Original paper

Association between sleep pattern, salivary cariogenic bacteria and fungi populations, pH and buffering capacity in children: A comparative study

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Dental and Medical Problems, ISSN 1644-387X (print), ISSN 2300-9020 (online) *Dent Med Probl*. 2024;61(2):217–224

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
None declared

Received on April 10, 2023
Reviewed on May 11, 2023
Accepted on June 4, 2023

Published online on April 26, 2024

Abstract

Background. Sleep quality has a significant impact on a child's health and is linked to oral and systemic diseases. It affects the circadian rhythm, which plays a crucial role in regulating the balance of the endocrine and hormonal systems. Current research has focused on exploring its role in the development of caries, which is influenced by inherent oral factors such as the composition of the oral microbiome and pH levels.

Objectives. This study aimed to investigate the relationship between bacterial population, pH, and buffering properties of saliva and sleep patterns in 8- to 12-year-old children.

Material and methods. This cross-sectional study was conducted on 85 elementary school children aged 8–12 years. After obtaining written consent, non-stimulating saliva samples were collected using the spitting method. The participants' sleep pattern information was obtained with the use of the Persian version of the Children's Sleep Habits Questionnaire (CSHQ). Based on the results of the CSHQ, the participants were divided into 2 groups: those with appropriate sleep patterns; and those with inappropriate sleep patterns. The study compared the bacterial population of *Streptococcus mutans*, *Lactobacillus* spp. and *Candida albicans*, as well as the buffering capacity and pH of the saliva between the 2 groups. The statistical analysis employed the χ^2 test, the independent samples *t*-test and Spearman's correlation.

Results. The group with inappropriate sleep patterns had significantly lower pH and buffering capacity ($p < 0.001$) and significantly higher colony counts of *Lactobacillus* and *S. mutans* ($p < 0.001$ and $p = 0.012$, respectively). There was no association between *C. albicans* and sleep patterns ($p = 0.121$).

Conclusions. Inappropriate sleep patterns increase the population of caries-causing bacteria and reduce salivary pH and buffering capacity. This can be a significant factor in the development of dental caries in children aged 8–12 years.

Keywords: *Candida albicans*, *Streptococcus mutans*, saliva, circadian rhythm, sleep disorders

Cite as
Mehdipour A, Fateh R, Fuladvand F, Aghaali M, Keykha E, Hadilou M. Association between sleep pattern, salivary cariogenic bacteria and fungi populations, pH and buffering capacity in children: A comparative study. *Dent Med Probl.* 2024;61(2):217–224. doi:10.17219/dmp/167411

DOI
10.17219/dmp/167411

Introduction

Sleep has a profound impact on the health and well-being of both adults and children. It plays a regulatory role in the body's physiological functions, including recovery from physical and mental fatigue, muscle growth and repair, enhancing the immune responses,[1] preserving endocrine and hormonal system balance,[2] and sustaining life. Insufficient sleep, which disrupts the circadian rhythm, has been linked to several systemic diseases, including cancer, diabetes, depression,[3] obesity, cardiovascular disease, periodontal disease, and an increased mortality rate.[4,5] Sleep deficiency hinders immune responses, resulting in elevated levels of inflammatory biomarkers (interleukin-6 (IL-6) and C-reactive protein (CRP)), increased white blood cell count and an increased risk of infectious diseases.[6] In addition, it disturbs the intestinal microflora composition by inducing Paneth cell failure, which controls intestinal flora by the secretion of antimicrobial peptides such as α-defensin and human α-defensin 5.[1] Chronic sleep deficiency may lead to obesity by altering appetite hormone secretion, energy intake and food preferences,[7,8] which in turn may result in the development of type 2 diabetes.[9] Several publications have explored the relationship between insufficient sleep and cancer risk.[10–12]

An individual's sleep pattern is a combination of sleeping behavior, duration and depth. Studies show that there is a growing concern about the consequences of insomnia in contemporary societies. Recent epidemiologic data indicates that a significant portion of the population experiences chronic sleep deficiency.[13] Insufficient sleep or sleeping late alters the quality and composition of saliva, as well as the microbial colony type.[14]

The secretion rate and composition of saliva follow a constant daily pattern. Saliva flow is low in the morning, increases in the afternoon and early evening, and decreases during sleep.[14] Saliva contains different types of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, and phosphate), proteins, enzymes, immunoglobulins, and other antimicrobial agents, including mucous glycoproteins, albumin, and other important polypeptides and oligopeptides. In addition to glucose, saliva contains nitrogen compounds such as urea and ammonia.[15,16] These substances are crucial in regulating the oral environment's pH, buffering capacity and microbial composition. Additionally, they play a role in the development of oral diseases such as carious lesions and periodontal diseases. The buffering capacity of saliva is essential in maintaining the pH of saliva and dental remineralization. The amount of saliva flow and pH levels play a significant role in the occurrence and spread of caries.[17,18] The pH of saliva at rest can be used to predict the likelihood of tooth decay and buffering capacity. In a healthy person, the resting saliva pH is approx. 7, indicating low caries activity. However, those with a resting saliva pH ranging between 5.5 and 7 have a higher incidence of caries.[19]

Various factors, including previous caries experience, patient health behaviors, socioeconomic factors, diet, and oral microbial flora, have been considered to predict the risk of tooth decay. Oral bacteria increase acid production, demineralization and tooth decay by metabolizing carbohydrates. The most prevalent strains involved in this process are *Streptococcus mutans* and *Lactobacillus* spp.[20,21] Therapeutic strategies that interfere with their colonization can significantly reduce the incidence of dental caries.[22] Studies have reported varying numbers of *Lactobacillus* spp. and *S. mutans* colonies in the saliva of patients from different age groups and communities.[23] On the other hand, studies show that the prevalence of fungal populations, particularly *Candida albicans*, is significantly higher in children with dental caries compared to those without.[24]

Given that the development of appropriate sleep habits should begin at a young age, few studies have been conducted on the effect of sleep patterns on children's oral and dental health, as well as the relationship between sleeping late, irregular sleep cycles, and sleep duration and oral health.[25] Consequently, the present study aims to examine the potential relationship between the sleep quality of 8- to 12-year-old schoolchildren in the city of Qom, Iran, and the populations of cariogenic bacteria and fungi, pH, and buffering capacity of their saliva. It is recommended that clinicians inquire about their patients' sleep patterns and consider sleep patterns a risk factor for dental and periodontal diseases. This risk factor can be eliminated by providing adequate instructions or referring patients to specialized clinicians.

Material and methods

Study design and participants

This descriptive, comparative, cross-sectional study was conducted in 2021 and 2022 at 3 schools located in downtown Qom, Iran. The Research Ethics Committee of Qom University of Medical Sciences approved the study protocol (approval No. IR.MUQ.REC.1401.143). A total of 40 samples were determined for each group, one with appropriate sleep patterns and the other with inappropriate sleep patterns. The alpha value used was 0.05, with a beta value of 0.2 and a prevalence of sleep disorders of 0.4%. The mean ± standard deviation ($M \pm SD$) values were calculated based on a previous study.[26] The sampling was conducted in 4 schools located in different parts of the city, chosen based on their willingness to participate in the study. The students were selected randomly from each of the 5 grade levels, with an equal number of male and female students in each group. The age and

gender of both groups were matched. The study protocol was explained to the parents and their children, and written consent was obtained.

The study included children between the ages of 8 and 12 who were physically and mentally healthy. The exclusion criteria were chronic systemic disease, sedative or hypnotic drug use, having at least 1 parent with a mental illness, drug abuse by at least 1 parent, congenital oral disease, history of radiotherapy or chemotherapy, and antibiotic use within the previous 2 weeks. Both groups received oral hygiene education 2 weeks before the start of the study to eliminate confounding variables. A questionnaire was employed to record demographic information of the parents and children, as well as the frequency of brushing, flossing and snack consumption. Data was collected by a trained dentist in coordination with school officials.

Measurement

Evaluation of sleep patterns

The sleep patterns of the participants were evaluated using the Persian version of the Children's Sleep Habits Questionnaire (CSHQ). The questionnaire was completed by parents. The CSHQ was designed by Owens et al. and contains 45 criteria that measure a child's sleep quality and habits. Based on the CSHQ results, the participants were divided into 2 groups: those with appropriate sleep patterns; and those with inappropriate sleep patterns.[27] The groups were matched by gender and age. Previous studies have confirmed the reliability and validity of the Persian version of the CSHQ.[28,29]

The questionnaire consists of 45 questions, including diagnostic and therapeutic questions that were not relevant to our research purposes; therefore, we only utilized 33 questions in this study. Each question was assigned a value between 1 and 3 (rarely, occasionally, frequently), with the exception of questions 1, 2, 3, 10, 11, and 26, which were scored inversely. The total score can range from 33 to 99. The score for each subscale was calculated based on the total number of related questions. The primary subscales were "bedtime resistance" (questions 1, 3, 4, 5, and 8), "sleep onset delay" (question 2), "sleep duration" (questions 9, 10 and 11), "sleep anxiety" (questions 5, 7, 8, and 21), "night wakings" (questions 16, 24 and 24), "parasomnias" (questions 12, 13, 14, 15, 17, 22, and 23), "sleep disordered breathing" (questions 18, 19 and 20), and "daytime sleepiness" (questions 26, 27, 28, 29, 30, 31, 32, and 33).

The total score on the CHSQ was calculated as the sum of all the section scores. Parents were given 3 response options for each question: frequently (5 to 7 nights per week); occasionally (2 to 4 nights per week); and rarely (0 to 1 night per week). A higher score on the CHSQ indicates a greater number of sleep issues. Children with a CHSQ score below 41 were considered to have appropriate sleep patterns, while those with a score above 41 were deemed to have inappropriate sleep patterns.[27]

Saliva collection

After completing the questionnaire, participants' unstimulated saliva samples were collected in the morning before eating breakfast, brushing their teeth, or washing their mouths. The sample was obtained by holding the patient's head down for 2–3 min and collecting 2 mL of unstimulated saliva in a sterile container.[30] The samples were stored on dry ice and immediately transferred to the microbiology laboratory of Qom University of Medical Sciences for bacteriological and fungal evaluation.

Salivary *S. mutans* level measurements

We thoroughly homogenized 0.5 mL of saliva with 5 mL of phosphate-buffered saline (PBS) using a shaker. Then, 20 µL of the solution was added to the mitis salivarius agar medium with bacitracin and 10% sucrose. The plates were incubated in an environment containing 5% CO_2 at a temperature of 37° for 48 h. Biochemical tests, including the mannitol, melibiose, sorbitol, raffinose fermentation, and arginine dihydrolase tests, as well as Gram staining, were conducted to isolate *S. mutans* from other species. Finally, the confirmed colonies of *S. mutans* were counted and graded.[31–33]

Salivary *Lactobacillus spp.* level measurements

A portion of the sample was cultured in the MRS Broth liquid medium for 48 h at 37°C under anaerobic conditions. Then, the bacterial growth in the MRS Broth medium was transferred to the MRS Agar medium. *Lactobacillus* spp. is an anaerobic microbe that requires a special environment to grow. The microbes were placed inside an anaerobic jar (Merck Chemicals GmbH, Darmstadt, Germany), which is an impermeable container with no gas exchange with the outside environment and is used to cultivate anaerobic bacteria. A MERCK gas pack (Merck Chemicals GmbH), an oxygen-absorbent chemical kit, was placed inside the jar to achieve these conditions. Next, we soaked it with 6 mL of normal saline and placed it in an incubator at 37°C. After keeping the samples in this environment for 1 day, a suspension of the microbes was prepared using a technique similar to that employed for *S. mutans*. *Lactobacillus* spp. colonies were differentiated and confirmed using morphological tests and Gram staining.[34]

Bacterial colonies were counted using the following criteria:

A – 0 colonies: no growth;
B – 1 colony: 1–10^3 bacteria per mL of saliva,
C – 2 colonies: 10^3–10^5 bacteria per mL of saliva; and
D – 3 colonies: more than 10^5 bacteria per mL of saliva.[35]

Salivary *C. albicans* level measurements

For *Candida* isolation, 0.1 mL of saliva was cultured on the suburban chloramphenicol dextrose agar medium and incubated at 37°C for 24–48 h. The presence of *C. albicans* colonies was confirmed by microscopic examination tests based on the colony color after 48 h.

Colony counting was performed using the following criteria:

A – 0 colonies: no growth;
B – 1 colony: 1–10 *C. albicans* isolates per mL of saliva;
C – 2 colonies: 10–100 *C. albicans* isolates per mL of saliva; and
D – 3 colonies: more than 100 *C. albicans* isolates per mL of saliva.[36]

Salivary pH measurements

To determine the pH of saliva, we analyzed the saliva samples using a pH meter (ISOLAB Laborgeräte GmbH, Wertheim, Germany) calibrated with 2 substances of pH 4 and 7. The device's electrode was washed with distilled water before being inserted into the sample. Salivary pH was expressed to 2 decimal places.

Salivary buffering capacity measurements

To determine the buffering capacity of the saliva, we added 0.1 mL of normal 0.05 hydrochloric acid to each saliva sample and measured the pH using a pH meter. This process was repeated multiple times to ensure consistency and continued until the pH of the saliva dropped sharply and the pH changes were minimal. According to this method, a solution with a greater resistance to acid had a greater buffering capacity, whereas a solution with a lower resistance to acid was considered to have a lower buffering capacity.[37]

Bias

The risk of bias was minimized by providing oral hygiene education 2 weeks before the study and using reliable methods to assess children's sleep patterns, salivary microbiota, pH, and buffering capacity in a dedicated laboratory in the city of Qom.

Statistical analysis

Data analysis was performed using the IBM SPSS Statistics for Windows software, v. 28.0 (IBM Corp., Armonk, USA) and descriptive statistics and percentages. Based on the type of variables, Spearman's correlation, *t*-test or χ^2 test was used. Correlation coefficient values were categorized as small (0.1–0.3), medium (0.3–0.5) and large (0.5–1.0).

Results

A total of 85 children participated in this study, with 41 having sufficient sleep and 44 having insufficient sleep. There was no significant difference in gender ($p = 0.450$) or age group ($p = 0.989$). The child's sleep pattern was not associated with the educational level of the mother or father ($p = 0.122$ and $p = 0.564$, respectively) (Table 1). Sleep patterns were linked to pH, buffering capacity, and colony counts of *Lactobacillus* spp. and *S. mutans*. Children with improper sleep patterns had significantly lower pH and buffering capacity ($p < 0.001$) and significantly higher colony counts of *Lactobacillus* spp. and *S. mutans* ($p < 0.001$ and $p = 0.012$, respectively). However, there was no significant association between sleep patterns and *C. albicans* (CFU/mL) ($p = 0.121$).

All of the subclasses correlated negatively with pH and salivary buffering capacity on medium to large scales (Table 2). There were no significant correlations between the subclasses and *S. mutans* colony counts, except for daytime sleepiness, which had a small positive correlation (correlation coefficient = 0.25, $p = 0.01$). A medium positive correlation was observed between the *Lactobacillus* spp. colony counts and sleep anxiety (correlation coefficient = 0.34, $p = 0.001$), parasomnias (correlation coefficient = 0.35, $p = 0.001$) and daytime sleepiness (correlation coefficient = 0.34, $p = 0.001$).

Table 3 presents the correlation between *Lactobacillus* spp., *S. mutans*, *C. albicans*, and buffering capacity, and the secondary variables recorded in this study. Two small correlations were identified: between daily snack consumption and *Lactobacillus* spp. colonies (correlation coefficient = 0.28, $p = 0.01$); and between daily fruit and vegetable consumption and *S. mutans* colonies (correlation coefficient = 0.27, $p = 0.01$).

Discussion

Sleeping habits have a significant impact on systemic health and play an essential immunoregulatory role in the oral cavity by influencing the flow rate and composition of saliva. Saliva, on the other hand, controls the pH and buffering capacity of the oral environment, as well as the types and abundance of oral microbial colonies,[14] including *C. albicans* fungus and *S. mutans* and *Lactobacillus* spp. These 2 bacteria are inherent in the oral cavity and are the major bacterial strains involved in caries development.[20,21]

The study revealed that children with inappropriate sleep patterns had lower salivary pH levels and higher saliva acidity compared to those with appropriate sleep patterns. Salivary *S. mutans* and *Lactobacillus* spp. loads were significantly higher in children with inappropriate sleep patterns. These 2 bacteria, in conjunction with the lower pH levels, can create an oral environment that is more susceptible to dental caries. Chen et al. found that

Table 1. Association between predictor variables and outcome variables

	Variables	Appropriate sleep pattern	Inappropriate sleep pattern	*p*-value
Participants, *n*		41	44	–
Age group, *n*	8 years	5	5	0.989[&]
	9 years	6	6	
	10 years	11	13	
	11 years	9	11	
	12 years	10	9	
Gender, *n*	male	19	24	0.450[&]
	female	22	20	
Single-parent child, *n*	yes	0	4	0.067[&]
	no	41	40	
Mother's education, *n*	not educated	4	1	0.122[&]
	up to secondary school	10	15	
	diploma	19	25	
	bachelor's and above	8	3	
Father's education, *n*	not educated	3	4	0.564[&]
	up to secondary school	12	18	
	diploma	14	14	
	bachelor's and above	12	8	
Brushing frequency [times/day]		1.02 ±0.68	0.84 ±0.68	0.207[#]
Flossing frequency [times/day]		0.24 ±0.62	0.06 ±0.25	0.133[#]
Daily brushing time [min]		2.19 ±1.72	1.63 ±1.41	0.189[#]
Snack consumption [times/day]		0.85 ±0.57	1.22 ±1.07	0.174[#]
Fruit and vegetable consumption [times/day]		1.75 ±1.01	1.54 ±1.06	0.231[#]
Dairy product consumption [times/day]		1.56 ±0.83	1.36 ±0.80	0.320[#]
pH		7.69 ±0.37	6.99 ±0.34	<0.001*[#]
Buffering capacity		7.46 ±0.37	6.77 ±0.42	<0.001*[#]
Lactobacillus spp. [CFU/mL]		13.04 ±9.50	32.45 ±21.14	<0.001*[#]
Streptococcus mutans [CFU/mL]		17.58 ±10.12	27.54 ±17.23	0.012*[#]
Candida albicans [CFU/mL]		4.17 ±5.83	14.45 ±24.08	0.121[#]

* statistically significant ($p < 0.05$); [&] χ^2 test; [#] independent t-test.

Table 2. Correlation between sleep quality and the average number of *S. mutans*, *Lactobacillus* spp. and *C. albicans* colonies, buffering capacity and pH

Variables	*Lactobacillus* spp.	*Streptococcus mutans*	*Candida albicans*	Buffering capacity	pH
Bedtime resistance	0.24[I] (0.027*)	0.20 (0.061)	0.14 (0.196)	–0.47[II] (<0.001*)	–0.53[III] (<0.001*)
Delayed sleep onset	–0.06 (0.530)	0.11 (0.310)	0.018 (0.090)	–0.20 (0.540)	–0.24[I] (0.020*)
Sleep duration	0.11 (0.287)	0.10 (0.361)	–0.06 (0.546)	–0.19 (0.070)	–0.29[I] (0.010*)
Sleep anxiety	0.34[II] (0.001*)	0.06 (0.537)	0.11 (0.314)	–0.47[II] (<0.001*)	–0.49[II] (<0.001*)
Night waking	0.22[I] (0.042*)	0.04 (0.654)	0.21[I] (0.044*)	–0.40[II] (<0.001*)	–0.42[II] (<0.001*)
Parasomnias	0.35[II] (0.001*)	0.10 (0.325)	0.25[I] (0.017*)	–0.38[II] (<0.001*)	–0.38[II] (<0.001*)
Sleep disordered breathing	0.18 (0.086)	0.08 (0.431)	0.09 (0.401)	–0.18 (0.099)	–0.14 (0.192)
Daytime sleepiness	0.34[II] (0.001*)	0.25[I] (0.017*)	0.10 (0.323)	–0.34[II] (0.001*)	–0.39[II] (<0.001*)

Data presented as Spearman's correlation coefficient (p-value); * statistically significant ($p < 0.05$); [I] small correlation; [II] medium correlation; [III] large correlation.

Table 3. Correlation between secondary variables and the average number of *S. mutans*, *Lactobacillus* spp. and *C. albicans* colonies and buffering capacity

Variables	*Lactobacillus* spp.	*Streptococcus mutans*	*Candida albicans*	Buffering capacity
Brushing frequency [times/day]	−0.06 (0.550)	−0.12 (0.250)	0.06 (0.530)	−0.03 (0.750)
Flossing frequency [times/day]	−0.09 (0.380)	−0.21 (0.060)	−0.11 (0.290)	0.06 (0.550)
Daily brushing time [min]	−0.08 (0.460)	−0.03 (0.730)	−0.06 (0.570)	−0.04 (0.680)
Snack consumption [times/day]	0.28[|] (0.010*)	−0.03 (0.770)	0.19 (0.070)	−0.07 (0.500)
Fruit and vegetable consumption [times/day]	−0.10 (0.320)	−0.27[|] (0.010*)	−0.00 (0.940)	−0.12 (0.260)
Dairy product consumption [times/day]	−0.10 (0.320)	−0.20 (0.060)	0.05 (0.580)	0.01 (0.860)

Data presented as Spearman's correlation coefficient (p-value); * statistically significant ($p < 0.05$); [|] small correlation.

insufficient sleep duration and delayed sleep onset were associated with an increased risk of dental caries in 3-year-old children.[38] In a study conducted by Roestamadji et al. on the risk of blood glucose, saliva levels and tooth decay in night shift workers, the night shift workers demonstrated a greater decrease in saliva pH levels than the control group.[18] Researchers believe that sleep deprivation activates the autonomic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis,[39] causing an increase in corticotropin-releasing factor and adrenocorticotropic hormone (ACTH) secretion from the hypothalamus, which in turn leads to cortisol secretion from the adrenal gland. Cortisol is associated with stress and affects saliva pH.[40] A study by Cohen and Khalaila demonstrated that stress can increase sympathetic nerve activity, leading to changes in the pH, acidity and buffering capacity of saliva.[41] This evidence confirms that sleep deprivation increases the acidity of saliva, which is a risk factor for tooth decay. Further research in this area is necessary.

The current study found that individuals with inappropriate sleep patterns had more *Lactobacillus* spp. and *S. mutans* colonies in their saliva; however, there was no significant difference in the number of *C. albicans* colonies between the groups. A study by Arvidsson et al. investigated the relationship between body mass index (BMI), eating habits, sleep, and the number of salivary *S. mutans* colonies in 4- to 11-year-old children in Sweden. The study found that a lack of sleep is associated with an increase in salivary *S. mutans* colonies.[42] Our findings are consistent with those of Alqaderi et al., who discovered a positive relationship between the lack of sleep and an increase in bacterial colonies in Kuwaiti children.[43] Chen et al., who investigated the relationship between insufficient sleep and dental caries in Japanese children, found a correlation between late sleep and elevated levels of IL-6, salivary *S. mutans* colonies, and an increased risk of dental caries in a child's primary and permanent teeth.[38] Sardana et al. investigated the effect of sleep on early childhood development of caries. Lack of sleep was cited as one of the causes of immune system weakness, which can increase bacterial colonies, including those that cause cavities, such as *S. mutans*.[44] The composition and properties of oral fluids play a significant role in the onset of dental caries, given that teeth are in contact with saliva. Saliva prevents caries in multiple ways by cleansing food and exhibiting buffering and antibacterial properties.[45]

In the current study, there was no significant difference in the number of *C. albicans* colonies between the 2 groups. There was only a slight positive correlation between night wakings and the parasomnia subclasses. The lack of differences between the 2 groups may be attributed to the small sample size, given the significant standard deviation in the *C. albicans* colony count values for both groups. In addition, there is no available evidence that evaluates the relationship between sleep patterns and the number of *C. albicans* colonies. This correlation should be investigated in future research.

Based on our study, there was a positive correlation between daily snack consumption and the number of *Lactobacillus* spp. colonies (Table 3), which suggests that snack consumption may increase the likelihood of caries development. In addition, daily consumption of fruits and vegetables was negatively correlated with *S. mutans*, indicating their potential role in preventing the progression of dental caries.

Limitations

Our findings were based on a relatively small number of participants. We focused on children living in 1 region, therefore, the generalizability of the findings to other populations may be limited. One of the limitations of the current study was the inability to match participants based on socioeconomic factors, which could influence a child's sleep, as well as their compliance with health and nutrition recommendations. The study's results should be interpreted with caution because filling out the questionnaires by parents was another limitation that could affect the results. However, this method appeared to be the most reliable, given the young age of the study participants.

Conclusions

There is a significant relationship between sleep patterns and salivary cariogenic bacteria, including *Lactobacillus* spp. and *S. mutans*. Participants with poor sleep had a higher salivary bacterial load. In addition, children with improper sleep patterns had more acidic saliva and a lower buffering capacity compared to children with normal sleep patterns. No correlation was found between sleep patterns and salivary *C. albicans* levels.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The Research Ethics Committee of Qom University of Medical Sciences approved the study protocol (approval No. IR.MUQ.REC.1401.143) All procedures were performed following relevant guidelines and regulations. Informed written consent was obtained from the children or their parents.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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