Micron 172 (2023) 103498

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Detection of bacteria in dental samples using the Periodic acid-Schiff (PAS) histological stain

Luciano Giardino [a], Luigi Generali [b], Massimo Del Fabbro [c,d], Gianluca Martino Tartaglia [c,d], Alessandro Bidossi [e], Paolo Savadori [c,d,*,1]

[a] Independent Researcher, 88900 Crotone, Italy
[b] Department of Surgery, Medicine, Dentistry and Morphological Sciences with Transplant Surgery, Oncology and Regenerative Medicine Relevance (CHIMOMO), University of Modena and Reggio Emilia, 41124 Modena, Italy
[c] Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, Università Degli Studi di Milano, 20122 Milan, Italy
[d] IRCCS Fondazione Cà Granda IRCCS Ospedale Maggiore Policlinico, 20133, Milano, Italy
[e] LTA S.r.l, 20041 Bussero, MI, Italy

ARTICLE INFO

Keywords:
Bacterial polysaccharides
Dentin
Glycoproteins
PAS stain
Root canals

ABSTRACT

The bacterial cell wall mainly consists of glycoproteins and polysaccharides, which could be detected in dental tissue with specific stain protocols. The present study aimed to investigate bacteria stainability in dental histological samples of human teeth by a histochemical method. Eight extracted teeth, because severely decayed, were decalcified, dehydrated, paraffin-embedded, and serially sectioned at 4 μm thickness each. The serial sections were then stained with Periodic acid-Schiff (PAS). Furthermore, SEM analysis was performed on the same slide of one previously histologically investigated tooth to acquire more details on the structures stained by the PAS method obtained from the histological procedures. Afterward, some American Type Culture Collection (ATCC) strains, smeared on glass slides, were stained following the staining method used in histological samples. Stained rod and cocci forms by PAS stain, observed under light microscopy, were predominantly detected inside dentinal tubules and root canal space of histologically examined specimens, suggesting their bacterial origin. Additional SEM analysis on the identical histological stained slide showed the precise nature of these forms (bacteria) and supplemental information regarding their vitality status. In addition, ATCC smeared strain samples showed variable PAS stainability of microorganisms investigated. Due to its properties, the PAS histochemical stain could be a valid and helpful aid for non- or weakly stainable microorganisms in infected tissues to be associated with other methods of investigation.

1. Introduction

The most frequently used technique to detect bacteria in paraffin-embedded tissues is the Gram stain (Gram, 1884), introduced one hundred forty years ago and first published a year earlier (Friedlander, 1883), modified only slightly since then (Woods and Walker, 1996). In its original method, certain bacteria, in the presence of the crystal violet-aniline dye and iodine, formed compounds insoluble in solvents such as alcohol (Gram-positive). At the same time, other types failed to retain the crystal violet-aniline in the presence of alcohol (Gram--negative). Therefore, some authors (Brown and Brenn, 1931) modified the original stain to simultaneously demonstrate Gram-positive and negative bacteria in tissue sections to overcome these drawbacks. However, the Brown-Brenn method does not regularly stain Gram-negative bacteria because this technique is more effective for detecting Gram-positive bacteria than Gram-negative ones, which may be undetected (Vera et al., 2012; Savadori et al., 2022); this is probably due to the degree of differentiation induced by the picric acid-acetone (Luna, 1968 pp. 222–223) and by operator's manual skills. Because Gram-negative bacteria are tough to visualize in tissue samples due to a lack of contrast between the bacteria and the counter stain (Gupta et al., 2009), a modification was suggested to detect these bacteria as an elective stain for these microorganisms (Brown and Hopps, 1973). Recently has been demonstrated that using isopropyl alcohol and a small

* Corresponding author at: Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, Università Degli Studi di Milano, 20122 Milan, Italy.
E-mail address: paolo.savadori@unimi.it (P. Savadori).
[1] ORCID: 0000-0001-8606-4694

https://doi.org/10.1016/j.micron.2023.103498
Received 24 March 2023; Received in revised form 30 May 2023; Accepted 1 June 2023
Available online 2 June 2023

amount of acetone (Savadori et al., 2022) instead of acetone as decoloring agent (Brown-Hopps's technique) because of its fast destaining (Bartholomew, 1962) was possible to enhance Gram-negative bacteria identification in paraffin-embedded samples. Today Gram staining procedure and its modifications are generally recognized as a fundamental diagnostic tool. However, many individual details influence the results, such as the concentration of dyes or iodine used, the procedure used for decolorization, and the nature of the counterstain, as previously reported (Bartholomew and Mittwer, 1952). Even though it is mainly used in clinical and histological laboratories, Gram stains have limitations. Indeed, some Gram-negative bacilli stain weakly and therefore are challenging to visualize, while mycoplasmas and spirochetes do not stain by those methods (Zanconati et al., 1994; Woods and Walker, 1996).

Some Gram-positive bacteria (i.e., *clostridia spp.*) instead, after decolorizing step, stained as Gram-negative, revealing a gradual, progressive increase toward gram negativity as the cultures aged (Beveridge, 1990). Due to these disadvantages, it could be helpful to consider a supplemental staining method such as Periodic acid-Schiff (PAS) to visualize fungal and some bacterial morphology under bright field microscopy (Mc Manus, 1948). Initially, this method was designed as a histochemical test to detect polysaccharides such as glycogen and mucin in tissues. The first chemical process is the oxidation of hydroxyl groups of cell wall polysaccharides to aldehydes; then, aldehydes combine with the Schiff reagent, staining in purple-magenta tissue structures rich in carbohydrates and microorganism walls. Formerly, some authors demonstrated that Gram-positive cocci and at least one species of Gram-negative rods were variably PAS-positive because the PAS method stains carbohydrate components in bacterial cell walls (Khavari et al., 1991). This result could be attributed to the fact that the peptidoglycan forms around 40–90% of the cell wall's dry weight of Gram-positive bacteria but only around 10% of Gram-negative strains (Misra et al., 2015). Based on this knowledge, the present study intended to verify PAS stain hypothesizing its practical use as a complementary tool in detecting bacteria in paraffin-embedded dental tissues and smear samples.

In one of eight histologically processed samples, the same slices, first stained positively by PAS and visualized with light microscopy, were also observed by scanning electron microscopy (SEM) to confirm the presence or no of bacteria inside dentinal tubules and root canal space.

2. Materials and methods

2.1. Samples preparation and staining

Eight extracted teeth from patients affected by severe caries and poor prognosis for their restoration were preliminarily used to test the staining capability of the PAS method on bacteria in root canal space and smears. All patients gave verbal consent to use their extracted teeth for scientific purposes. According to the institutional and national research committee, this type of research (in-vitro study based on teeth extracted because of the disease) does not require additional consent from the committee as it is not an in-vivo medical experiment.

First, teeth were fixed in 10% buffered formalin solution (Carlo Erba reagent, Milano, Italy), then decalcified, embedded in paraffin, and serially sectioned according to an established and previously reported method (Giardino et al., 2019a; Savadori et al., 2022).

Briefly, after fixation, the samples were decalcified in a solution of 50:50 of 22.5% formic acid and 10% sodium citrate for about 3–4 weeks, then dehydrated through ethanol solution baths and infiltrated with paraffin. Samples embedded in paraffin blocks were cut into 4 μm slices with a rotary microtome model RM2245 Leica (Leica biosystem, Nussloch - Germany) and placed in SuperFrost® microscope glass slides (V. W.R. International, Milano, Italy) to ensure a good adherence between the sections and the glass.

Sections were then stained with Periodic acid-Schiff (PAS) according to the AFIP Manual of Histologic Staining Techniques (Luna, 1968, pp. 158–160). Subsequently, 0.1% Fast green FCF solution (Carlo Erba reagent, Milano, Italy) was used as a counterstain to improve the background contrast between PAS-positive and PAS-negative structures, following a protocol suggested (Baum, 2008). The images were acquired with an Olympus CX43 light microscope coupled with ad Olympus LC30 camera (sensor: color CMOS ½ inch; resolution: 2048 ×1532 pixels, pixel size 3.2 ×3.2 μm; camera adapter: Olympus U-TV0.5XC-3) (Evident-Olympus, Tokyo, Japan) and elaborated with CellSense software (Evident-Olympus, Tokyo, Japan). Moreover, images taken at 1000x magnification (Olympus 100x/1.25 Oil PlanC N UIS 2, ∞/-/FN22. Eyepiece: Olympus WHB10x/20) were obtained by stacking 3 different shots with different depth filed to have a single focused shot. This visual elaboration was done using the plug-in z-stack of ImageJ software ((N.I.H., Bethesda, Maryland. https://imagej.nih.gov/ij/index.html) and allowed increasing the detail level. Finally, the images refiguring the whole tooth section were obtained by sampling the slide at 40x magnification (Olympus 4x/0.10 P Plan N UIS 2, ∞/-/FN22. Eyepiece: Olympus WHB10x/20) and then assembled with AutoStitch software, as seen in Fig. 1A (Brown and Lowe, 2007; http://matthewalunbrown.com/autostitch/autostitch.html).

A further study was also performed in a slide containing PAS-stained sections of one tooth forming part of this investigation, previously observed under light microscopy and then visualized by scanning electron microscopy (SEM). The slide was first immersed in xylene to float off the coverslip. Herein, the time spent detaching the coverslip from the slide immersed in xylene was three days. Immersion of slides in xylene usually ranges from 30 min to several days, depending on when they were prepared and mounted (Geissinger, 1971). The specimen was mounted on metal stubs (right side of Fig. 1), using a graphite-based conductive tape and gold sputter coated, then observed by scanning electron microscopy (SEM: Nova NanoSEM 450 equipped with a field emission gun – FEG – source: FEI – ThermoFischer Scientific, Hillsboro, OR, USA) working at 20 kV and images were obtained at different magnifications from 103x to 16.000x.

2.2. PAS method stainability on bacterial strains

Validation of the PAS method for bacteria staining was performed on two different American Type Culture Collection (ATCC) bacterial strains: *Enterococcus faecalis (E. faecalis* ATCC 29121) and *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27853). Bacteria were provided from L. T.A. S.r.l. (Bussero, Milano, Italy) and stored at − 80 °C (Savadori et al., 2022). Before use, the strains were thawed and cultured in Brain Heart Infusion broth (B.H.I., Difco, Kansas City, MO, U.S.A.) for 24 h at 37 °C in aerobic conditions (Giardino et al., 2019b). Individual and mixed strains of both microorganisms were smeared on microscope slides following a previously described procedure (Petersen and Mclaughlin, 2016) and then stained by the PAS method. Additional glass slides were smeared with *Treponema denticola* (*T. denticola*) strain ATCC 35405 obtained by American Type Culture Collection (10801 University Boulevard Manassas, VA, U.S.A.). It was grown for 48–96 h at 37 °C in anaerobic conditions and established through the broth culture's turbidity visualization, then smeared on glass slides and stained by the PAS technique. This test was additionally used to verify the stainability by the PAS method on a microorganism that is notoriously poorly or difficult to stain with conventional staining techniques (Zhou et al., 2015). Some authors (Woods and Walker, 1996) reported that these thin spiral bacilli do not stain by Gram, then further samples were prepared and stained by Gram's method (Luna, 1968 pp. 222–223) to confirm or not their Gram-negativity. Image acquisition was performed the same way as for tissue sections. In addition to light microscopy, *T. denticola* smears were acquired and observed by dark-field, phase-contrast, and SEM microscopy.

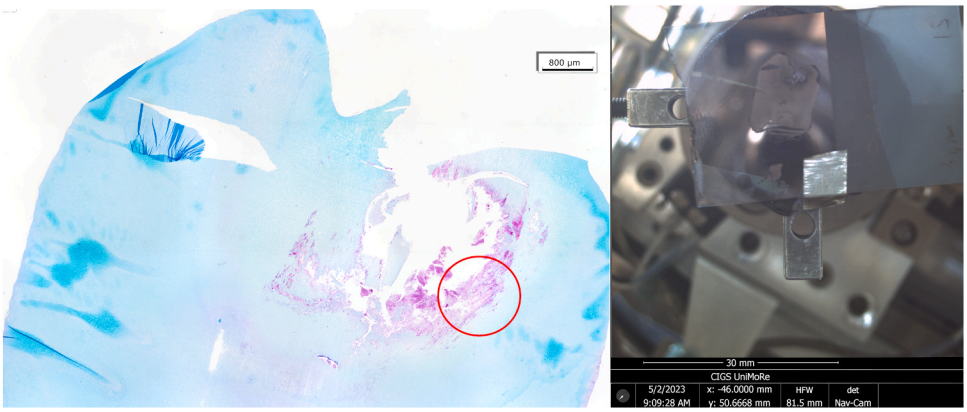

Fig. 1. To the right side of the image, a view of the slide PAS stained acquired by light microscopy at 40x; to the left side instead, a micrograph of the same histological slide after detaching the coverslip from the slide, placed in the SEM vacuum chamber, adequately prepared for SEM analysis. The circled area to the left indicates the slide zone investigated by light and SEM microscopy.

3. Results

Fig. 1 visualizes a panoramic image of the coronal portion of a third mandibular molar formed by thirteen fields assembled with AutoStitch software acquired by light microscopy at 40x; in the right part of the figure, the same histological sample, after detaching the coverslip from the slide and gold coated immediately before SEM analysis.

Fig. 2 shows the histological slice stained by the PAS-Fast green FCF method (Fig. 2A). At higher magnifications (Fig. 2C, D), intense bacterial colonization can be seen in the dentinal tubules and root canal space. Rod and cocci were the dominant morphological forms detected (Fig. 2E, F). Due to a counterstain with Fast green FCF, a green uniform background is observed, improving the contrast between PAS-positive and PAS-negative structures.

In Fig. 3, correlative scanning electron microscopy analysis of the same slide view in Fig. 2 confirmed previous findings. Fig. 3A documents the same area processed by light microscopy in Fig. 2A at 103x, while Fig. 3B and C represent the same area magnified at 300x and 1000x, respectively. Fig. 3E-F gives a better-defined image resolution of bacteria detected containing some new information as their living status (bacteria in the phase of division). Moreover, microorganisms appear well-demarcated from surrounding structures such as dentinal tubules and root canal space (3D).

Both certified smeared ATCC bacteria strains (Gram-positive *E. faecalis* and Gram-positive *P. aeruginosa*) using this staining protocol and observed under bright field microscopy at 1000x magnifications, were variably colored from weak to intense purple-magenta likely due to different amounts of carbohydrates of their cell walls (Fig. 4A, B, C). Bacterial suspension of *T. denticola,* smeared on slides, besides that be visualized by phase-contrast and dark-field microscopy (Fig. 5A, B), was differentiated by the Gram stain as Gram-negative (Fig. 5C) and identified as PAS-positive after using PAS stain and their turning purple-

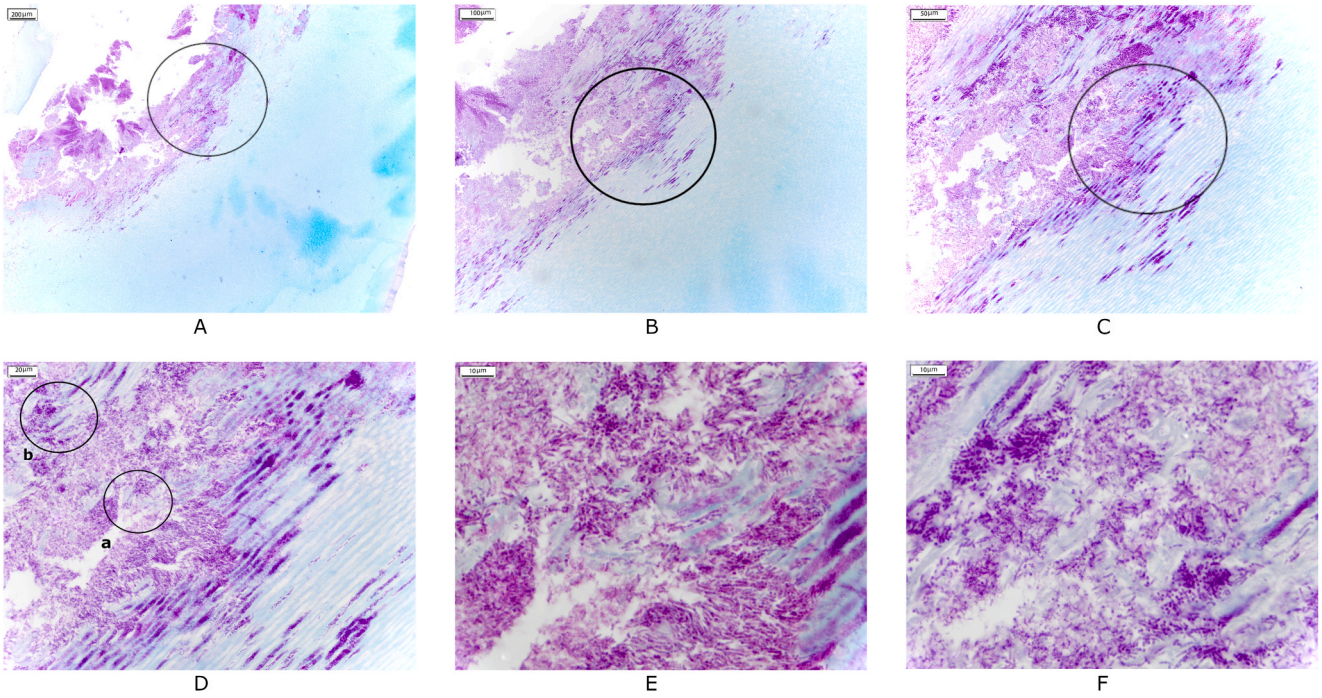

Fig. 2. Images panel of tooth histological section stained with PAS-Fast green FCF and acquired through light microscopy. 2A 40x image of the coronal portion of the sectioned tooth. B, C, and D represent further enlargements to 100x, 200x, and 400x. Microorganisms and biofilm can be seen on the dentin wall and within the dentinal tubules in the two circled areas **a** and **b** of Fig. 2D. Rod and cocci were found within the dentinal tubules (2E) and the root canal space (2 F). The 2E and 2 F images at 1000x magnification were generated by stacking multiple images with different depth fields; the morphology of the bacteria is better visible.

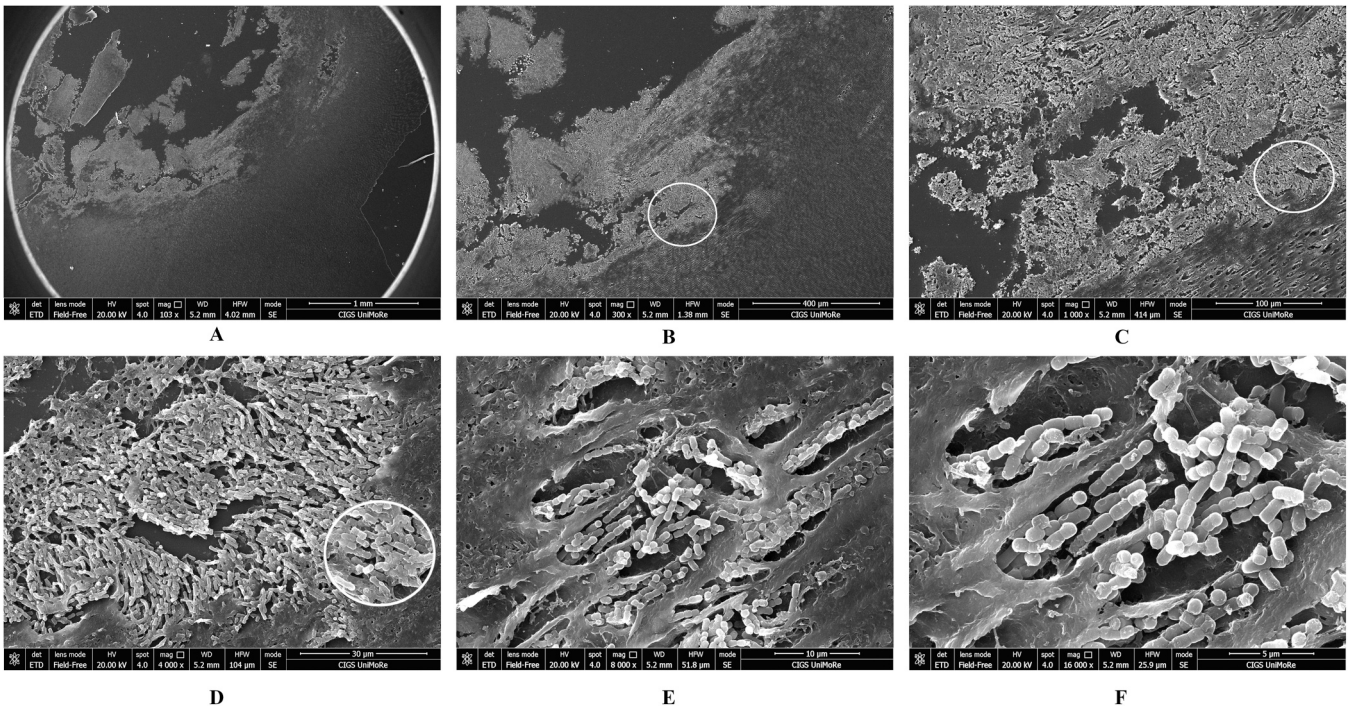

Fig. 3. Image panel acquired by SEM microscopy of the same histological section stained with PAS-Fast green FCF (Fig. 1) to obtain an image correlation. 3A micrograph shows the section previously obtained in Fig. 2A by light microscopy. SEM acquired images 3B-3F at different magnifications from 300 to 16.000x. These magnified pictures clearly show the bacteria morphology and localization in root canal space and dentinal tubules (3D, 3E, 3F); the bottom right of Fig. 3D is an enlarged detail of bacteria rod morphology. Furthermore, comparing the PAS - Fast green FCF sample taken with light microcopy and the same section analyzed with SEM shows that PAS-positive substances are bacteria.

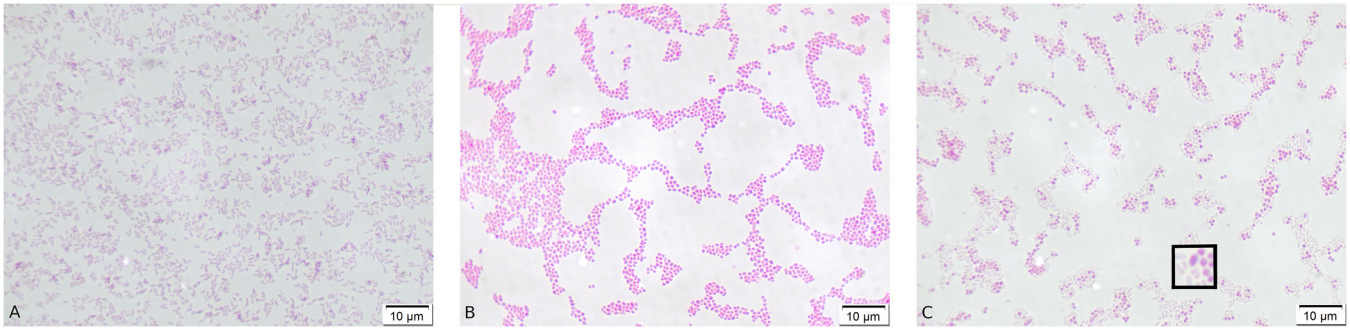

Fig. 4. Bacteria smears after staining by PAS method: A *E. faecalis*, B *P. aeruginosa*, C *E. faecalis* and *P. aeruginosa* mixed smear. In C, a squared detail shows more intense colorability in the magenta of E. faecalis compared to the more faded one of *P. aeruginosa*. Magnification, 1000x.

magenta (Fig. 5D) similarly to the other two bacterial strains. Fig. 5A, C, and D were acquired at 1000x magnifications instead Fig. 5B at 400x magnifications. A magnified morphological view of *T. denticola* on a smeared glass slide is shown in Fig. 6 (A, B, C). A better visualization was possible at magnifications greater than 3000x (B, C), confirming its slim structure challenging to identify in embedded histological tissues.

4. Discussion

Introduced since the middle of the last century to demonstrate glycogen, glycolipids, mucopolysaccharides such as mucin, mucoproteins, and glycoproteins in paraffin-embedded samples (Mc Manus, 1948), the PAS stain has also been widely used to visualize fungi in tissues (Kligman and Mescon, 1950). By a previous report (Mc Manus, 1948) using this staining method, it has been confirmed that all naturally occurring biological compounds in histologic sections stained positively from pink to purple-red with the PAS technique belong to the carbohydrate class (Hotchkiss, 1948). The reason why this staining method is superior to any other employed resides in the structure of fungal cell walls, mostly made up of carbohydrates and glycoproteins (Latgé, 2007; Ruiz-Herrera and Ortiz-Castellanos, 2019), producing intense staining of fungi in tissues (Hotchkiss, 1948). Similarly to fungi, glycoproteins are also present on the bacterial cell surface of both Gram-positive and negative microorganisms such as glycoproteins, exopolysaccharides (EPSs), capsular polysaccharides (CPSs), lipopolysaccharides (LPSs), lipooligosaccharides (LOSs), lipoglycans, peptidoglycan (PG), teichoic acids (TAs), and other glycosylated secondary cell wall polymers (Tytgat and Lebeer, 2014). Structurally, Gram-negative bacteria are enclosed by two cell membranes separated by a thin peptidoglycan layer and display lipopolysaccharides (LPSs) embedded in the outer membrane (Campanero-Rhodes et al., 2020). Lipopolysaccharide (LPS) is the major component (75%) of the outer membrane surface of Gram-negative bacteria (Le Brun et al., 2013). Instead, Gram-positive bacteria only have one cell membrane coated with a thick peptidoglycan layer, usually displaying teichoic acids (TAs) or lipoteichoic acids (LTAs) and capsular polysaccharides anchored to the

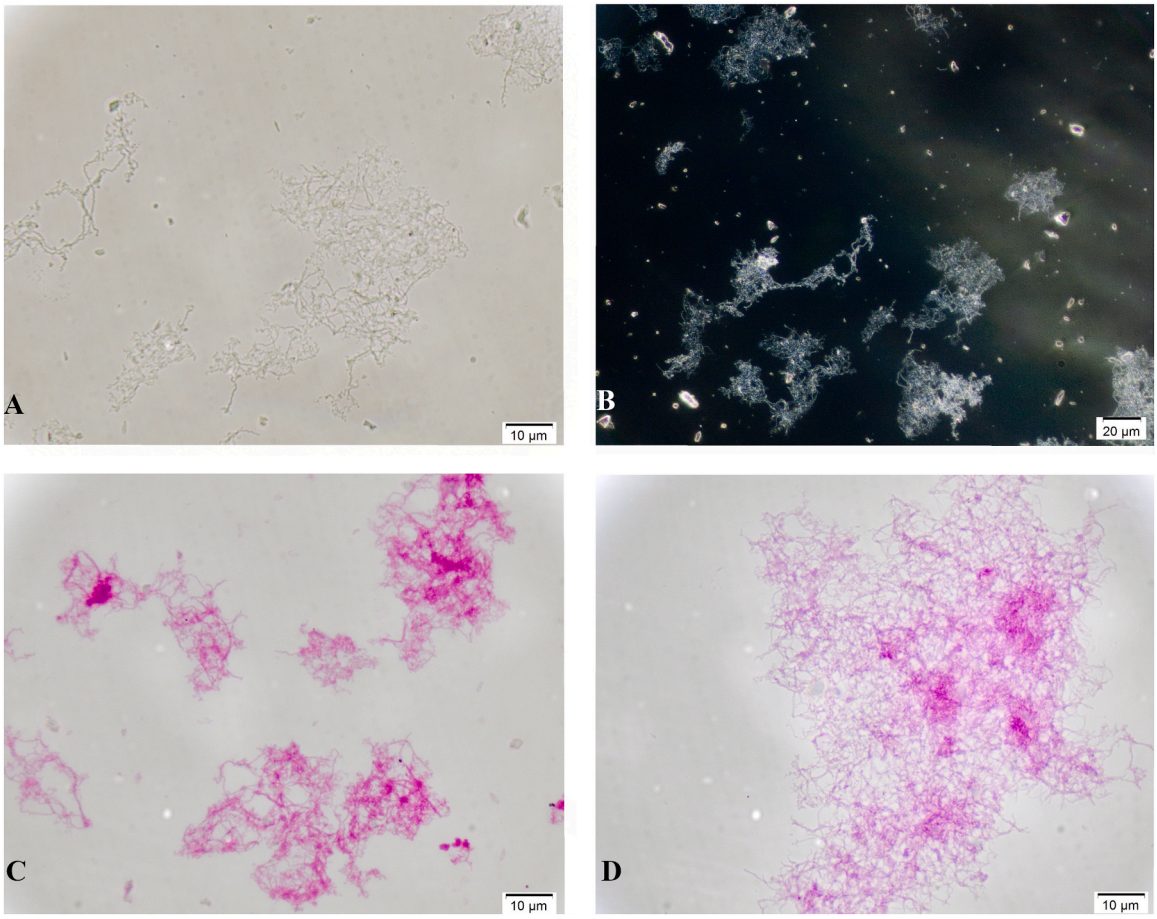

Fig. 5. Composite image showing *T. denticola* under phase contrast and dark field on unstained smears (5 A, 5B). C and D show a smear stained by Gram and PAS under a bright field.

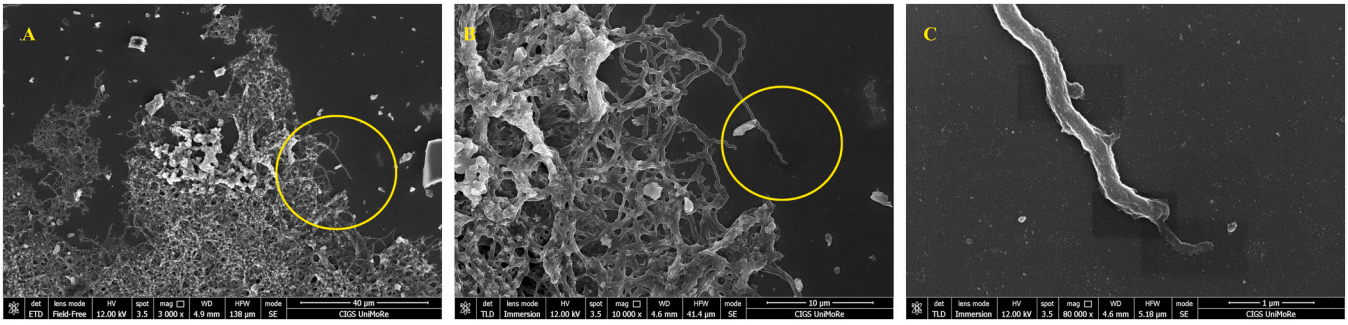

Fig. 6. Scanning electron microscope micrographs of smeared *T. denticola* bacterial aggregate showing its spiral morphology at different magnifications (3000x, 12000x, 80000x).

membrane (Schäffer and Messner, 2001). Peptidoglycan, consisting of sugars and amino acids, is another component of the bacterial cell wall mainly found outside the cytoplasmic membrane of almost all bacteria and forms 90% of the dry weight of Gram-positive bacteria but only 10% for Gram-negative strains (Vollmer et al., 2008). Because cell walls of both classes of bacteria are composed of polysaccharides, which, upon oxidation, could form aldehyde groups detectable by PAS stain, this staining method could be a valuable diagnostic tool to visualize bacteria in paraffin-embedded and smear samples. As reported above, only one study found PAS positivity in some Gram-positive and one negative bacteria (Khavari et al., 1991). Lipopolysaccharide (LPS) from Gram-negative bacteria is the major virulent factor involved in primary endodontic infections, contrary to secondary endodontic infections sustained mainly by lipoteichoic acid (LTA) endotoxin of Gram-positive microorganisms (Gomes et al., 2021). Currently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis is one of the principal methods used to separate, identify, and characterize bacterial components, including LPS stained by PAS stain (Maskell, 1991; Zhan et al., 2021). Based on electrophoresis assay and positive PAS staining, some authors investigated and detected the presence of glycoproteins purple-stained in LPS gels of Gram-negative *Borrelia burgdorferi* (Sambri et al., 1992), a microorganism challenging to detect by Gram stains (Zanconati et al., 1994). Other authors reported that PAS staining in electrophoresed LPS samples, while showing a lower sensitivity than silver staining, is simple and selective for LPS, then adequate for the analysis (Takeshi and Akira, 1991). Thus, extracting LPS by

DOC-PAGE, followed by visualization by PAS staining, appears to be effective for identifying LPS in gram-negative bacteria (Takeshi and Akira, 1991).

Moreover, PAS stain is a valuable way to distinguish between glycoproteins and non-glycosylated proteins separated by gel electrophoresis; it stains carbohydrates a magenta color but does not stain proteins (Benz and Schmidt, 2002). In addition, it is a practical control for the presence of non-LPS components which could appear in the silver stain, and it is free of the indiscriminate staining characteristics of that stain (Diedrich et al., 1983). In the end, the findings of these studies indicated that the PAS histochemical method could be a valuable aid in identifying not routinely Gram-stained or weakly visible bacteria because they are too small to be seen under a light microscope. Still today, PAS stain is the most common identification method for fungi due to the high carbohydrate content in these organisms' cell walls (Shalin et al., 2020), while it is poorly or not considered to detect bacteria in infected tissues.

Unfortunately, only one published study (Khavari et al., 1991) appears in the literature on 6.550 results (https://pubmed.ncbi.nlm.nih.gov/?term=PAS%20stain&size=100, accessed on 31 January 2023) concerning its use in infected paraffin-embedded tissues. This bibliographic research confirmed its poor consideration concerning its usage for bacterial detection in infected tissue samples. Presently, the identification of bacteria was investigated by a correlative study based on histopathological examination and scanning electron microscopy analysis of the same dental sample stained by the PAS technique (root canal space, dentinal tubules, and pulpal chamber). Thanks to this combined methodology, it was possible to detect and confirm the presence of bacteria and their presumable living status on the same slides, as shown by our results. To our knowledge, this is the first study performed with a combined methodology, using the same histological slide, firstly PAS-stained, and subsequently examined by SEM tool on dental hard tissues (teeth). Previously, this method has been used in tissue samples affected by periodontal disease to better identify in detail bacteria morphology in diseased gingiva (Saglie et al., 1985; Saglie, 1988). However, in the investigations mentioned above, the authors, by using Gram-stained instead of the PAS staining, as first used herein, and SEM tool in the same histological sections of teeth extracted with a portion of their periodontium attached, identified cocci, rods, filaments, and spirochetes in the junctional epithelium, showing that this method may be a simple, helpful and definitive method for assessing the presence and identifying bacteria within the gingival tissues.

Furthermore, an advantage of combining light and scanning electron microscopy (SEM) allows the study of the same sample paraffin-embedded and stained tissue section by SEM tool. In this way, even when the bacterial nature of material stained with Gram is considered doubtful, it is possible confirming or not the true bacterial origin of previously stained and examined material under a light microscope thanks to SEM's more significant depth of focus and its better-resolving power (Saglie et al., 1985).

Moreover, the PAS procedure confirmed its utility on smears of stained *E. faecaslis* and *P. aeruginosa* strain cultures while showing a different colorability (weak to intense purple-magenta) of both investigated cell walls. The higher carbohydrate content of Gram-positive cell walls (Misra et al., 2015) explains its more intense stainability than Gram-negative, not compromising the visualization of this class of bacteria. *E. faecalis* and *P. aeruginosa* strains were selected in the present study because they were most frequently isolated in root canals, causing persistent endodontic infections and their notorious ability to form biofilm (Savadori et al., 2022). This data was confirmed by observing *T. denticola* strain, a thin spiral-shaped Gram-negative difficult to stain, showing its Pas-positivity. It is, therefore, conceivable that the quantity of carbohydrates present in the walls of the various Gram-negative strains determines their greater or lesser PAS-dyeability.

Then, results obtained currently on dental tissues and smears validate our hypothesis concerning the stainability of bacteria with this method. Thanks to that observation method, a best-defined resolution of rod and viable cocci forms (bacteria in the division phase) in dentinal tubules, dentinal walls, and root canal lumen were obtained. *Treponema denticola*, herein investigated, is a common oral bacteria detected in root canal systems (30.8%), causing primary and secondary endodontic infections (Cavrini et al., 2008; Nóbrega et al., 2013). Its bacteria cell is similar to Gram-negative and mainly composed of lipids, glycoproteins, and carbohydrates (Zhou et al., 2015). However, even if the Treponema genus is classified as Gram-negative, most species stain poorly, or not at all, with Gram or Giemsa stain because they are too thin to be visualized when investigated by light microscopy (Zhou et al., 2015).

Instead, it may be examined in slide smears, by darkfield, and by phase contrast under light and scanning electron microscopy or stained with silver impregnation methods (Zhou et al., 2015), as confirmed by our observations. In addition, some supplemental slides identified these spiral-shaped microorganisms as Gram-negative using the Gram method; others showed their Pas-positivity when stained by the PAS method. A more detailed morphology was appreciated thanks to SEM investigation.

As mentioned above, many factors and limitations can affect the Gram stain and its modified methods, probably dependent on the cell wall composition of both classes of Gram bacteria, dyes, and reagents as decolorizers used (Bartholomew, 1962). In addition, a histological procedure such as decalcification of hard tissues (bone, dentin) with strong acids (i.e., formic acid) negatively influences bacteria stainability. For example, using the Brown and Brenn method, only one out of 15 microorganisms were stained when this Gram-modified stain was applied (Wijnbergen and Van Mullem, 1987). Furthermore, some bacteria cannot be or are not routinely Gram stained because they are too thin or lack a cell wall to be visualized, as mentioned above (Woods and Walker, 1996). Instead, PAS stain is a valuable indicator of the presence of carbohydrates (mucin and glycogen) in Gram-positive or negative bacteria's tissues and bacterial cell walls (Herget et al., 2008). Based on this evidence, in the present study, using the PAS technique for the reasons outlined above (Herget et al., 2008), it was possible to detect different bacteria species in smears, root canal space, and dentinal tubules of teeth affected by endodontic pathology sustained by microorganisms.

On the contrary, Gram staining, an empirical stain method obtained by accidentally spilling a Lugol's iodine solution on samples subsequently removed with alcohol (https://digitalcommons.liberty.edu/cgi/viewcontent.cgi?article=1197&context=bio_chem_fac_pubs), is based solely on the appearance of the stained cells and their morphological forms. Light microscopy offers a simple and inexpensive way to detect bacteria in tissues when stainable, including dental samples. However, advanced microscopy techniques such as scanning electron microscopy, fluorescence in situ hybridization (FISH), and confocal laser scanning microscopy (CLSM) supplemented with the live/dead assay staining solution (a mixture of two fluorescent dyes that differentially label live and dead cells) could provide better information about their viability and structures. A possible limitation of this study is that few insufficient samples have yet been analyzed to give a complete answer to this topic.

A future study aimed at deepening PAS histochemical stain, conducted with more advanced means of investigation such as CLSM, SEM, or FISH, in addition to standard light microscopy, with a more significant number of samples than that of the present study, would help to establish the practical utility of this staining method as a diagnostic method of investigation in clinical microbiology and histology laboratory.

5. Conclusion

Detection of bacteria in histological samples represents a challenge for pathologists and researchers. Gram stain, to date, is the most frequently used technique for histological samples, but not all bacteria stain with this method due to their too-thin thickness, the lack of cell

walls, and sample age. In addition, histological decalcification and decolorization processes negatively affect microbial integrity and stainability. The PAS stain aid in the objective visual inference of bacteria in histopathological smears. Bacteria challenging to stain and visualize, such as *T. denticola*, were identified with this technique, while it does not distinguish Gram-positive from Gram-negative microorganisms. The carbohydrate content, common to all bacterial cell walls, serves as a target for this methodology initially devised as a histochemical test to detect polysaccharides, such as glycogen, in tissues, coloring them red-magenta. PAS staining technique has proven to be efficient and reproducible in detecting bacteria and provides a high selectivity due to the Periodic acid-Schiff (PAS) reaction mechanism; moreover, it manages to stain bacteria that show issues when stained with other staining methods.

CRediT authorship contribution statement

Luciano Giardino: Conceptualization, Methodology, Writing – original draft, Visualization. **Luigi Generali:** Investigation. **Massimo Del Fabbro:** Supervision. **Gianluca Martino Tartaglia:** Formal analysis. **Alessandro Bidossi:** Investigation. **Paolo Savadori:** Conceptualization, Methodology, Writing – original draft, Visualization.

Declaration of Competing Interest

The author declares that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

The authors thank Centro Interdipartimentale Grandi Strumenti (CIGS) of the University of Modena and Reggio Emilia, Italy.

References

Bartholomew, J.W., 1962. Variables influencing results, and the precise definition of steps in Gram staining as a means of standardizing the results obtained. Stain Technol. 37, 139–155. https://doi.org/10.3109/10520296209117723.

Bartholomew, J.W., Mittwer, T., 1952. The Gram stain. Bacteriol. Rev. 16, 1–29. https://doi.org/10.1128/br.16.1.1-29.1952.

Baum, S., 2008. The P.A.S. Reaction for Staining Cell Walls. C.S.H. Protoc., 1, pdb. prot4956. doi: 10.1101/pdb.prot4956.

Benz, I., Schmidt, M.A., 2002. Never say never again: protein glycosylation in pathogenic bacteria. Mol. Microbiol. 45, 267–276. https://doi.org/10.1046/j.1365-2958.2002.03030.x.

Beveridge, T.J., 1990. Mechanism of gram variability in select bacteria. J. Bacteriol. 172, 1609–1620. https://doi.org/10.1128/jb.172.3.1609-1620.1990.

Brown, J., Brenn, L., 1931. A method for the differential staining of gram-positive and gram-negative bacteria in tissue sections. Bull. John Hopkins Hosp. 48, 69–73.

Brown, M., Lowe, D.G., 2007. Automatic panoramic image stitching using invariant features. Int. J. Comput. Vis. 74, 59–73. https://doi.org/10.1007/s11263-006-0002-3.

Brown, R.C., Hopps, H.C., 1973. Staining of bacteria in tissue sections: a reliable gram stain method. Am. J. Clin. Pathol. 60, 234–240. https://doi.org/10.1093/ajcp/60.2.234.

Campanero-Rhodes, M.A., Palma, A.S., Menéndez, M., Solís, D., 2020. Microarray strategies for exploring bacterial surface glycans and their interactions with glycan-binding proteins. Front Microbiol. 10 (10), 2909. https://doi.org/10.3389/fmicb.2019.02909.

Cavrini, F., Pirani, C., Foschi, F., Montebugnoli, L., Sambri, V., Prati, C., 2008. Detection of Treponema denticola in root canal systems in primary and secondary endodontic infections. A correlation with clinical symptoms. N. Microbiol 31, 67–73.

Diedrich, D.L., Domenico, A.R., Fralik, J.A.J., 1983. Influence of urea on the resolution of lipopolysaccharides in sodium dodecyl sulfate polyacrylamide gels. Microbiol. Methods 1, 245–251. https://doi.org/10.1016/0167-7012(83)90029-5.

Friedlander, C., 1883. Die Mikrokokken der Pneumonie. Fortschr. Med. 1, 715–733.

Geissinger, H.D., 1971. Correlated light optical and scanning electron microscopy of Gram smears of bacteria and paraffin sections of cardiac muscle. J. Microsc. 93, 109–117. https://doi.org/10.1111/j.1365-2818.1971.tb02272.x.

Giardino, L., Grande, N.M., Savadori, P., Del Fabbro, M., Plotino, G., 2019a. Clinical and histological findings of post-treatment infection in the presence of vertical root fracture and apical periodontitis: case reports. Eur. Endod. J. 4, 45–48. https://doi.org/10.14744/eej.2018.14622.

Giardino, L., Del Fabbro, M., Morra, M., Pereira, T., Bombarda de Andrade, F., Savadori, P., Generali, L., 2019b. Dual Rinse® HEDP increases the surface tension of NaOCl but may increase its dentin disinfection efficacy. Odontology 107, 521–529. https://doi.org/10.1007/s10266-019-00436-4.

Gomes, B.P.F.A., Francisco, P.A., Godoi Jr, E.P., Endo, M.S., Barbosa-Ribeiro, M., Delboni, M., G., Pecorari, V.G.A., 2021. Identification of culturable and nonculturable microorganisms, lipopolysaccharides, and lipoteichoic acids from root canals of teeth with endodontic failure. J. Endod. 47, 1075–1086. https://doi.org/10.1016/j.joen.2021.04.011.

Gram, H.C., 1884. Über die isolierte Färbung der Schizomyceten in Schnittund Trocken präparaten. Fortschr. der Med. 2, 185–189.

Gupta, E., Bhalla, P., Khurana, N., Singh, T., 2009. Histopathology for the diagnosis of infectious diseases. Indian J. Med. Microbiol. 27, 100–106. https://doi.org/10.4103/0255-0857.49423.

Herget, S., Toukach, P.V., Ranzinger, R., Hull, W.E., Knirel, Y.A., von der Lieth, C.W., 2008. Statistical analysis of the Bacterial Carbohydrate Structure Data Base (BCSDB): characteristics and diversity of bacterial carbohydrates in comparison with mammalian glycans. B. M. C. Struct. Biol. 8, 35. https://doi.org/10.1186/1472-6807-8-35.

Hotchkiss, R.D., 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem. 16, 131–141.

Khavari, P.A., Bolognia, J.L., Eisen, R., Edberg, S.C., Grimshaw, S.C., Shapiro, P.E., 1991. Periodic acid-Schiff positive organisms in primary cutaneous Bacillus cereus. Case report and an investigation of the periodic acid-Schiff staining properties of bacteria. Arch. Dermatol. 127, 543–546. https://doi.org/10.1001/archderm.1991.04510010111013.

Kligman, A.M., Mescon, H., 1950. The periodic acid-Schiff stain for the demonstration of fungi in animal tissue. J. Bacteriol. 60, 415–421. https://doi.org/10.1128/jb.60.4.415-421.1950.

Latgé, J.P., 2007. The cell wall: a carbohydrate armour for the fungal cell. Mol. Microbiol. 66, 279–290. https://doi.org/10.1111/j.1365-2958.2007.05872.x.

Le Brun, A.P., Clifton, L.A., Halbert, C.E., Lin, B., Meron, M., Holden, P.J., Lakey, J.H., Holt, S.A., 2013. Structural characterization of a model gram-negative bacterial surface using lipopolysaccharides from rough strains of Escherichia coli. Biomacromolecules 14, 2014–2022. https://doi.org/10.1021/bm400356m.

Luna, L.G., 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology 3rd ed. Blakiston Division, McGraw-Hill, New York, 158–160 and 222–223.

Maskell, J.P., 1991. The resolution of bacteroides lipopolysaccharides by polyacrylamide gel electrophoresis. J. Med. Microbiol. 34, 253–257. https://doi.org/10.1099/00222615-34-5-253.

Mc Manus, J.F., 1948. Histological and histochemical uses of periodic acid. Stain Technol. 23, 99–108. https://doi.org/10.3109/10520294809106232.

Misra, S., Sharma, V., Srivastava, A.K., 2015. Bacterial Polysaccharides: An Overview. In: Ramawat, K., Mérillon, J.M. (Eds.), Polysaccharides. Springer, Cham. https://doi.org/10.1007/978-3-31.

Nóbrega, L.M., Delboni, M.G., Martinho, F.C., Zaia, A.A., Ferraz, C., C., Gomes, B., P., 2013. Treponema diversity in root canals with endodontic failure. Eur. J. Dent. 7, 61–68.

Petersen, J., Mclaughlin, S., 2016. Laboratory exercises in microbiology: discovering the unseen world through hands-on investigation. CUNY Queensborough Community Coll. 37–41. ⟨https://academicworks.cuny.edu/qb_oers/16/⟩.

Ruiz-Herrera, J., Ortiz-Castellanos, L., 2019. Cell wall glucans of fungi. A review. Cell Surf. 5, 100022 https://doi.org/10.1016/j.tcsw.2019.100022.

Saglie, F.R., 1988. Scanning electron microscope and intragingival microorganisms in periodontal diseases. Scanning Microsc. 2, 1535–1540 (Available at). ⟨https://digitalcommons.usu.edu/microscopy/vol2/iss3/29⟩.

Saglie, F.R., Sa Ferreira, J.C., Smith, C.T., Valentin, P.L., Carranza Jr., F.A., Newman, M. G., 1985. Identification of bacteria by studying one section under light microscopy, scanning, and transmission electron microscopy. J. Electron. Microsc. Tech. 2, 581–588. https://doi.org/10.1002/jemt.1060020609.

Sambri, V., Stefanelli, C., Cevenini, R., 1992. Detection of glycoproteins in Borrelia burgdorferi. Arch. Microbiol. 157, 205–208. https://doi.org/10.1007/BF00245150.

Savadori, P., Dalfino, S., Piazzoni, M., Parrini, M., Del Fabbro, M., Tartaglia, G.M., Giardino, L., 2022. A simplified method for detecting Gram-positive and Gram-negative bacteria in dental histological samples: a preliminary and comparative study. Acta Histochem 125 (1), 151992. https://doi.org/10.1016/j.acthis.2022.151992.

Schäffer, C., Messner, P., 2001. Glycobiology of surface layer proteins. Biochimie 83, 591–599. https://doi.org/10.1016/s0300-9084(01)01299-8.

Shalin, S.C., Ferringer, T., Cassarino, D.S., 2020. P.A.S. and G.M.S. utility in dermatopathology: review of the current medical literature. J. Cutan. Pathol. 47, 1096–1102. https://doi.org/10.1111/cup.13769.

Takeshi, S., Akira, Y., 1991. Rapid method for lps analysis with doc-page and pas stain. Inst. Ferment. Inst. Ferment. Osaka Res. Commun. 15, 42–51.

Tytgat, H.L., Lebeer, S., 2014. The sweet tooth of bacteria: common themes in bacterial glycoconjugates. Microbiol. Mol. Biol. Rev. 78, 372–417. https://doi.org/10.1128/MMBR.00007-14.

Vera, J., Siqueira Jr., J.F., Ricucci, D., Loghin, S., Fernández, N., Flores, B., Cruz, A.G., 2012. One- versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J. Endod. 38, 1040–1052. https://doi.org/10.1016/j.joen.2012.04.010.

Vollmer, W., Blanot, D., de Pedro, M.A., 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev. 32, 149–167. https://doi.org/10.1111/j.1574-6976.2007.00094.x.

Wijnbergen, M., Van Mullem, P.J., 1987. Effect of histological decalcifying agents on number and stainability of gram-positive bacteria. J. Dent. Res. 66, 1029–1031. https://doi.org/10.1177/00220345870660050701.

Woods, G.L., Walker, D.H., 1996. Detection of infection or infectious agents by use of cytologic and histologic stains. Clin. Microbiol. Rev. 9, 382–404. https://doi.org/10.1128/CMR.9.3.382.

Zanconati, F., Cattonar, P., Grandi, G., 1994. Histochemical and immunohistochemical methods for demonstration of spirochetes in skin biopsies. Acta Derm. Venereol. (Ljubl.) 3, 99–104.

Zhan, X., Hakoupian, M., Jin, L.W., Sharp, F.R., 2021. Lipopolysaccharide, identified using an antibody and by P.A.S. Staining, is associated with corpora amylacea and white matter injury in Alzheimer's Disease and aging brain. Front. Aging Neurosci. 13, 705594 https://doi.org/10.3389/fnagi.2021.705594.

Zhou, X., et al., 2015. Subgingival Microbes. In: Zhou, X., Li, Y. (Eds.), Atlas of Oral Microbiology: From Healthy Microflora to Disease. Springer, Singapore, pp. 92–93. https://doi.org/10.1016/B978-0-12-802234-4.00004-5.