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Relationship between cariogenic bacteria and molar incisor hypomineralization in Brazilian schoolchildren

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Background: Teeth with defects in their structure, such as the ones affected by molar-incisor hypomineralization (MIH), are more susceptible to carious lesions. Caries is a complex and multifactorial disease highly prevalent in childhood. The present research evaluated the relationship between the stages of MIH and cariogenic bacteria in children.

Methods: After examining 566 schoolchildren, four groups of 10 children each were formed: healthy (G1), mild MIH (G2 and G3), and severe MIH + caries (G4). Dental biofilm was assessed to quantify *Streptococcus mutans* (SM) and *Lactobacillus* spp. (LB) using real-time polymerase chain reaction (RT-PCR).

Results: LB counting in biofilm samples of healthy children (G1) and those with mild MIH characterized by white opacities (G2) were not significantly different. The same happened when the ones with yellow opacities (G3) were compared with severe MIH + caries (G4) ($P>0.05$). The *post hoc* Tukey test proved that G4 had greater levels of SM and LB when compared with G2 ($P<0.05$); however, the control group did not diverge from the others considering SM ($P>0.05$). Increased LB enhanced the severity of MIH [rate ratio (RR): 7.706; $P=0.035$].

Conclusions: LB was influenced by different degrees of MIH and the presence of caries and could guide clinical decisions and patients' recommendations to prevent carious lesions in MIH children.

Keywords: Molar-incisor hypomineralization (MIH); dental caries; *Lactobacillus*; bacteria

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Introduction

Molar-incisor hypomineralization (MIH) is a qualitative developmental defect of the enamel of genetic predisposition in association with a multifactorial origin (1), affecting obligatorily one, at least of the first permanent molars, involving or not the incisors (2). The permanent first molars and incisors formation begins in the final period of pregnancy and ends around three to five years of the child's age (3). Identifying the factors that can affect the enamel-producing cells in this period is challenging. Pre-

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and post-natal risk factors have been suggested, such as complications in pregnancy, alcohol consumption/smoking, breastfeeding period, high fever episodes, illnesses, antibiotic intake, diarrhea, chickenpox, asthma, pneumonia (4), and organic pollutants (5).

The clinical finding that defines MIH is the presence of demarcated opacities, and the tooth affected by MIH may present a mild degree, characterized by changes in the color of the enamel (white to yellow/brownish) or severe with post-eruptive enamel breakdown (6). The defective enamel is highly susceptible to post-eruptive enamel breakdowns due to masticatory forces and provides a perfect environment for biofilm accumulation and carious process development. In addition, these teeth could show high sensitivity (6).

MIH treatment may involve using glass ionomer cements restorations or sealants (7), fluoride-based materials applications, resin infiltration (8), complex restoration performance, and even tooth extraction. Promising technologies have recently been studied for desensitizing and remineralizing MIH-affected sites, such as paste based on zinc-hydroxyapatite. A newly published randomized clinical trial involving Italian school-age children showed a desensitizing effect of biomimetic zinc-hydroxyapatite when used to treat MIH (9). A recent review also revealed that calcium phosphate-based approaches could remineralize MIH teeth (10).

The relationship between dental caries and MIH has already been evidenced, and a recent study showed that the presence of MIH was associated with 6.15 times higher caries prevalence in the first permanent molars (11).

In the scientific literature, studies involving MIH, oral microbiota, especially the classical acidogenic and aciduric related to caries [*Streptococcus mutans* (SM) and *Lactobacillus* spp. (LB)], and molecular biology techniques are incredibly scarce (12-14). However, MIH is a topic widely discussed nowadays, and there have been about 328 articles published in PubMed in the last five years. A systematic review revealed that MIH is expressively prevalent worldwide (10.7–15.3%) (15). Thus, the present research aimed to assess the relationship between the stages of MIH and cariogenic bacteria in children. We present this article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-23-48/rc).

Highlight box

Key findings

- *Lactobacillus* spp. (LB) was influenced by different degrees of molar-incisor hypomineralization (MIH) and the presence of caries.

What is known, and what is new?

- MIH teeth are more susceptible to carious lesions development than non-affected ones.
- MIH-yellow opacities presented higher levels of LB than MIH-white opacities, similar to those found in the MIH-caries lesion group.

What is the implication, and what should change now?

- LB levels could guide clinical decisions and patients' recommendations to prevent carious lesions in MIH children.

Methods

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the University São Francisco (USF), Bragança Paulista, São Paulo, Brazil (protocol No. 10408119.0.0000.5514). Guardians who agreed with their child's participation in this study signed an informed positive consent form.

Sampling characteristics

The present cross-sectional research included Brazilian children of both sexes, aged 6 to 8 years, with fully erupted first permanent molars, attending the most significant public schools in the central area of Bragança Paulista, São Paulo, Brazil. Bragança-Paulista has about 172,346 inhabitants, a human development index of 0.776, and an amount of fluoride in drinking water of 0.69 mg/L.

As part of a more extensive study encompassing 566 children (MIH prevalence: 30%), forty children were divided into the following groups (after caries and MIH diagnosis), according to a convenience sampling strategy: G1 (n=10), healthy first permanent molars; G2 (n=10), mild MIH with white opacity and free of caries; G3 (n=10), mild MIH with yellow opacity and free of caries; G4 (n=10), severe MIH with white/yellow opacities and presence of caries (*Figure 1*). The MIH groups (G2, G3, and G4) account for 166 affected teeth with different degrees of severity (75.3% first permanent molars and 24.7% incisors). Specifically, G2 accounts for 32 first permanent molars + 7 incisors, G3 for 35 first permanent molars + 21 incisors, and

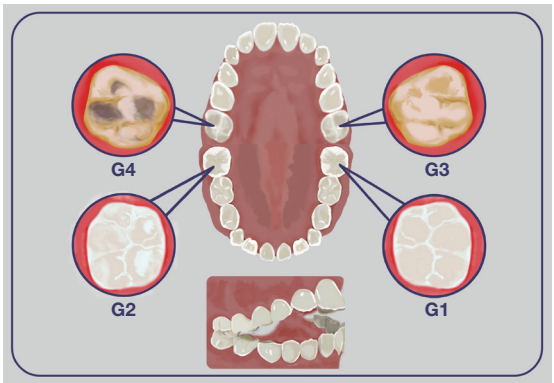

Figure 1 Degree of severity of molar incisor hypomineralization versus sound enamel. Sound enamel—G1; mild MIH with white opacity and free of caries—G2; mild MIH with yellow opacity and free of caries—G3; severe MIH with white/yellowish opacities and presence of caries—G4. MIH, molar incisor hypomineralization.

G4 for 38 first permanent molars + 13 incisors.

Enrolled schoolchildren were from similar socioeconomic backgrounds (low to mid) and used fluoridated toothpaste (1,100 ppm) to brush their teeth. Children taking antibiotics or pre/probiotics at the time of the biofilm collection or in the 30 days preceding the biofilm collection were excluded. Also, the ones with special needs or chronic non-respiratory systemic diseases were kept out. The criteria for exclusion still comprise children with carious lesions only, without hypomineralization or with other enamel defects, and those free of caries that were not included in the healthy group (G1).

Assessment of the daily frequency of sugar exposure

The daily frequency of sugar exposure was assessed using the "Food Questionnaire of the Previous Day - QUADA-3" (16). According to illustrative images, children were asked to show the food groups eaten in all meals on the previous day (at school or home), increasing the fidelity of the data collected. Based on QUADA-3, the frequency of daily sugar exposure in the liquid and solid forms was estimated.

Assessment of health conditions

The child's history of pre-and post-natal conditions was evaluated using a structured and self-administered questionnaire to parents or guardians. The following questions were asked: "Did you have problems in the last trimester of pregnancy, considering fever/urinary infection/antibiotics usage?", "What were the baby's perinatal conditions considering delivery mode/respiratory difficulties/incubator necessity/weight/pre-term birth?" and "Did your child have pneumonia, asthma, sinusitis, rhinitis, or use antibiotics until the second year of life?". The questions were settled based on the study of Lima *et al.* (17).

Assessing caries and MIH through clinical examination

Dental caries and MIH diagnosis

Children's teeth were cleaned and dried with gauze. Diagnosis of MIH and caries was made by visual inspection using a focusable flashlight with a mirror and a ball-ended dental probe. The criteria of the European Academy of Paediatric Dentistry (EAPD) (6,18) and World Health Organization criteria modified by including active white spot lesions (19) were used. A calibrated dentist performed all clinical examinations. Prior to the beginning of the study, the examiner received all theoretical-practical guidelines on the criteria to be used. Then, replicate examinations were carried out on ten children, randomly selected, with at least a one-week interval period. Intra-examiner agreement measured by calculated kappa values were: 0.82 for MIH and 0.99 for dental caries.

Biofilm collection

Biofilm samples were collected from all surfaces of the affected first permanent molars in the experimental groups (G2, G3, and G4) and from one healthy first molar in the control group (G1). The first permanent molar was the teeth of choice for collection, as MIH affects these teeth obligatorily. The samples were placed into microcentrifuge tubes inside an icebox during the collection period. In the Laboratory of Microbiology of the USF, the collected biofilm was frozen at –80 ℃ until DNA extraction and real-time polymerase chain reaction (RT-PCR) analysis to quantify the species: SM, LB, and the Firmicutes phylum (FP).

Bacterial detection using RT-PCR

DNA extraction from the collected dental biofilm was made using the Lucigen/Epicentre kit (MasterPure™ Complete DNA Purification Kit, MC85200, Middleton,WI, USA), and the DNA levels were assessed in the Biodrop equipment (Biodrop µLite Spectrophotometer, Biochrom US Inc., Holliston, MA, USA). Concisely, the biofilm was

Table 1 Phylum/species, target genes, and primers for identifying and quantifying bacteria by real time polymerase chain reaction

Phylum/species	Target genes	Amplicon size base pairs (bp)	Primers
Streptococcus mutans (20)	*gtfB* gene	114	FP: GCCTACAGCTCAGAGATGCTATTCT
			RP: GCCATACACCACTCATGAATTGA
Lactobacillus spp. (21)	*16S rRNA*	126	FP: GAGGCAGCAGTAGGGAATCTTC
			RP: GGCCAGTTACTACCTCTATCCTTCTTC
Firmicutes* (22)	*16S rRNA*	126	FP: GGAGYATGTGGTTTAATTCGAAGCA
			RP: AGCTGACGACAACCATGCAC

*, Firmicutes primers were based on conservative *16S rRNA* gene sequences of a multiplicity of oral species (GenBank; http://www.ncbi.nlm.nih.gov/genbank/).

resuspended in 300 µL of a Tissue and Cell Lysis Solution (containing proteinase K) and incubated at 65 ℃ for 15 minutes. After placing samples on ice for 5 min, 150 µL of an MPC protein precipitation reagent was inserted, and centrifugation was performed (10 min, 10,000 ×g, 4 ℃). The supernatant was transferred to a clean microcentrifuge tube, and the pellet was discarded. Isopropanol (500 µL) was added to the supernatant and mixed gently by inversion. After centrifugation (10 min, 10,000 ×g, 4 ℃), total nucleic acids were obtained, rinsed twice with 70% ethanol, and resuspended in Tris-EDTA (TE) Buffer (35 µL). The DNA was used to identify the SM, LB, and FP through RT-PCR.

Real-time analyses were performed on the 7300 Real-Time System (Applied Biosystems, Foster City, CA, USA), using the SYBR Green Power up (Thermo Fisher Scientific, Carlsbad, CA, USA) reagent. Primer sequences, genes, and amplicons are described in *Table 1*.

A total of 1.5 µL of the DNA extracted from biofilm samples was used for each assay, together with 5 µL of SYBR Green Power up (Thermo Fisher Scientific), 2.9 µL of H_2O, 0.3 µL of forwarding primer, and 0.3 µL of the reverse primer.

The steps for the detection of SM, LB, and FP included 50 ℃ for 2 min, 95 ℃ for 10 min, and 40 cycles of 95 ℃ for 15 s followed by 60° for 1 min.

For all experimental samples, the absolute quantity was determined from the standard curves using the following species in serial dilutions (10^1–10^5 ηg DNA/µL): SM (UA159 - ATCC 70061) (20), *Lactobacillus casei* (clinical strain previously identified at the Laboratory of Clinical and Molecular Microbiology - USF) (LB) (21), and *Clostridium perfringens* (ATCC 13124) (FP) (22). These bacteria were also used as positive controls. Based on the standard curves, the software (Sequence Detection Software version 1.3.1, Applied Biosystems, Foster City, CA, USA) interpolates the absolute quantity of the target content in the test samples.

The threshold cycle value was the number at which the detectable fluorescence was above the background fluorescence (standard threshold: 0.2). RT-PCR analyses were performed in duplicates, and bacterial levels were expressed by ηg DNA/µL.

Statistical analysis

Data was statistically analyzed using the SPSS package for Windows, version 21.0 (SPSS, Inc., Chicago, IL, USA). Gaussian distribution was tested using the Shapiro-Wilk test and Quantile-quantile-plot (QQ-plot) analysis. Levene's test was used to prove the homogeneity of variance. SM and LB were the dependent variables and were adjusted by dividing them by FP levels.

The exploratory statistics consisted of medians and interquartile ranges. Data were transformed using normal logarithmic expression to adhere to the analysis of variance premises. The one-way analysis of variance followed by the Tukey test was used, considering a 0.05 significance level. In addition, a model assessing the significant risk indicators in different stages of MIH concerning bacterial parameters was performed (Poisson regression analysis).

Results

Characteristics of the school children

The mean age of the schoolchildren included was 7.38 years (±0.87). Boys and girls showed an equivalent distribution inside the groups. In children with severe MIH with white/yellowish opacities and the presence of caries (G4), the mean number of decayed missing or filled primary teeth

Table 2 SM and LB levels according to different severity degrees of MIH

Groups	SM (ηg DNA/μL)	LB (ηg DNA/μL)
Healthy first permanent molars (Group 1)	0.038 (0.034)[ab]	0.004 (0.003)[a]
Mild MIH with white opacity and free of caries (Group 2)	0.028 (0.013)[b]	0.002 (0.001)[a]
Mild MIH with yellow opacity and free of caries (Group 3)	0.057 (0.038)[ab]	0.012 (0.010)[b]
Severe MIH with white/yellow opacities and presence of caries (Group 4)	0.095 (0.084)[a]	0.016 (0.008)[b]
ANOVA P value	0.023*	<0.001*
Partial eta squared	0.296	0.692
Power	0.806	1.000

Statistical analyses were performed with a sample of 40 cases, 10 per group. Data were expressed in the median (interquartile range). Data were transformed using normal logarithmic expressions. SM and LB were adjusted by dividing them by Firmicutes phylum levels. Different lower letters ([a], [b] or [ab]) represent statistically significant differences according to groups (comparison in column). *, statistically significant at P<0.05. SM, *Streptococcus mutans*; LB, *Lactobacillus* spp.; MIH, molar incisor hypomineralization; ANOVA, analysis of variance.

Table 3 Risk indicators regarding SM and LB levels in school children with MIH

Risk indicators	Different severity degrees of MIH in children	
	Rate ratio (95% CI)	P value
Lactobacillus spp. (ηg DNA/μL)	7.706 (1.153–51.518)	0.035*
Streptococcus mutans (ηg DNA/μL)	1.056 (0.983–1.134)	0.134

Poisson Regression Model; main outcome: degrees of MIH. *, statistical significance: P<0.05. n=40; Omnibus Test: likelihood ratio Chi-square =4.887. The rate ratio was used as the effect size: 1.22 (small); 1.86 (medium); 3.00 (large). SM and LB were adjusted by dividing them by Firmicutes phylum levels. SM, *Streptococcus mutans*; LB, *Lactobacillus* spp.; MIH, molar incisor hypomineralization; CI, confidence interval.

was 1.10 (±0.49), and the mean number of decayed missing or filled permanent teeth was 1.66 (±0.56).

Cariogenic bacteria in different severity degrees of MIH

SM and LB levels according to different severity degrees of MIH are shown in *Table 2*. Significant distinctions in LB counting in biofilm samples of healthy children (G1) and those with mild MIH characterized by white opacities (G2) were not found (P>0.05). The same happened when the ones with yellow opacities (G3) were compared with severe MIH + caries (G4) (P>0.05). The *post hoc* Tukey test proved that G4 had greater levels of SM and LB when compared with G2 (P<0.05); however, the control group did not diverge from the others considering SM (P>0.05). As shown in *Table 3*, increased LB enhanced the severity of MIH [rate ratio (RR): 7.706; P=0.035].

Health conditions in MIH children

Health conditions according to different severity degrees of MIH are displayed in *Table 4*. In the present study, systemic diseases occurring during the last trimester of pregnancy, in the perinatal period, and post-natal (0 to 2 years) were more frequent in MIH-affected teeth (G2, G3, and G4; *Table 4*). The same happened with sugar intake.

Discussion

The present investigation showed that LB levels were shaped by different degrees of MIH and caries (*Table 2*). Of interest, there is no information in the scientific literature considering cariogenic bacteria and MIH degrees. LB counting in biofilm samples of children with healthy first permanent molars (G1) and those with mild MIH

Table 4 Health conditions according to different severity degrees of MIH

Period/behavior	Conditions	Group 1	Group 2	Group 3	Group 4
Last trimester of gestation	Fever/infection (yes)	0	0	1 (10%)	0
	Urinary infection (yes)	0	0	3 (30%)	1 (10%)
	Antibiotic (yes)	0	1 (10%)	3 (30%)	1 (10%)
Perinatal conditions	Hypoxia (yes)	0	2 (20%)	0	0
	Delivery (C-section)	6 (60%)	5 (50%)	5 (50%)	4 (40%)
	Dyspnea (yes)	0	1 (10%)	0	1 (10%)
	Incubator (yes)	0	2 (20%)	0	2 (20%)
	Birth (premature)	2 (20%)	3 (30%)	0	1 (10%)
	Birth weight (<2.5 kg)	2 (20%)	3 (30%)	3 (30%)	3 (30%)
Post-natal conditions until 2 years	Pneumonia (yes)	1 (10%)	0	1 (10%)	2 (20%)
	Asthma (yes)	0	1 (10%)	0	0
	Sinusitis/rhinitis (yes)	0	1 (10%)	2 (20%)	1 (10%)
	Fever (yes)	2 (20%)	6 (60%)	7 (70%)	6 (60%)
	Antibiotics (yes)	1 (10%)	4 (40%)	6 (60%)	6 (60%)
Eating behavior	Sugar intake (>3 times/day)	2 (20%)	5 (50%)	6 (60%)	7 (70%)

Group 1: healthy first permanent molars (n=10); Group 2: mild MIH with white opacity and free of caries (n=10); Group 3: mild MIH with yellow opacity and free of caries (n=10); Group 4: severe MIH with white/yellow opacities and presence of caries (n=10). MIH, molar incisor hypomineralization.

characterized by white opacity (G2) were not significantly different. In the same way, a lack of statistical divergence occurred when LB levels in children with mild MIH represented by yellow opacity (G3) were compared with the severe MIH condition (G4). Besides, yellow opacity (G3) or severe MIH, together with caries (G4), had greater LB when compared with G2 or G1 (P<0.05) (*Table 2*). White opacities in the first permanent molars affected by MIH characterized the minor degree of the defect. This way, expecting the enamel with closer characteristics to the sound enamel is plausible. On the other hand, the yellow opacity could be suggested as a step forward in the severity scale, being more porous than the white ones, with worse prism organization and more prone to acid attacks (23). This way, yellow areas may resemble a stricter stage involving post-eruptive enamel breakdowns and dental caries (24). Notably, niche retentiveness is important for LB accumulation (25). SM does not need a rough surface to adhere, probably explaining why the control group did not diverge from the others considering this microbe (P>0.05).

Remarkably, our investigation found that every one-unit increase in LB enhanced seven times the severity of MIH (RR: 7.706; P=0.035) (*Table 3*). LB are Gram-positive rods, acidogenic and aciduric anaerobes. Although they are secondary colonizers in pre-existing carious lesions (because they need a retentive niche, such as the rough yellow opacities, rapidly turning into enamel breakdown), they are of prime importance in the progression of these lesions (26). Their capacity of organic acid production by dietary sugar fermentation promotes enamel dissolution and degradation of the dentinal organic tissues. Consequently, bacteria reach dentinal tubules, and their penetration is enhanced by the wide tubules of young teeth and the hypomineralized dental tissues, which is the case of MIH (27). This way, the pulp injury/inflammatory response mechanisms, as well as the hypersensitiveness of these teeth, are favored.

The microbial load is also enhanced when the first permanent molar appears in the oral cavity due to increased biofilm accumulation (28), as they are underneath the occlusal plane without contact with the antagonist's teeth during chewing and are difficult to reach with the toothbrush. If biofilm accumulation is potentialized, the same occurs with caries susceptibility, especially in hypomineralized tissues. The chronic pulp inflammation

and great sensitivity of teeth affected by MIH hampered the performance of clinical procedures, prejudicing the oral health-related quality of life (29,30). Without proper therapeutic intervention, caries lesions turn deeper. They might lead to tissue necrosis, allowing root canal systems invasion by bacteria together with alveolar bone via apical foramen (31), reaching the circulatory system and being able to spread to the role body, maybe contributing to systemic diseases (32).

In the present study, systemic diseases occurring during the last trimester of pregnancy, in the perinatal period, and post-natal (0 to 2 years) were more frequent in MIH-affected teeth (G2, G3, and G4; *Table 4*), as well as sugar intake. Remarkably, the genesis of the first permanent molars and incisors occurred during these periods, precisely the dental teeth affected by MIH. These findings reinforce the production of hypomineralized enamel with concomitant medical conditions (4), as dental tissues cannot be separated from the rest of the body. The link between MIH and systemic childhood diseases supports the importance of a multidisciplinary and holistic approach favoring early diagnosis.

The identification of MIH soon after the eruption of the first permanent molars in the oral cavity, as performed in the present research, enables assertive strategies for the affected children, avoiding complex and expensive rehabilitation, which are challenging during childhood and probably impacts the future quality of life.

Despite some limitations, such as limited sample size and the popular methodology, this pioneering study warns of the importance of developing well-designed future studies exploring the microbiota and MIH, mainly using robust methods such as genome sequencing. Curiously, there is only one paper available in the scientific literature involving DNA sequencing proposing an association of MIH with periodontal pathogenic bacteria but without considering different degrees of MIH (13). Prospective studies would also be valuable, especially considering the link between the defect to medical conditions and the prolonged use of biomimetic hydroxyapatite to improve enamel integrity and reduce tooth sensitivity.

Conclusions

In conclusion, LB was influenced by different degrees of MIH and the presence of caries. Specifically, MIH-yellow opacities presented higher levels of LB than MIH-white opacities, similar to those found in the MIH-caries lesion group. This finding could guide clinical decisions and patients' recommendations to prevent carious lesions in children with MIH.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-23-48/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-23-48/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-23-48/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the University São Francisco, Bragança Paulista, São Paulo, Brazil (protocol No. 10408119.0.0000.5514), which classified it as minimal-risk research. Guardians who agreed with their child's participation in this study signed an informed positive consent form.

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