced by bacteria in the dental plaque. The main microorganisms involved are *Streptococcus mutans* and *S. sobrinus*, although *Lactobacill*us and *Actinomyces* also participate. Of these, the first one is a pathogen most associated with caries (1), since it causes tooth enamel demineralization and, in some cases, even its loss. This is due to an imbalance in the composition of dental enamel as a consequence of carbohydrate metabolism, which produces acids, as well as the activity of the bacterial biofilm formed, leading to pain, infection or tooth loss (2-5).

S. mutans is a Gram-positive diplococcus, arranged in chains, facultative anaerobe, catalase negative and non-motile. It can produce lactic, formic and propionic acids due to the fermentation of glucose, lactose, sucrose, raffinose, mannitol, inulin and salicin, which reduces the pH from 7 to 4.2 in approximately 24 hours, thereby causing tooth enamel demineralization (6). In culture media such as mitis salivarius agar (MSA), enriched with bacitracin (200 U/uL), 1% potassium tellurite, and 10% sucrose, *S. mutans* colonies exhibit a mucoid, convex morphology with clear edges and a dark spot. This species has been subclassified serologically into several serotypes based on its immunological and biological properties, the main ones being c, e, f and k (6, 7).

Research suggests that serotype c is the progenitor of *S. mutans*, while e and f would have emerged from mutations (7). These serotypes are composed of a rhamnose backbone and glucose side chains (8). Regarding the k genotype, recent studies indicate that it is distinguished by a significant reduction in the amount of glucose side chains, resulting in lower cariogenicity due to alterations in several surface protein antigens (8). In addition, it exhibits greater survival in the bloodstream due to its lower susceptibility to phagocytosis (9).

On the other hand, propolis is a natural resinous and sticky substance collected and processed by *Apis mellifera* bees from tree and plant exudates (10). This substance has been widely recognized for its antiseptic properties, due to its antibacterial, antiviral and antifungal activity. Its most prominent active component, caffeic acid phenethyl ester, is responsible for its antimicrobial and anti-inflammatory effects. In addition, it has immunostimulant, antiallergic, remineralizing and antioxidant properties.

Numerous scientific studies have identified more than 100 components of propolis that act synergistically, with flavonoids being the most relevant due to their biological and therapeutic activity (11). Some authors suggest that this substance inhibits the enzymatic activity of various proteins essential for the growth and development of oral microorganisms responsible for dental caries, such as *S. mutans* and, to a lesser extent, *Lactobacillus acidophilus* (12, 13). Furthermore, other studies have shown that the methanolic extract of cinnamon and clove has antibacterial activity against microorganisms (14). Similarly, the methanolic fraction of Oxapampa propolis showed *in vitro* activity against the biofilm of *S. gordonii* ATCC ® 51656™ and *F. nucleatum* ATCC® 10953™ (15).

In the present study, we aimed to determine the inhibitory effect of the methanolic fraction of propolis from Oxapampa, Peru, in different genotypes of *S. mutans* isolated from children with dental caries.

MATERIALS AND METHODS

This study is an *in vitro* experimental study. The collection of samples from children with dental caries was conducted with the approval of the Institutional Research Ethics Committee of Universidad Peruana Cayetano Heredia (CIEI-UPCH), under Certificate No. 324-13-18 and Registration Code No. 102214, dated 18 June 2018. The sample size was calculated using EPIDAT v. 3.1. A total of 250 children who attended the UPCH Teaching Dental Center for the first time were included, with a 95% confidence interval and an expected proportion of 70%.

A total of 250 children without prior treatment for caries were recruited. From this group, 150 children of both sexes, between the ages of 6 and 12 were selected. Each of them underwent a swabbing of the buccal mucosa and a scraping of the soft dental plaque from a caries-affected tooth (with at least one lesion), during August 2018. The samples were transported in test tubes containing 3 mL of thioglycolate medium (Merck® 108190) to the bacteriology laboratory of the Research and Development Laboratories at UPCH, where they were incubated at 37 °C for 48 hours.

Isolation and identification of *S. mutans*

Seeding was performed on mitis salivarius (MS) agar with bacitracin (200 U/µL), 1% tellurite and 10% sucrose for 48 hours under anaerobic conditions at 37 °C. Subsequently, mucoid, convex colonies with light clear edges and a dark spot, characteristic of

S. mutans, were identified. Each colony was transferred to a brain-heart agar (BHA) medium and incubated at 37 °C for 24 hours for maintenance and subsequent identification. Identification was performed using Gram staining, catalase testing and carbohydrate fermentation, including sorbitol, mannitol, raffinose and sucrose. To confirm the presence of *S. mutans*, the Api20 Strep (bioMérieux®) identification system was used. Colonies were preserved in brain-heart infusion medium (BHI, Merck®). Genotypes were determined by conventional PCR, using *S. mutans* genotype c (ATCC® 25175™) as the control strain. The GenElute Bacterial Genomic DNA kit (Merck®) was used for genomic DNA extraction®. The identification of the *S. mutans* species was performed using smut-specific primers, followed by genotyping with c, e, f and k primers (Table 1) (16).

Table 1. Primers used for the identification of *Streptococcus mutans* genotypes.

Genotype	First	Pb
Streptococcus mutans	SMUT5: TGA AAC CTT GTC TAT CTC CTC TTT ACC SMUT3: TCA GTT TTC AAA GGG CTC TG	137 pb
c	SC-F CGG AGT GCT TTT TAC AAG TGC TGG SC-R AAC CAC GGC CAG CAA ACC CTT TAT	727 pb
e	SE-F CCT GCT TTT CAA GTA CCT TTC GCC SE-R CTG CTT GCC AAG CCC TAC TAG AAA	517 pb
f	SF-F CCC ACA ATT GGC TTC AAG AGG AGA SF-R TGC GAA ACC ATA AGC ATA GCG AGG	316 pb
k	CEFK-F ATT CCC GCC GTT GGA CCA TTC C K-R CCA ATG TGA TTC ATC CCA TAC C	294 pb

The 150 *S. mutans* strains were amplified using smut in a thermocycler under a 25-cycle program, which included denaturation at 96 °C for 15 seconds, hybridization at 61 °C for 30 seconds and extension at 72 °C for 1 minute (Figure 1) (17).

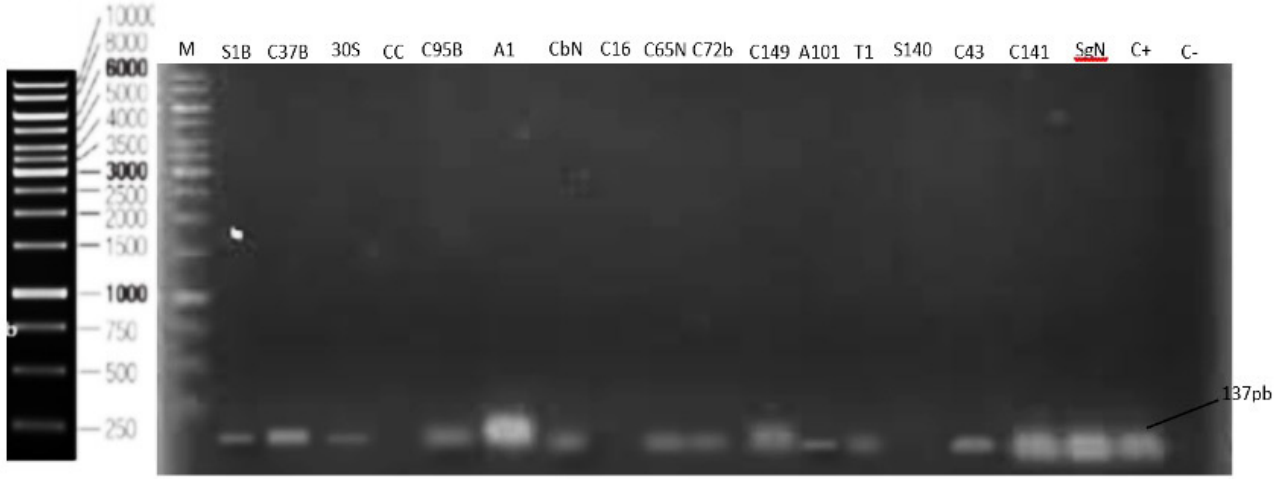

Figure 1. 1% agarose gel electrophoresis of amplification products with smut primers. Fragment of 137 pb.

For the genotyping of the strains, primers c, e and f were used, which were amplified in the thermocycler for 25 cycles, with denaturation at 96 °C for 15 seconds, hybridization at 61 °C for 30 seconds and extension at 72 °C for 1 minute. For genotype k, a 25-cycle protocol was performed with denaturation at 95 °C for 30 seconds, hybridization at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds (Figure 2) (18).

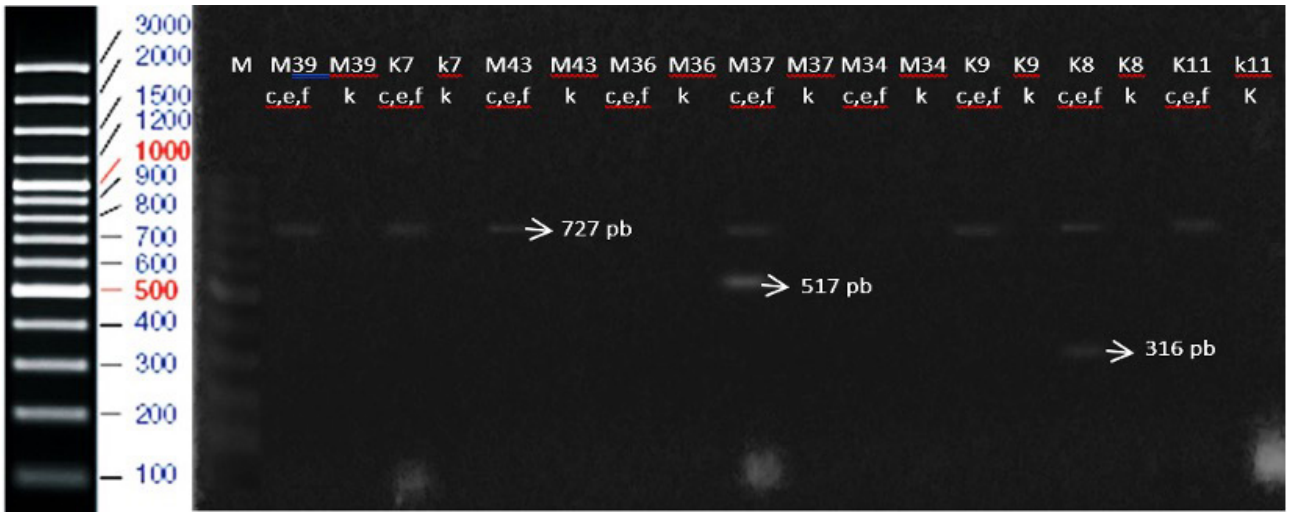

Figure 2. 1% agarose electrophoresis of amplification products with primers c, e, f and k. Fragment of 727 pb genotype c, 517 pb genotype e, and 316 pb genotype f.

Subsequently, for species determination and genotyping, PCR products were placed in a 1% agarose gel with tris-acetate-EDTA buffer (TAE 1X), and electrophoresis was performed at 94 V for 1 hour. The bands were revealed with 1% ethidium bromide and were visualized with a UV transilluminator (High Performance UV Transilluminator TFM-40V model), comparing them with the control. Finally, 40 mL of the methanolic fraction of propolis was used at a concentration of 0.78 mg/mL, provided by the Bacteriology Laboratory of UPCH. This fraction was obtained through the methanolic extraction of propolis from Oxapampa, in the city of Cerro de Pasco, Peru (15).

Determination of the inhibitory activity of genotyped *S. Mutans* strains

Genotyped strains and *S. mutans* ATCC® 25175™ were cultured in BHI medium for 24 hours at 37°C under microaerophilic conditions. The antibiotic susceptibility test was applied using the disk diffusion method to evaluate the inhibitory activity of the methanolic propolis extract (19). The genotyped strains and the *S. mutans* ATCC® 25175™ strain were adjusted to a turbidity equivalent to the 0.5 McFarland scale. Subsequently, they were seeded in BHA medium (Merck®), and 6 mm filter paper discs (Whatman 3) impregnated with 10 µL 0.12% chlorhexidine (positive control), 10 µL of DMSO and Milli-Q water (1:1) as negative control, and 10 µL of the propolis methanolic fraction at a concentration of 0.78 mg/mL were placed. The plates were incubated at 37 °C for 48 hours under microaerophilic conditions. Finally, the inhibition halos were measured in millimeters (mm) using a calibrated Truper® vernier.

Statistical analysis

The data obtained from the conventional PCR test were analyzed using descriptive statistics. The results of inhibition halos were presented in terms of means, standard deviations and medians. The Student's t test was applied with a 95% confidence level (CL = 95%) and an α error = 0.05 to compare the different serotypes of *S. mutans* between the control strain *S. mutans* ATCC ® 25175™ and the samples of children with dental caries.

RESULTS

Of the 150 samples collected from children diagnosed with caries, 138 strains of *S. mutans* were identified using the smut-specific primer (Figures 1 and 2). These strains were then genotyped using the different specific primers (c, e, f and k). It was found that 63.77% belonged to genotype c; 21.73% to genotypes c and e; and 14.50% to genotypes c and f, indicating the presence of multiple genotypes and the absence of genotype k (Figure 3).

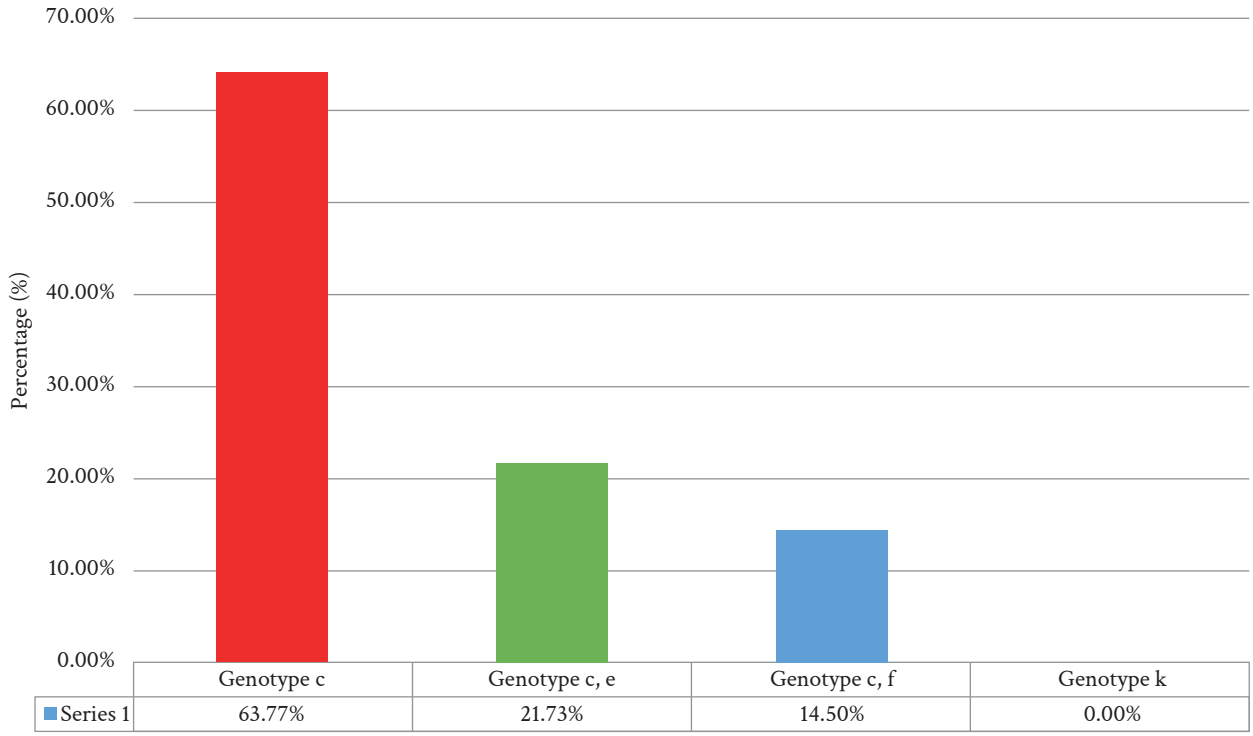

Figure 3. Percentage of individual and multiple genotypes found in the study.

Table 2 shows the mean, standard deviation and median of the inhibition halos for *S. mutans* ATCC® 25175™ and the strains from pediatric patients diagnosed with dental caries. It was observed that the methanolic fraction of propolis responded similarly to chlorhexidine against *S. mutans* strains isolated from children with dental caries. Additionally, it was also found that the inhibition diameter of the extracted strains against the methanolic fraction showed greater diameter (14.13 mm) in relation to the *S. mutans* ATCC ® 25175™ strain (10.16 mm) with statistically significant differences.

Table 2. Comparison of the mean, standard deviation and median of the inhibition halos (in millimeters) of *Streptococcus Mutans* ATCC ® 25175™ and strains from pediatric patients diagnosed with dental caries.

Impregnated substances	*Streptococcus mutans* strains					
	S. mutans ATCC® 25175™			Genotyped strains of *S. mutans*		
	Mean	SD	Median	Mean	SD	Median
DMSO + Milli Q water	0.00	0.00	0.00	0.00	0.00	0.00
Chlorhexidine	14.83	0.28	15.00	10.16	0.28	10.00
Methanolic fraction	16.83	5.35	15.50	14.13	5.27	11.50
p value*		0.005			0.850	

*Student's t test with CL = 95%.
*One p value < 0.05 indicates statistical significance.
SD: standard deviation.

DISCUSSION

Several studies have linked tooth caries with the presence of *S. mutans* (20). Children under 12 years old are the most affected, largely due to a higher consumption of carbohydrates, which leads to acid production that can cause caries. Currently, different plant extracts and natural products are being investigated to evaluate their inhibitory effect on *S. mutans*, as well as their prevalence and circulating genotypes. Among the genotypes of this microorganism, c, e, f and k are the most notable. In the

study by Momeni et al. (21), conducted in 129 patients, genotypes c and k were found to be the most prevalent, followed by genotypes e and f. However, in this study, whose sample consisted of 138 patients under 12 years old, genotype c was the most frequent, followed by genotypes e and f, while genotype k was not detected. Momeni et al. (21) suggest that the presence of genotype k is related to strains containing collagen-binding proteins (CBP, Cnm and Cbm), which are associated with systemic diseases.

The identification of the *S. mutans* species, using conventional PCR in soft dental plaque in the cheek, had a prevalence of 92.00% (138 out of 150 children), which was higher than that reported by Delgadillo et al. (22), in Peru, who found 75.60% (59 out of 78 children) in saliva. Sánchez-Pérez & Acosta (23) in Mexico found a prevalence of 32% bacterial plaque of the fissure, while Arévalo-Ruano et al. (24) in Colombia reported a 14.90% prevalence in saliva. This difference may be due to the multiple techniques used for sampling, isolation and identification, as well as diet, antibiotic consumption, carious lesions, and the classification of the different types of caries present in children.

Several studies indicate that genotype c of *S. mutans* is the main bacterium associated with caries (7, 25, 26), which aligns with the findings in this study. Additionally, other research studies have found genotypes e in 20%, f in 10%, and k in less than 5% (27), results that are consistent with this study, which found genotypes c and e in 21.73%, genotypes f in 14.50% and no genotypes k were observed. Furthermore, the previously mentioned study found that more than half of a population of 129 children presented multiple genotypes, with the most frequent being c and k at 31%, followed by c, e and k at 11%, and c and e at 7.75% (27), which is consistent with the findings of this study.

The inhibition halos generated by the methanolic fraction of propolis against *S. mutans* showed a larger diameter compared to those observed in the *S. mutans* ATCC® 25175™ strain. This could be attributed to the characteristics of methanolic fractions because, according to Galgowski et al. (28), this fraction could contain a higher concentration of non-polar bioactive compounds with higher antimicrobial activity.

Among the main limitations of this study, it is highlighted that genotyping was conducted during the pandemic period, which caused logistical difficulties with suppliers due to the shortage of supplies. The delivery times of orders reached up to six months to arrive in Peru, which significantly delayed the process. At the same time, the isolation of *S. mutans* presented further challenges, as this bacterium can easily be confused with *Streptococcus sobrinus*, which required the use of advanced molecular techniques to ensure adequate and accurate identification.

CONCLUSIONS

The methanolic fraction of Oxapampa propolis exhibits inhibitory activity in isolated and genotyped *S. mutans* strains in children diagnosed with dental caries, of which 100% belonged to genotype c, 21.58% to genotype e, and 14.39% to genotype f, with no genotype k detected. In addition, the methanolic fraction of Oxapampa propolis shows higher inhibitory activity against the strains obtained from children, compared to *S. mutans* ATCC® 25175™.

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