INTRODUCTION

Global populational studies document that dental caries represents the most frequently manifested civilizational disease (1, 2). The etiopathogenesis of dental caries includes several causes, and has not been completely clarified (3). Currently, the principal etiological agent initiating dental caries is known to involve cariogenic bacteria (cariopathogens). At this preliminary stage of the process, the principal role is played by oral streptococci, above all, by *Streptococcus mutans* (3). This species exopolysaccharides (EPS) (mostly glucans), which play the key role in the formation of the matrix of dental plaque (4). In parallel with this process, acids, generated by *S. mutans* from fermentable sugars are accumulated within the EPS matrix, and this creates a highly acidic, caries-initiating microenvironment (5). In turn, the significance of oral lactobacilli is recognized in the development of the caries process, particularly in the dentine environment.

The bacteria manifest a significant acidophilic character, and this promotes their rapid growth, inducing progress in dental caries (6, 7). Nevertheless, strains of *Lactobacillus* spp. can produce hydrogen peroxide and such bacteria can act antagonistically toward potential pathogenic species, inhibiting their growth (8, 9). In our earlier studies, we showed that oxygen peroxide-producing oral lactobacilli may counteract progression of chronic periodontitis (10). Human saliva contains unique acidic proline-rich proteins (APRPs). Currently, 10 different phenotypes of these proteins have been distinguished, of which APRP-1/2 isoforms are particularly physiologically important in the oral cavity. The phosphoproteins show high affinity for hydroxyapatite, but they may also bind oral bacteria, supporting bacterial colonization of dental surfaces (11, 12).

A previous study shows that levels of acidic salivary proline-rich phosphoproteins-1/2 (APRP-1/2) increase with caries severity. The aim of this study was to examine whether this relationship also depends on the presence of H$_2$O$_2$-producing strains of *Lactobacillus* spp. Adults with severe caries (decayed, missing, and filled teeth (DMFT) > 13.9, n = 28) were compared with similarly aged adults who had minimal caries (DMFT < 5, n = 20). A total of 48 samples of whole unstimulated saliva were collected in the morning and centrifuged. *Lactobacillus* spp. were isolated from the sediment in Rogosa agar and peroxide (H$_2$O$_2$) production was determined by growing the isolates on TMB-Plus agar. Salivary APRP-1/2 content in the saliva supernatant was estimated using an enzyme-linked immunosorbent sandwich assay (ELISA). Lactobacilli were present in 67% of both caries groups but were H$_2$O$_2$ positive only in the minimal caries group. Irrespective of the presence of Lactobacilli, the total content of APRP-1/2 proteins was 34.5 ± 4.9 ng/ml in severe caries but just under half this in minimal caries. We conclude that *Lactobacillus* spp. was absent from about a third of the severe and minimal caries groups, and H$_2$O$_2$-producing strains were present only in the minimal caries group. The severe caries group possessed twice the content of salivary APRP 1/2 proteins as the minimal caries group. The implications of these findings for caries development are discussed.

**Key words:** acidic proline-rich proteins, oral health, dental caries, oral lactobacilli, bacterial colonization, hydrogen peroxide, saliva

MATERIALS AND METHODS

Patients

The study was approved by the Medical Ethics Committee at University of Medical Sciences in Poznan, Poland. All subjects gave written informed consent as defined by the Declaration of Helsinki.
Dental studies and isolation of saliva samples from the qualified patients were conducted in the Department of Preclinical Conservative Dentistry and Preclinical Endodontics, University of Medical Sciences in Poznan, Poland.

The studies were conducted on 48 patients (25 – 42 years of age), where dental examination and calculation of the DMFT (decayed, missing, and filled teeth) index permitted the evaluation of the intensity of dental caries (caries experience). Patients qualified for studies manifested minimal (DMFT < 5) or severe dental caries (DMFT > 13.9), in line with WHO criteria (14). Also, dental caries (DT) was evaluated in all the patients, calculating the number of decayed surfaces as related to the surfaces of all teeth. Moreover, the PL.I (plaque index), was calculated, representing an exponent of dental plaque presence and thickness (15). Group 1 included 20 persons (25 – 40 years of age, mean 32.5 ± 3.8 years; 12 men and 8 women; DMFT = 2.3 ± 1.0) with minimal caries. Group 2 included 28 patients (26 – 42 years of age, mean 36.2 ± 4.9 years; 18 men and 10 women; DMFT = 19.5 ± 3.8) with severe dental caries.

The patients qualified for the studies were healthy, with no general or chronic diseases in anamnesis. Moreover, the exclusion criteria included fungal infection in the oral cavity, destructive periodontal diseases, bruxism and smoking of cigarettes. In the three weeks preceding the study, the patients were not subjected to hygienization procedures or to the use of anti-bacterial mouth washes.

**Salivary sample collection**

Samples of saliva from the patients were collected between 8:00 a.m. and 11:00 a.m. All subjects abstained from eating and drinking for 2 hours. Unstimulated whole saliva was collected for 10 min by the spitting method. Saliva samples were homogenized and clarified by centrifugation at 3000 × g for 15 min at room temperature. The aliquots of clarified supernatants were stored at −70°C for the APRP-1/2 measurements. In turn, the remaining supernatant and the sediment were used to prepare a homogenous suspension, which was tested for the manifestation of oral lactobacilli.

**Microbiological analyses**

*Lactobacillus* spp. was cultured on Rogosa agar, and the isolates obtained in anaerobic conditions were identified using API 50 CHL (bioMerieux). The capacity for hydrogen peroxide production among *Lactobacillus* spp. strains was defined in the cultures of the obtained isolates in the presence of 5% CO₂ at a temperature of 37°C for 48 hours in a differentiating medium, TMB-Plus agar, prepared according to Rabe and Hillier (16). Development of an altered color for the growing colonies (appearance of a blue color) indicated production of hydrogen peroxide. Examples of the obtained results are presented in Fig. 1 and Fig. 2.

**Determination of APRP-1/2**

In turn, salivary APRP-1/2 was quantitated using an immunoenzymatic technique (ELISA). A PRH2 ELISA kit (MyBioSource, San Diego) was applied, manifesting high specificity for detection of salivary APRP-1/2 and showing high sensitivity: 0.55 ng/ml. The tests were performed as recommended by the manufacturer. Values of absorbance, depending on estimated APRP-1/2, were read at the wavelength of A = 450 nm using a Reader 250 (bioMerieux). The results were obtained from standard curves. Every estimation of salivary APRP-1/2 was repeated three times, and the obtained mean represented the individual result for the patient.

Salivary samples collection, microbiological tests and determinations of salivary APRP-1/2 were conducted in the Department of Medical Microbiology, University of Medical Sciences in Poznan, Poland.

**Fig. 1.** H₂O₂-producing *Lactobacillus* strain (note the blue color of grown-up colonies on TMB-Plus agar in a 48 h aerobic culture with supplementation of 5% CO₂, at 37°C).
Data analysis

Results obtained in the studies were analyzed using STATISTICA v. 12.0. In the analysis of quantitative characters, we used mean arithmetic values, standard deviations and median values. The Shapiro-Wilk test demonstrated the absence of a normal distribution for the data. Consequently we employed the non-parametric Mann-Whitney test in the statistical analysis. Differences with P-values higher than 0.05 were considered insignificant.

RESULTS

*Lactobacillus* spp. was detected in saliva in 14 persons in group 1, manifesting minimal dental caries and in 18 patients of group 2 with severe dental caries. In both groups of patients, the proportion of *Lactobacillus* strains amounted to 67%. Based on the detected presence of oral lactobacilli in the two examined groups, subgroups were distinguished: 1A: including 14 persons with detected salivary *Lactobacillus* spp.; 1B: including the remaining 6 persons from group 1. 2A: including 18 patients with detected *Lactobacillus* spp. and 2B: including the remaining 10 persons from group 2. In parallel, it was demonstrated that a capacity for H\(_2\)O\(_2\) production was manifested by all the obtained isolates of *Lactobacillus* spp. from subgroup 1A. On the other hand, 16 of the obtained isolates of *Lactobacillus* spp. in subgroup of 2A failed to produce H\(_2\)O\(_2\), while in the remaining two isolates production of hydrogen peroxide posed difficulties in interpretation.

The data, together with results for DMFT, DT and PL.I indices, are listed in Table 1. Mean values for DMFT, DT and PL.I in subgroup 1A amounted to, respectively, 2.43 ± 0.76, 0.58 ± 0.19, and 0.51 ± 0.14.
Comparing the obtained results across the studied groups, statistically significant differences were revealed in terms of the mean DMFT values across subgroups of group 1 (1A and 1B) and subgroups of group 2 (2A and 2B). However, mean DT values in the subgroups of 1A and 1B were significantly lower than those in group 2A, but they did not significantly differ from the mean value in subgroup 2B. At the same time, mean PL.I values in subgroups 1A and 1B were significantly lower than the mean values in subgroup 2A, but they manifested no statistical difference from the mean value in subgroup 2B. The results of comparative analysis of DMFT, DT, and PL.I values across individual subgroups are listed in Table 2. In turn, mean values for salivary APRP-1/2 in 1A and 1B subgroups amounted to, respectively, 15.6 ± 2.9 ng/ml, 15.8 ± 3.2 ng/ml, while in 2A and 2B subgroups these were significantly higher, amounting to, respectively, 32.4 ± 4.6 ng/ml and 36.5 ± 5.1 ng/ml. The obtained results are listed in Table 3.

**Table 2. Comparison of DMFT, DT, and PL.I across the studied subgroups.**

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>P-values</th>
<th>DMFT</th>
<th>DT</th>
<th>PT.I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A &amp; 1B</td>
<td></td>
<td>0.6023</td>
<td>0.5048</td>
<td>0.6487</td>
</tr>
<tr>
<td>1A &amp; 2A</td>
<td>&lt; 0.0001*</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001*</td>
<td></td>
</tr>
<tr>
<td>1A &amp; 2B</td>
<td>&lt; 0.0001*</td>
<td>0.1056</td>
<td>0.6589</td>
<td></td>
</tr>
<tr>
<td>1B &amp; 2A</td>
<td>&lt; 0.0001*</td>
<td>&lt; 0.0001*</td>
<td>&lt; 0.0001*</td>
<td></td>
</tr>
<tr>
<td>1B &amp; 2B</td>
<td>0.0012*</td>
<td>0.1366</td>
<td>0.5928</td>
<td></td>
</tr>
<tr>
<td>2A &amp; 2B</td>
<td>0.0001*</td>
<td>&lt; 0.0001*</td>
<td>&lt; 0.0001*</td>
<td></td>
</tr>
</tbody>
</table>

Asterisks mark statistically significant differences between the examined subgroups in terms of DMFT (decayed, missing, and filled teeth), DT (dental caries) and PL.I (plaque index).

**Table 3. Results of estimating salivary APRP-1/2 in patients with minimal caries (group 1) - subgroup 1A and 1B or with severe caries (group 2) - subgroup 2A and 2B.**

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>APRP-1/2 mean values ± SD (median values)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (n = 14)</td>
<td>15.6 ± 2.9 [16]</td>
<td>1A vs. 1B P = 0.353</td>
</tr>
<tr>
<td>1B (n = 6)</td>
<td>15.8 ± 3.2 [15.4]</td>
<td>1A vs. 2A P &lt; 0.001*</td>
</tr>
<tr>
<td>2A (n = 18)</td>
<td>32.4 ± 4.6 [39.2]</td>
<td>1B vs. 2B P &lt; 0.001*</td>
</tr>
<tr>
<td>2B (n = 10)</td>
<td>36.5 ± 5.1 [39.6]</td>
<td>2A vs. 2B P = 0.389</td>
</tr>
</tbody>
</table>

Asterisks mark statistically significant differences between the examined subgroups. 1A: denotes the subgroup of patients with minimal caries, in whom oral lactobacilli were detected; 1B: denotes the subgroup of patients with minimal caries, in whom oral lactobacilli were not detected; 2A: denotes the subgroup of patients with severe caries, in whom oral lactobacilli were detected; 2B: denotes the subgroup of patients with severe caries, in whom oral lactobacilli were not detected.

**DISCUSSION**

The studies presented in this paper were conducted on patients with minimal dental caries (group 1) or with severe dental caries (group 2), as distinguished by DMFT index values, in line with WHO criteria (14). The above index expresses the dynamic nature of ongoing dental caries. Therefore, evaluation of DMFT values allows for an objective analysis of the obtained data in the context of the intensity manifested by the caries process. In parallel, to perform complex analyses of dental caries intensity in all participants the degree of dental caries (DT), and the plaque index (PL.I) were evaluated (15). PL.I value hygienic reflex condition of the oral cavity, which plays a significant role in the development and intensity of dental caries (17).

Based on the results of bacteriological studies, subgroups of patients were distinguished: 1A and 2A - with the presence of salivary Lactobacillus spp., and 1B and 2B - in whom no such bacteria were detected. Lactobacilli are considered to comprise just 0.1 – 1% of microflora in the oral cavity (18). A marked reduction of the microbial contents in saliva is also possible, and this seems to explain the negative results obtained in this study from cultures, the traditional method of detecting bacteria. In turn, the human oral microbe identification microarray (HOMIM) technique used in recent years does not detect Lactobacillus spp. more frequently in full saliva (19). Moreover, use of the culture method alone allows bacterial isolates to be obtained which are required to analyze their properties, including their ability to produce hydrogen peroxide.

The proportion of detectable Lactobacillus strains in both caries groups amounted to 67%. The data seem to contradict the results of Caufield et al. (20): these authors suggested that oral lactobacilli are mainly associated with caries progression, since the bacteria may accumulate in the arising precaries lesions, playing the role of ‘retentive nitches’. However, no data are as yet available which would specify the correlation between the number of lactobacilli in caries lesions and their manifestation in saliva. The conducted dental studies indicate that the two subgroups, 1A and 1B, did not significantly differ in terms of the mean values of DMFT, DT and PL.I; they were associated with minimal dental caries, corresponding to the criteria for good oral cavity hygiene. At the same time, saliva samples from patients of subgroup 1A were found to contain hydrogen peroxide-producing lactobacilli while no such bacteria were detected in the 1B subgroup. Therefore, no oral lactobacilli were detected in subgroup 1B possibly due to their low numerical force. Nevertheless, it is difficult to exclude the possibility of the absence of such bacteria in the whole saliva of some patients. Therefore, the obtained data allow the conclusion that in the prevention of the development of dental caries it may not only be the presence of hydrogen peroxide-producing oral lactobacilli, as indicated earlier, that is significant (21). This conclusion, at least in part, may be supported by Karaoglanoglu et al. (22), who demonstrated a lack of any correlation between DMFT values and lactobacilli levels in adults. In turn, mean PL.I values did not differ between subgroups 1A and 1B. Thus, the data may provide evidence for the significance of good oral hygiene level in prevention against dental caries, independent of the manifestation of hydrogen peroxide-producing oral lactobacilli.

The subgroups of patients distinguished in this study, 2A and 2B, differed significantly in terms of mean values for DMFT, DT and PL.I. Nevertheless, both subgroups manifested a high intensity of dental caries, in line with WHO criteria. In turn, present caries was approximately 4.5-fold more intense in...
subgroup 2A than in 2B. At the same time, saliva samples from patients of subgroup 2A were found to contain lactobacilli not producing hydrogen peroxide (only in 2 isolates was production of hydrogen peroxide difficult to interpret), while no such bacteria were detected in subgroup 2B. Moreover, mean PL.I values corresponded to poor oral cavity hygiene in subgroup 2A and good hygiene in the 2B. The high detected DMFT values, accompanied by low DT in subgroup 2B point to inhibition of the present caries. Such data may also confirm the significance of good hygiene in the oral cavity in prevention against dental caries. However, the significantly higher DT values in subgroup 2A as compared to 2B subgroup allow, for the first time, the conclusion that progression of dental caries seems to be linked to strains of Lactobacillus spp., which do not produce hydrogen peroxide. In parallel, Lactobacillus strains produce acids, representing cariogenic bacteria, independent of their ability to release H₂O₂. Nevertheless, in patients with minimal caries, the detected lactobacilli were H₂O₂-positive only. Thus, the obtained results may indicate that H₂O₂ released by Lactobacillus strains acts antagonistically toward mutants streptococci, restricting progression of caries. In parallel, the activity of saliva antioxidant enzymes - peroxidase and catalase, remains reduced in part due to excessive production of H₂O₂. (25). Thus, development of caries is linked not only to microbial specificity, but also depends on microbial activity, as earlier noted by Marsh (24).

The estimations of salivary APRP-1/2 conducted in this study demonstrated that the mean values were significantly higher in patients manifesting severe dental caries (group 2) than those in patients with minimal dental caries (group 1). Structural studies indicate that APRP-1/2 involve two positional isoforms, each involving a single polypeptide chain which consists of 150 residues (25). Its N-terminal domain consists of 30 residues including two phosphoserines but only one proline. In turn, the sequence from residue 31 to the C-domain represents a proline-rich region, including repeats high in proline, glycine and glutamine. At present, the unique structure of APRP-1/2 is thought to determine its specific properties (26). The N-terminal domain ensures coupling of APRP-1/2 to hydroxypatite and formation of the acquired enamel pellicle. In parallel, the C-terminal domain of ARP-1/2 binds to oral bacteria promoting bacterial colonization of dental surfaces, which may play a significant role in the pathogenesis of caries (12, 13).

The presented results were analyzed in the distinguished subgroups: 1A, 1B and 2A, 2B. In the two subgroups 2A and 2B mean values for salivary APRP-1/2 were significantly lower than the mean values in the two subgroups 1A and 1B. However, no significant differences were revealed between ARP-1/2 values across the individual subgroups 1A and 1B, and 2A and 2B.

These results suggest that elevated levels of APRP-1/2 may cooperate in caries processes. However, the good hygienic condition of the oral cavity and the presence of hydrogen peroxide-producing oral lactobacilli seem to be of significance in the prevention of caries processes.

Author contributions: A.K. Szkarakiewicz-Karpinska designed the study concept; A.K. Szkarakiewicz-Karpinska, A. Zeidler, O. Goslinska-Kuzniarek and K. Uram performed the material collection and the experiments; A.K. Szkarakiewicz-Karpinska and A. Szkarakiewicz analyzed the data and wrote the paper.

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