PHYSICOCHEMICAL SALIVARY PROPERTIES, *LACTOBACILLUS*, MUTANS STREPTOCOCCI COUNTS AND EARLY CHILDHOOD CARIES IN PRESCHOOL CHILDREN OF COLOMBIA

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ABSTRACT

Dental caries is a multifactorial disease, in which saliva has an important role. Saliva properties affect the growth of cariogenic microorganisms, so variations below threshold levels are considered risk factors for the development of dental caries. Salivary tests and microbiological counts of cariogenic bacteria are often used for clinical purposes in order to reveal problems that could explain caries development and have been evaluated in multiple studies, but these studies have mainly been carried out on adults. Thus, the aim of this study was to identify physicochemical salivary properties, mutans streptococci and Lactobacillus counts among preschool children, and their relationship with ECC in a cross-sectional study in Medellín, Colombia. Caries was determined using ICDAS criteria for a sample of 201 preschool children. Stimulated saliva samples were processed for bacterial detection, mean flow rate, pH and buffer capacity calculation. Risk variables for ECC were assessed individually and adjusted using a binary logistic regression model. The results showed that 71.14% of study subjects had ECC. Flow rate, final pH and Buffer capacity increased with age, but none of them were related to the presence of ECC. Although OR analysis detected correlations between ECC, gender, age, dental eruption stage and Lactobacillus presence, binary logistic regression analysis only showed gender and Lactobacillus as strongly associated with ECC. It can be concluded that gender and Lactobacillus presence are key variables for the presence of ECC in these children; whilst age and dental eruption stage play a minor role. Further studies are required to clarify the role of mutans streptococci counts in this population and to establish normal salivary parameters as well as threshold levels for hyposalivation in preschool children, as results showed differences with normal parameters used for adults.

Key words: dental caries, saliva, Streptococcus mutans, Lactobacillus, preschool children.

CARIES DE LA INFANCIA TEMPRANA, PROPIEDADES FISICOQUÍMICAS DE LA SALIVA Y RECUENTO DE ESTREPTOCOCOS DEL GRUPO MUTANS Y *LACTOBACILLUS* EN NIÑOS PRE-ESCOLARES DE COLOMBIA

RESUMEN

La caries dental es una enfermedad multifactorial en la cual la saliva tiene un impacto importante. Las propiedades de la saliva afectan el crecimiento de microorganismos cariogénicos y por lo tanto las variaciones por debajo de los niveles umbral son consideradas factor de riesgo para el desarrollo de caries. Las pruebas salivares y los recuentos de microorganismos cariogénicos son utilizadas con frecuencia en la clínica para identificar factores que puedan explicar el desarrollo de lesiones cariosas y han sido evaluados en múltiples estudios, pero éstos han sido desarrollados principalmente en adultos. El objetivo de éste trabajo fue identificar propiedades fisicoquímicas de la saliva y recuentos de estreptococos del grupo mutans y Lactobacillus, en relación con la Caries de la Infancia Temprana (CIT). Para esto, se realizó un estudio descriptivo transversal. Se registraron las lesiones de caries dental con el sistema ICDAS en 201 niños pre-escolares de hogares infantiles de estrato socioeconómico medio-bajo. Se obtuvieron muestras de saliva estimulada, que fueron procesadas para detectar estreptococos del grupo mutans y Lactobacillus; se calculó la tasa de secreción salivar, pH y capacidad Buffer. Se analizaron posibles variables indicadoras de riesgo para CIT, mediante el cálculo de OR individuales por variable y OR ajustadas mediante un modelo de regresión logística binario. Los resultados indicaron que el 71.14% de los niños presentaban CIT. Las propiedades de la saliva mostraron un mejor comportamiento con la edad, con valores diferentes a los parámetros definidos para poblaciones adultas, pero ninguna tuvo relación con la enfermedad. El género y la presencia de Lactobacillus mostraron asociación fuerte en el análisis de regresión sugiriéndolos como principales indicadores de riesgo para CIT en esta población. Se requieren estudios prospectivos y analizar variaciones biológicas, para clarificar el papel de los estreptococos del grupo mutans y revisar los valores umbral para la evaluación de la secreción salivar en preescolares.

Palabras clave: caries dental, saliva, Streptococcus mutans, Lactobacillus, niños preescolares.

INTRODUCTION

Early Childhood Caries (ECC)[1] constitutes a burden for the dental profession and public health administrators, as many authors report that the situation in primary dentition has not improved over the last decades, impacting children's quality of life and future oral health[2-4]. Biological as well as socioeconomic factors are related to the development of dental caries, and are both relevant in understanding the disease process and designing individual or population-oriented preventive approaches.

The cariogenic capacity of oral microorganisms is essential, but does not alone determine the presence of dental caries. It is necessary to have appropriate substrates and host-related physiological conditions to enable colonization and survival, creating an imbalance that facilitates the development of caries lesions[5]. Saliva has an important impact, through functions relying on physicochemical characteristics such as flow rate, pH and buffering capacity, so variations under threshold levels are considered risk factors for the development of dental caries[6].

Salivary tests and microbiological counts of cariogenic bacteria are used for clinical and research purposes[7]. However, studies of salivary flow rate, pH and buffer capacity have mainly been carried out on adults and there are limited data on stimulated salivary flow rates in children, especially in younger children, although there is a clear relationship with dental caries[8-10]. In Colombia, there are no available data on dental caries in preschool children, although the reduction of dental caries levels is more evident in permanent than in primary dentition. The latest National Oral Health Survey showed that 60.4% of five-year old children had experienced dental caries[11], and recent studies show no improvement[12].

The aims of this study were: (1) to identify stimulated salivary flow rate, buffer capacity, pH and, bacterial counts of mutans streptococci and *Lactobacillus,* among a preschool children population and (2) to establish their correlation with ECC.

MATERIAL AND METHODS

Population and sample

The sample was taken from children at five kindergartens selected from among a total of twelve centers located in northeastern zone of the city of Medellín, Colombia, South America. These private institutions receive government aid for attending to children living in an urban area with middle and middle-low socioeconomic conditions, according to the classification by the Mayor of Medellín, taking into account housing characteristics. Saliva samples were taken and clinical evaluations performed on 201 preschool children. This study was approved by the Ethics Committee at Faculty of Dentistry of Antioquia University, and children's parents submitted written informed consent.

Clinical examination

Caries levels were recorded using the International Caries Detection and Assessment System (ICDAS) criteria. A single examiner was trained by a pediatric dentist not belonging to ICDAS Committee, who was trained by an ICDAS Coordinating Committee member. The training included theoretical and clinical sessions. Cohen's Kappa were 0.72 (IC 0.65 – 0.78) inter-examiners and 0.77 (IC 0.70 – 0.83) intra-examiner. The examination took place in a portable dental chair under halogen light, installed at the selected centers. Teeth were cleaned, dried, and cotton rolls were placed to isolate the examination area; visual inspection was performed with a plain mouth mirror and WHO 11.5 probe to check for surface discontinuity[13]. For classification of the study group according to the diagnosis of caries, levels 1 and 2 of ICDAS were taken together and included in the group of children with ECC. In other words, children diagnosed without dental caries had to be free of restorations, cavitated carious lesions and any white spot lesion suggestive of dental caries. ECC and severe caries were diagnosed following the criteria of the American Academy of Pediatric Dentistry. Tooth eruption stages were classified as incomplete in children with less than 20 temporary teeth present in mouth.

Saliva sampling

Stimulated salivary samples were collected between 8:00 a.m. and 11:00 a.m., at least one hour after a meal. Each child was seated in a school chair and the procedure was carried out under close supervision. Stimulation was induced by chewing 1 gr. of wax for one minute until soft. Then the child spit the saliva intermittently into a sterile 50-mL graduated plastic centrifuge tube (Greiner Bio-one®, Frickenhausen, Germany) for 5 minutes. After collection, samples were transported to the laboratory in refrigerated conditions, within the following 3 hours.

Saliva analysis

The total volume of saliva was calculated avoiding foam, with a sterile micropipette. Mean flow rate was calculated taking into account the total time of sample collection and the volume of saliva. The pH of the samples was measured in a digital pHmeter (Metrohm Ion Analysis, Sweden). One experienced examiner performed all the observations according to Ericsson's method[14]. Briefly, 1 mL of saliva was mixed with 3 mL of 0.005 N hydrochloric acid, subject to bubbling, and after 20 minutes, final pH values were determined again. Buffer capacity was calculated by the difference between initial and final pH.

For mutans streptococci and *Lactobacillus* counts, samples were vortexed for 30 sec (IKA® VibroFix, Staufen, Germany) at maximal setting. Then the samples were serially diluted from 10^{-1} to 10^{-6} with Brain Heart Infusion (Difco Laboratories, Detroit, MI). The 10^{-4}, 10^{-5}, and 10^{-6} dilutions were plated on Mitis Salivarius Agar (Difco, USA) supplemented with 0.001% potassium tellurite, 0.2 U/mL bacitracin (Sigma, USA), and 10% (w/v) sucrose (CJ Co., Korea) (MSB agar). To determine mutans streptococci, four typical colonies were randomly selected from the plate cultures of each child exhibiting bacterial growth after 48 hours of anaerobic incubation, based on their morphology, size, and color. Then they were identified using the API-20 STREP system (bioM'erieux Vitec, Inc., Hazelwood, MO). Rogosa agar was used to culture *Lactobacillus spp*[15]. The 10^{-3} dilution was plated and incubated anaerobically at 37°C for at least 48 hours. After incubation, agar plates were screened at 10-50X magnification using a Stemi 2000® stereomicroscope (Carl Zeiss, Oberkochen, Germany). CFU/mL of individual species were calculated by a single trained investigator (MM).

Statistical Analysis

Data collected were analyzed using Statistical Package for the Social Sciences (SPSS) 15.0® (SPSS, Chicago, IL, USA). The proportion of children with ECC and Severe Early Childhood Caries condition (S-ECC) was calculated taking into account the evidence of dental caries experience[16]. Demographic characteristics and cultured microorganisms were analyzed based on the frequency distributions and were compared to identify differences in the prevalence of ECC using two-sided Fisher's exact test for variables with two categories and Pearson's Chi square test (χ^2) for variables with more than 2 categories. To identify differences between age and levels of salivary factors such as salivary flow rate, initial salivary pH, final salivary pH and buffer capacity, Pearson's Chi square test was applied.

Median values and range were obtained for salivary flow rate, initial pH, final pH and buffer capacity according to the clinical groups for all subjects. The Kolmogorov-Smirnov test for large sample size did not show normal distribution, so variables were analyzed using a nonparametric Mann-Whitney *U*-test to compare between groups with and without ECC. Cultured microorganisms were categorized into four groups for mutans streptococci, according to CFU/mL, and two groups for presence or absence of *Lactobacillus*, to be entered in a logistic regression model.

Odds ratio (OR) with 95% confidence interval was calculated for variables related to dental caries development. Variables with OR values greater than 2, a confidence interval not including 1.0, and a *P*-value < 0.05, were considered risk indicator variables. Finally, a binary logistic regression model was used; each candidate risk variable was evaluated after adjusting for the other candidate risk variable and for potential confounding variables. In the stepwise logistic regression analysis, $P < 0.05$ was used as the entry criterion while $P > 0.10$ was the removal criterion. The Hosmer-Lemeshow goodness-of-fit test statistic was set at $P > 0.05$. Significance for all tests was established at a *P*-value < 0.05.

RESULTS

This study obtained information about salivary properties, cariogenic bacteria and ECC in 201 (97 female and 104 male) preschool children living in a middle-low and low income urban area in Medellín, Colombia. Demographic and clinical characteristics according to presence or absence of ECC in the study subjects are shown in Table 1.

Table 1: Demographic characteristics of the children and clinical findings (n=201). *

	Without ECC	With ECC	Total	*P-value*
Gender				**0.031†**
Female	35 (17.4)	62 (30.8)	97 (48.2)	
Male	23 (11.4)	81 (40.3)	104 (51.7)	
Total	**58 (28.8)**	**143 (71.1)**	**201 (100)**	
Age (years)				**<0.001‡**
3 or younger	27 (13.4)	25 (12.4)	52 (25.8)	
4	17 (8.5)	52 (25.9)	69 (34.4)	
5	14 (7.0)	66 (32.8)	80 (39.8)	
Total	**58 (28.9)**	**143 (71.1)**	**201 (100)**	
Tooth eruption stage				**<0.001†**
Complete	19 (9.5)	131 (65.3)	150 (74.8)	
Incomplete	39 (19.4)	12 (6.0)	51 (25.4)	
Total	**58 (28.9)**	**143 (71.3)**	**201 (100)**	

* Values are given as n (%) of subjects; † Two-sided Fisher's exact test
‡ Two-sided Chi square test

Table 2: Physicochemical salivary properties of the children by age (n=201).

Variable	Age					
	2	3	4	5	Total	*P*-value
Salivary Flow rate						**<0.001†**
Low < 0.5	19 (90.5)	22 (71.1)	51 (73.9)	35 (43.8)	127(63.2)	
Médium 0.5 – 0.9	2 (9.5)	7 (22.6)	13 (18.8)	31 (38.8)	53(26.4)	
High ≥ 1	0 (0)	2 (6.5)	5 (7.2)	14 (17.5)	21(10.4)	
Initial salivary pH						**0.070†**
Low < 6.5	0 (0)	0 (0)	3 (4.3)	0 (0)	3 (1.5)	
Medium 6.5 – 7.5	1 (4.8)	8 (25.8)	7 (10.1)	13 (16.3)	29 (14.4)	
High ≥ 7.6	20 (95.2)	23 (74.2)	59 (85.5)	67 (83.8)	169 (84.1)	
Final salivary pH‡						**0.006†**
Low < 5.5	10 (47.6)	11 (35.5)	25 (36.2)	12 (15)	58(28.9)	
Medium 5.5 – 5.9	2 (9.5)	2 (6.5)	12 (17.4)	9 (11.3)	25(12.4)	
High ≥ 6	9 (42.9)	18 (58.1)	32 (46.4)	59 (73.8)	118(58.7)	
Buffer capacity§						**0.002†**
Low < 1.2	0 (0)	0 (0)	1 (1.4)	2 (2.5)	3(1.5)	
Medium 1.2 – 1.5	1 (4.8)	5 (16.1)	16 (23.2)	37 (46.3)	59(29.4)	
High ≥ 1.6	20 (95.2)	26 (83.9)	52 (75.4)	41 (51.3)	139(69.2)	

* Values are given as n (%) of subjects; † Two-sided Chi square test; ‡ pH values of the samples after application of Ericsson's method; § Difference between initial and final pH.

Table 3: Counts (cfu/ml) of microorganisms and salivary properties in stratified clinical groups (n=201)

	Without ECC	With ECC	*P*-value
Mutans streptococci (CFU/ml)*			0.048‡
$\leq 10^4$	27 (13.4)	47 (23.4)	
10^5	19 (9.5)	44 (21.9)	
10^6	12 (6.0)	40 (19.9)	
$\geq 10^7$	0 (0.0)	12 (6.0)	
***Lactobacillus* present (CFU/ml) ***			<0.001§
Yes	2 (1.0)	39 (19.4)	
No	56 (27.9)	104 (51.7)	
Salivary Flow rate (ml/min) †	0.33 (1.0)	0.40 (2.0)	0.12**
Initial salivary pH†	8.0 (3.0)	7.99 (2.0)	0.61**
Final salivary pH†	6.21 (4.0)	6.12 (4.0)	0.96**
Buffer capacity†	1.87 (4.0)	1.82 (4.0)	0.70 **

* Values are given as n (%) of subjects; † Values are given as mean (range)
‡ Two-sided Chi square test; § Two-sided Fisher's exact test; ** Mann-Whitney U test

ECC was detected in 71.14% of study subjects, whereas 56.7% had severe forms of the disease. Categories established for gender and dental eruption stage showed significant difference ($P < 0.05$) in relation to presence/absence of ECC. The proportion of children with dental caries was higher for males than for females, and there was also a significant difference between age groups and ECC ($P < 0.05$), with 5-year-old children having the highest proportion of affected subjects (39.8%).

Salivary properties related to flow rate, final pH and buffer capacity showed significant differences by age, whereas initial salivary pH showed no statistical difference (Table 2). It was found that salivary flow rate increased with age; nevertheless, a high proportion of children (63.2%) were in the low rate group, defined as < 0.5 ml/min according to accepted threshold. Similarly, final pH increased with age, children reaching ≥ 6 value varying from 42.9 % by age two, to 73.8% at five years. Buffer capacity, defined as the difference between initial and final pH, showed an improvement with age, reflected in lower variations.

Table 3 presents microorganism counts and salivary properties, including variations between initial and final pH (buffer capacity) in relation to ECC presence. None of the salivary factors analyzed was significantly related to caries presence, showing similar mean values in both groups. In contrast, significant relationship with mutans streptococci levels (*P*-0.048) and *Lactobacillus* presence (*P*-0.00) were found.

Table 4 shows frequency, percentage and estimated odds ratios (OR) and 95% confidence intervals (CI) for gender, age, eruption stage, mutans streptococci levels and *Lactobacillus* presence. OR for development of dental caries was significantly greater ($P < 0.05$) for male children older than 3 years and complete primary dentition. Although mutans streptococci appeared to be a positive indicator of caries risk in the previous analysis (Table 3), when each category is compared in the OR analysis to the reference group (mutans streptococci $\leq 10^4$), the mutans streptococci loses strength as risk indicator for the development of dental caries. Calculation of OR with $\geq 10^7$ category was not possible because of the absence of children without ECC with this amount of bacteria. The OR of development of dental caries was significantly greater ($P < 0.05$) for children with presence of *Lactobacillus*.

Based on the OR analysis, four variables were considered as candidate risk variables: gender, age, dental eruption stage and presence of *Lactobacillus*. These variables were evaluated in a binary logistic regression analysis using a stepwise variable selection; the model with adjusted OR is shown in Table 5. The age and denture eruption stage changed and became non-significant, only gender and presence of *Lactobacillus* remained significant in the model, adjusting for the other variables; although they presented only a slight increase of the adjusted OR, the model indicated that male gender and presence of *Lactobacillus* were 2.09 and 11.7 times more likely to have ECC. None of the variables included in the regression model presented interacting relationships (Wald test. *P* value > 0.05).

Table 4: Univariate analysis of indicative factors for ecc in children.

	Number of cases*	OR (95% CI) †	*P*-value‡
Gender			
Male	81 (56.6)	1.99 (1.07 ~ 3.70)	0.031
Female (reference)	62 (43.3)	1	
Age group			
5 years	66 (46.1)	5.09 (2.30 ~ 1.25)	<0.001
4 years	52 (36.4)	3.30 (1.52 ~ 7.15)	0.002
3 or younger (reference)	25 (17.5)	1	
Tooth eruption stage			
Complete	131 (8.4)	5.32 (2.38 ~ 11.91)	<0.001
Incomplete (reference)	12 (91.6)	1	
Mutans streptococci			
$\geq 10^7$	12 (8.4)	---§	---
10^6	40 (28.0)	1.92 (0.86 ~ 4.26)	0.122
10^5	44 (30.7)	1.33 (0.65 ~ 2.72)	0.472
$\leq 10^4$ (reference)	47 (32.8)	1	
***Lactobacillus* present**			
Yes	104 (72.7)	10.5 (2.44 ~ 45.11)	<0.001
No (reference)	39 (27.3)	1	

* The values are given as n (%) respect to total among of children with ECC
† Odds ratio (95% confidence interval); ‡ Two-sided Fisher's exact test
§ Impossible to calculate due to the absence of children without ECC with $\geq 10^7$ UFC/ml

Table 5: Binary logistic regression analysis: significant risk factors for development of ECC‡.

	Unadjusted OR (95% CI)*	Adjusted OR (95% CI)*	*P*-value†
Gender	1.99 (1.07 ~ 3.70)	2.09 (1.05 ~ 4.18)	0.036
Age group (5 y 4 years)	5.09 (2.30 ~ 1.25)	2.55 (0.95 ~ 6.90)	0.065
	3.30 (1.52 ~ 7.15)	1.71 (0.74 ~ 3.96)	0.208
Tooth eruption stage	5.32 (2.38 ~ 11.91)	2.89 (0.98 ~ 8.54)	0.054
Lactobacillus present	10.5 (2.44 ~ 45.11)	11.70 (2.55 ~ 53.71)	0.002

* Odds ratio (95% confidence interval); †Wald test; ‡ Hosmer & Lemeshow: P > 0.822

DISCUSSION

Using ICDAS criteria, the children studied showed a high prevalence of dental caries (71.14%), considering their early age and the high proportion of subjects with incomplete dental eruption (84.7%). Although dental caries is more prevalent in developing countries and low-income communities[17], it is a complex disease with multiple contributing factors, some of which may present variations from one population to another, creating unique environments. In fact, some demographic and clinical characteristics, as well as laboratory findings, showed statistically significant differences between children with and without ECC in a population sharing other characteristics.

It is well known that dental caries has cumulative characteristics, so lesions are more easily identified in older age groups. In this study, the relationship between age and tooth eruption stage with dental caries was consistent with other studies showing that dental caries severity increases with age[16,18,19], as well as the finding that Early Childhood Caries was more frequent in males[20]. However, results related to stimulated saliva properties in the children examined requires detailed analysis, considering the conflicting results among authors and the lack of inclusion of values that appear frequently in samples from children when compared to established thresholds for adults, which makes the clinical use of these variables as clinical risk indicators a complex field.

Saliva properties showed statistically significant differences by age, reflecting a tendency to increase flow rate as well as mean values of final pH and buffer capacity, constituting potential protective factors. However, no statistically significant differences were found in salivary flow rate, initial and final pH, and buffer capacity between children with and without dental caries, contrary to some previous studies[8, 21], a finding supporting the ambiguous evidence available in relation to salivary properties and dental caries in children.

While some authors have found that chronically low salivary flow rate (<0.8-1.0 ml/min stimulated whole saliva) is a strong indicator of an increased risk of caries[21-25], others were not able to demonstrate a relationship or predictive value for individuals with normal salivary flow rate[26-30]. Similarly, some studies report a correlation between low levels of salivary pH and buffer capacity and caries[21,23,31], but others conclude that salivary factors are poor indicators of caries risk and caries experience[28,32-35].

An additional problem for concluding studies about saliva and ECC is the fact that there are few reports on stimulated salivary properties such as flow rate in young children[27,36] so normality parameters or threshold limits are not totally defined in scientific literature. While analyzing the relationship between salivary variables, age and dental caries, we noted that it was necessary to adjust threshold levels for salivary flow rate, as 174 children (79.8%) presented values below 0.7 ml/minute, considered as the reference value for hyposalivation in adults[37,38]. This finding shows the need to establish proper reference values for preschool children through large population samples and analytical studies in this specific age group.

Determining the role of mutans streptococci and *Lactobacillus* as caries risk indicators is a complex matter, as they can also be part of normal oral flora in healthy people, or in contrast, people with carious lesions may have no detectable counts of these bacteria in their mouths[39-42]. Nevertheless, the evidence indicates both microorganisms as important cariogenic agents, promoting their usefulness in risk assessment, as they are associated with a considerable increase in caries risk[39,43].

In this study both, high levels of mutans streptococci and presence of *Lactobacillus* showed significant statistical differences between children with and without ECC ($p < 0.05$). However, OR analysis showed no association between ECC and mutans streptococci counts, in agreement with some authors[39,44], although they are recognized in the literature as an important variable in caries risk assesment[45]. So we can hypothesize that the high counts of this group of microorganisms found in children without ECC in this study may indicate that a carious process may be present at a subclinical level, a limitation of this cross-sectional study in comparison with prospective ones, or biological variations within bacteria present in this population that need to be explored in through more specific tests.

In contrast, presence of *Lactobacillus* proved to be a good candidate risk variable, as did complete temporary teething, age above four years and male gender, but in the binary logistic regression analysis, tooth eruption stage and age showed a reduction in their effect when adjusted. So *Lactobacillus* and gender were the only variables analyzed considered to be strong risk factors for ECC, in agreement with other studies[46,47].

Our findings show limitations for using conventional risk assessment models for this preschool population, and that some key variables show no relationship with ECC, suggesting that further studies are needed on aspects such as salivary properties in children under six. This initial information may provide guidelines for future studies seeking a more complete understanding of risk factors related to the physicochemical properties of saliva affecting the development of ECC in children living in developing countries.

CONCLUSIONS

- Only male gender and *Lactobacillus* presence could be considered strong indicators for ECC presence in the population studied.

- Physicochemical salivary properties showed no relationship with ECC presence or absence, but results showed the need to conduct studies leading to the establishment of normal parameters as well as threshold levels for hyposalivation and saliva alteration in preschool children.

- Prospective studies, as well as genetic microbiological analyses, are required to elucidate controversial results for mutans streptococci counts in relation to ECC in this population.

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