METHODOLOGICAL APPROACHES FOR THE BACTERIOME ANALYSIS OF THE ROOT CANAL SYSTEM OF TEETH AFFECTED BY APICAL PERIODONTITIS

Literature review

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SUMMARY

Introduction and aim: Apical periodontitis (AP) is an inflammatory disease of the dental periradicular tissues, caused by a bacterial infection. Various methodological approaches can be used to determine the bacteria inhabiting the root canals; however, the analysis of the entire root system of a tooth affected by AP remains a challenge. Our study aimed to perform literature search focused on sample collection procedures and methods for bacteriome analysis and, based on these, to propose a suitable methodological approach facilitating the study of the etiopathogenesis of this disease.

Methods: PubMed database was searched for original articles in which the bacterial DNA of human teeth was analyzed.

Results: The studies differ greatly in terms of methods – from sample collection and DNA isolation to bacterial DNA analysis itself. The use of sterile endodontic paper points is a common method of sample collection. Although this method of sampling is suitable in clinical practice, it is considered insufficient for a comprehensive analysis of the environment of the root canal system, due to the morphology of the tooth itself and the presence of ramifications. Resection of the root tip using sterile burs and subsequent grinding of the apex or smearing with sterile endodontic paper points is another method of sampling. Only the apical part of the tooth is used to determine the bacteriome; bacteria that colonize the coronal part of the tooth and participate in the etiopathogenesis of the disease, therefore, cannot be analyzed. Some recent studies used cryogenic grinding of the entire extracted AP-affected tooth into fine homogeneous powder, facilitating the determination of complex bacteriome of the root canal system and the pulp chamber from the dust of a crushed tooth. From the experimental point of view, this method seems to be optimal for the sample preparation. For DNA isolation, most studies use solid-phase extraction with various purification kits. Subsequent DNA analysis is most commonly performed using the polymerase chain reaction-based methods, with the sequencing of the hypervariable regions in the gene for 16S rRNA becoming the gold standard for categorizing bacteria and characterizing bacterial communities.

Conclusion: The use of extracted tooth samples and immediately frozen without further preanalytical steps seems to be most appropriate for studying the AP bacteriome. Ground teeth are a suitable matrix for the isolation of microbial DNA with commercially available kits, provided that sterile conditions are maintained during cryogenic grinding. Next-generation sequencing is currently the best choice for determining the bacteriome and obtaining information about the relative abundance of bacterial genera, both analytically and economically.

Key words: apical periodontitis, root canal, cryogenic grinding, bacteriome, sequencing

Cerulová S, Száraz D, Gachová D, Kapitán M, Bořilová Linhartová P.
Methodological approaches for the bacteriome analysis of the root canal system of teeth affected by apical periodontitis.
Čes. stomatol. Prakt. zub. lék. (Czech Dental Journal). 2023; 123(4): 105–114. doi 10.51479/cspzl.2023.008

INTRODUCTION

Apical periodontitis (AP) is a disease of the periradicular tissues caused by microbial infection. The pulp chamber of a non-vital tooth becomes infected with oral microorganisms that penetrate from dental caries or through cracks caused by trauma to the hard dental tissues or from periodontal pockets. This leads to the gradual infection of the root canals (RC). The presence of oral microbiota in the RC system and the penetration of bacteria or their metabolites through the apical foramen initiate an inflammatory response in the body. Under the conditions of chronic inflammation, a periapical granuloma can develop. Bacteria that are present and colonizing the apical root system directly contribute to the etiopathogenesis of this inflammatory disease.

RC infections have a heterogeneous composition; therefore, no single bacterial species or strain can be considered the primary endodontic pathogen. Dysbiosis and the presence of multiple bacteria with pathogenic potential in the periradicular tissues can be considered the main causes of AP. The microbial community in the RC system of a tooth affected by AP comprises both aerobic and anaerobic bacteria. Aerobic bacteria are primarily found in the coronal part of the root due to the sufficient oxygen supply necessary for their survival and reproduction. Anaerobic bacteria predominantly prevail in the apical part of the root in the RC system of a tooth with AP. The most common anaerobic bacteria colonizing the infected RC include *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella baroniae*, and *Olsenella uli*. Facultative anaerobes such as *Gemella morbillorum* and *Streptococcus mitis* are also often present. Fungi, such as *Candida* sp., can also occur in non-vital teeth with infected RC (gangrene).

Comprehensive microbiota analysis in the areas of the tooth that are difficult to access with instruments and irrigants, such as the apical part of the RC, lateral canals, ramifications, isthmuses, and dentinal tubules, is challenging. Bacteria colonizing these areas can be in direct contact with the periodontium, leading to persistent AP. Other factors contributing to persistent AP include inadequate aseptic control, failure to detect all RC during treatment, poorly performed debridement (removal of necrotic tissue), insufficient RC wall preparation, inadequate root system disinfection, and poorly sealed fillings. AP treatment involves treating the infected RC of the affected tooth. However, this treatment is not always successful even after adequate RC preparation and filling placement due to factors such as extra-radicular infection or the presence of biofilm, leading to persistent AP. Recommended treatment for persistent AP involves removing the source of inflammation through surgical procedures, including root-tip resection with retrograde filling or tooth extraction.

With advances in molecular biology methods, the analysis of bacteria in the RC of teeth affected by AP involves genome analysis (specifically the bacteriome, i.e., the collective bacterial genetic information in a sample). The main goal of our article was to summarize the used methodological approaches, discuss their advantages and disadvantages, and then propose the most effective method for determining the comprehensive bacteriome of the RC system of a tooth with AP in the study of the etiopathogenesis of this disease.

METHODS

The PubMed database was used for literature search using the following keywords: (apical OR periapical) AND (periodontitis OR granulom* OR lesion OR cystogranulom*) AND (microb* OR bacter* OR infect* OR pathogen) AND (isolation OR sampling). We included only articles that i) were published from 2010 onwards, due to the relevance of the molecular biology approaches used in the studies, ii) are of the type “original articles” and “case reports”, iii) deal with the determination of the microbiome in patients with AP (primary, secondary, and persistent). The search was independently conducted by two co-authors, based on the established criteria. The results were compared and merged after reviewing and resolving of disputed items. According to the agreed criteria, we further excluded publications that i) were duplicates of an included publication, ii) focused on the mycobiome rather than the bacteriome, iii) included samples from patients with AP but did not analyze the tooth or RC, and iv) did not provide a sufficient methodological description of the RC bacteriome analysis.

Studies that described the analysis of the comprehensive RC bacteriome as well as those that aimed to identify only one or a few selected bacteria were included in this review.

RESULTS

A total of 67 articles, summarized in **Supplementary Table S1 (available online at www.cspzl.dent.cz)**, were suitable for inclusion in this literature review. For each study, we recorded: i) the sample collection method – how the sample for the tooth bacteriome analysis was collected, ii) sample preparation for analysis – how the sample was processed, iii) how DNA isolation was performed, iv) bacteriome analysis, and v) how many samples were analyzed. The workflow for biological material collection and subsequent analysis of the RC bacteriome is summarized in **Figure 1**.

A total of 2027 teeth were analyzed in these studies, ranging from 5 to 231 teeth per study. On average, 30 samples per study were analyzed. In 66 articles, it was reported that disinfection of the oral cavity or of the tooth was performed prior to sample collection. In 52 (78%) of all retrieved studies, endodontic paper points were used for sample collection. In 13 out of those 52 studies, other endodontic or surgical tools were used to collect additional samples. Approximately in one-fifth of all studies, an invasive sampling method involving tooth extraction and/or root resection was used. In nine studies (13% of all publications included in the review), the cryogenic grinding method was used to crush the tooth; these studies are summarized in **Table 1** [10, 15–22]. In eight out of these nine studies, the extracted teeth were divided into apical and coronal parts before grinding to determine and compare the bacteriome in individual parts of the teeth.

In 54 studies, a commercial kit was used for DNA isolation; in 65% of those, the QIAamp DNA Mini Kit (Qiagen, Germany) was used. In 44 studies, 16S rRNA amplicon sequencing was performed. Other relatively common approaches for analyzing bacterial DNA included quantitative polymerase chain reaction (qPCR), which was used in nearly 18% of studies, and DNA hybridization method, which was performed in 16% of studies.

DISCUSSION

Taking a representative sample from the RC of a tooth affected by AP using non-invasive methods is not easy. The reason lies in the morphology of the RC system itself and the possible presence of filling material if the tooth has already undergone RC treatment. Bacteria located in areas such as accessory canals and the apical part of the RC may, therefore, be difficult to detect. Limited amount of infectious material collected from the RC and/or low sensitivity of the method used for identification may lead to the failure in recognizing many bacteria during the analysis [10, 23].

SAMPLE COLLECTION METHODS FROM TEETH AFFECTED BY AP

The method of sample collection from teeth affected by AP varies across studies. In general, the methods can be classified as non-invasive (using endodontic paper points and other endodontic instruments), which can be used in clinical practice, and invasive (extraction and/or apicectomy), for which specific indications must be met. Studies mention that before surgical procedures, the oral cavity can be rinsed with disinfectant solutions, and the tooth can be washed with a saline solution, similar to sample collection using endodontic paper points [10, 19, 21, 25].

Non-invasive Sample Collection from Infected Teeth and Their Storage

This method is the most commonly used due to its simplicity and non-invasive approach during collection, meaning the affected tooth is endodontically treated with the aim of being preserved. In teeth affected by persistent AP, the root canal filling is removed before sample collection from the root canal. According to the literature, microbiome analysis of the root canal is conducted on samples collected using swabs, endodontic paper points, or aspirates. Non-invasive sample collection methods also include the use of endodontic instruments (e. g., K-files) [3, 14, 26].

When collecting samples from the root canal using endodontic paper points, it is important to ensure that the paper point is inserted as deeply into the root canal as possible and that the collection is conducted under strict aseptic conditions. Published studies use different disinfectants and solutions to clean the oral cavity and the tooth sample itself, such as 0.12% chlorhexidine or solutions of H_2O_2, NaClO, or $Na_2S_2O_3$ [3, 26, 27]. Swabs from the tooth surface are used to ensure sufficient aseptic work [22]. Collection using endodontic paper points is conducted according to protocols described in previous studies [3, 24, 29, 30], meaning that the access cavity for sample collection is prepared using a high-speed sterile carbide

bur [24] and the tooth is cleaned before exposing the pulp. The contents of the main root canal are absorbed into the endodontic paper point which is then transferred into a sterile tube [30, 31]. Endodontic paper points can be stored in various solutions or buffers, such as Tris-EDTA buffer, PBS, etc. [23, 24, 25–27]. The storage temperature of collected samples is most commonly -20 °C or -80 °C. The freezing speed of the sample after collection must be also standardized to prevent the growth of certain strains in the sample between collection and storage which would affect the analysis results.

Invasive Methods of Sample Collection from Infected Teeth and Their Storage

Due to the technical limitations of sampling resulting from the morphology of the RC, analyzing the RC microbiota using extracted teeth affected by AP is more suitable for research purposes. In extracted teeth, it is possible to detect bacteria present throughout the entire RC system and simultaneously determine the bacteria in all parts of the tooth – the apical, central, and coronal parts [21, 22]. A limitation of this approach lies in its applicability only to teeth indicated for periapical surgery or extraction. Extraction of a tooth affected by AP is indicated if there is no prospect of sufficient long-term survival of the tooth post-treatment or in the case of the formation of a radicular cyst that needs to be extirpated. The sample from the extracted tooth is usually subjected to cryogenic grinding to obtain a suitable matrix for subsequent analysis. In one study, however, RC material from an extracted tooth was collected using a K-file and an endodontic paper point retrogradely inserted after the resection of the root tip [32].

PREPARATION OF A SAMPLE FROM A TOOTH AFFECTED BY AP FOR FURTHER ANALYSES

Sample preparation includes its processing into a matrix suitable for further analyses (e. g., cryogenic grinding of the tooth) and/or culturing the infectious material under specific conditions.

Cryogenic Grinding

Cryogenic grinding of teeth or their fragments is a method designed for obtaining a representative sample for selective and

Tab. 1 Overview of methodological procedures for the analysis of the bacteriome of a tooth affected by apical periodontitis – selection of only studies involving cryogenic grinding of the sample.

1st and 2nd step Sampling and Sample Preparation for Analysis							**3rd step DNA Extraction**					**4th step Bacteriome Analysis**				
Mouth rinse	Tooth cleaning	Sterility check	Removal of surrounding tissues around the tooth	Tooth extraction	Tooth resection	Cryogenic tooth grinding	QIAamp DNA Mini Kit	Norgen bacterial DNA Extraction Kit	AGOWA mag Mini DNA Isolation Kit	CTAB	Fast DNA SPIN Extraction Kit	16S rRNA sequencing	qPCR	DNA hybridization	Number of teeth examined in the study	Reference
-	yes	yes	-	yes	yes	yes	-	-	-	-	yes	yes	-	-	10	[15]
yes	-	-	-	yes	yes	yes	-	-	yes	-	-	yes	-	-	41	[10]
yes	yes	yes	yes	yes	-	yes	-	yes	-	-	-	yes	-	-	41	[16]
-	yes	yes	-	yes	yes	yes	-	-	-	yes	-	yes	yes	-	26	[17]
yes	yes	yes	yes	yes	yes	yes	yes	-	-	-	-	yes	-	-	10	[18]
ano	ano	ano	yes	yes	yes	yes	yes	-	-	-	-	yes	yes	-	27	[19]
-	yes	yes	-	yes	yes	yes	-	-	-	yes	-	yes	-	-	25	[20]
-	yes	yes	yes	yes	yes	yes	yes*	-	-	-	-	yes	-	yes	15	[21]
-	yes	yes	yes	yes	yes	yes	yes*	-	-	-	-	yes	-	-	10	[22]

* incubation with lysozyme

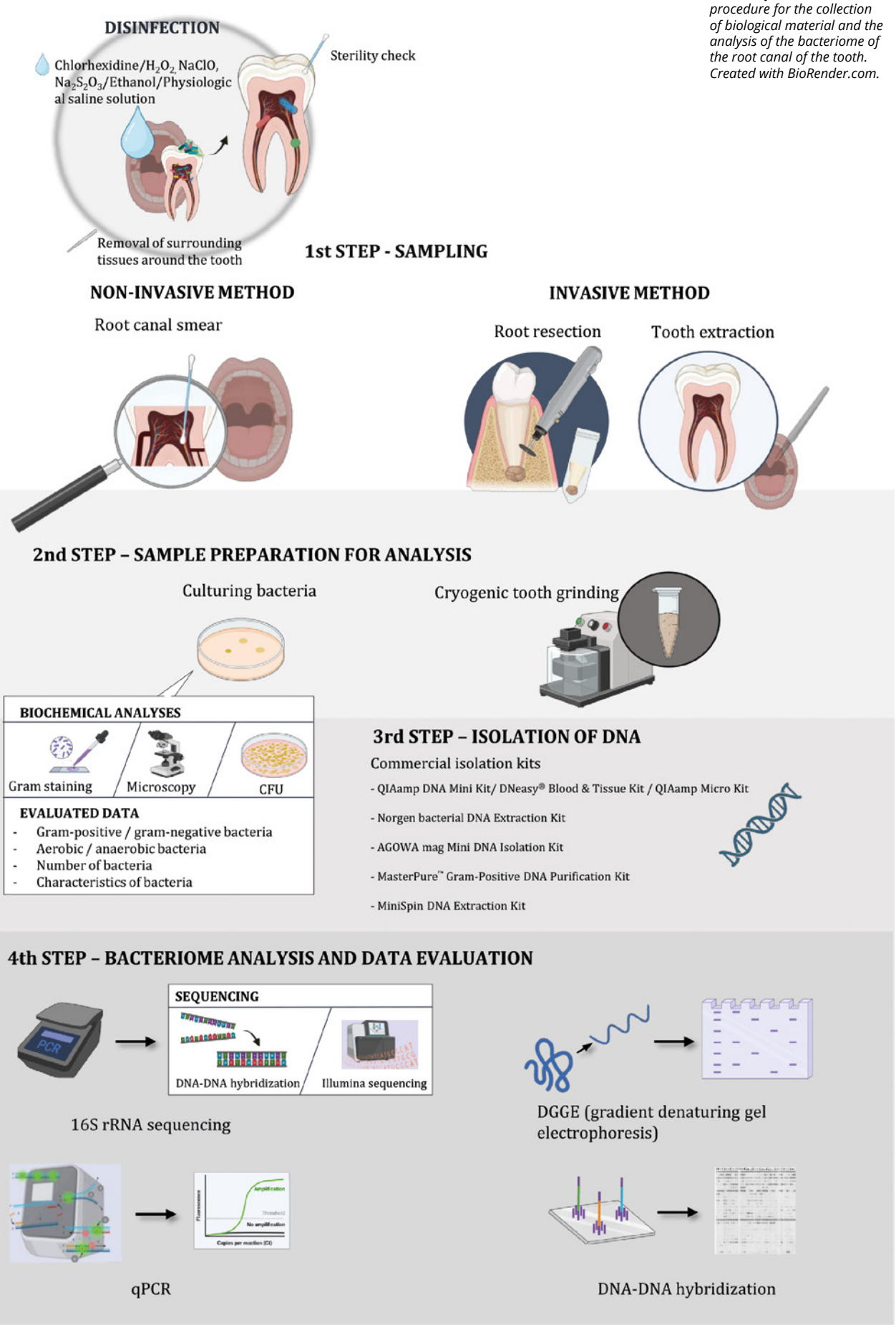

Fig. 1
Scheme of the methodical procedure for the collection of biological material and the analysis of the bacteriome of the root canal of the tooth. Created with BioRender.com.

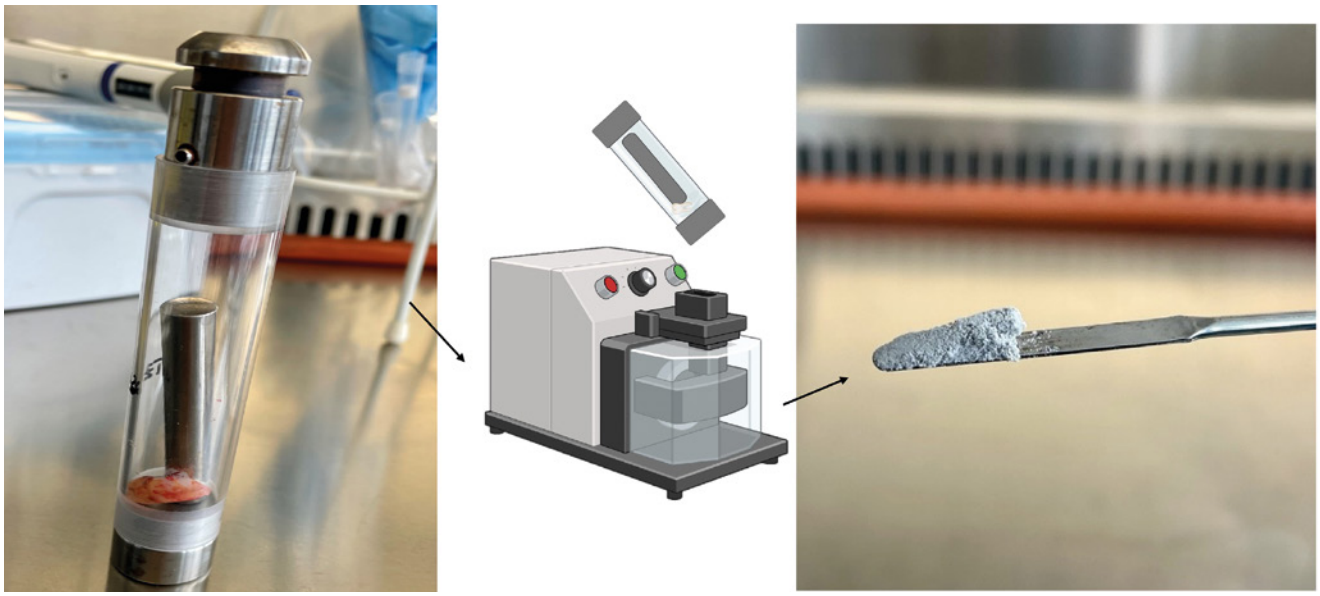

Fig. 2
Extracted tooth in a grinding cartridge before cryogenic grinding and crushed tooth in the form of a homogeneous powder.

comprehensive analysis of the RC microbiome [7, 8, 12, 16, 35]. The entire extracted tooth or its specific segment intended for analysis is placed into a cryogenic grinder. If required, the tooth may be separated into apical and coronal fragments before cryogenic grinding to determine the microbiota in specific parts of the root. However, the more the tooth is manipulated, the greater is the risk of introducing microbial contamination into the sample and compromising it. The microbiota of the apical fragment was shown to differ from that of the coronal fragment [20, 25]. In the apical part of the root, there are slow-growing microorganisms that exhibit a greater dependence on interspecies interactions compared to those colonizing the coronal fragment [33].

The principle of tooth grinding in a cryomill was first described by Alves et al. in 2009 [19]. Cryogenic grinding is performed in cryomills (e. g., Spex, USA), operating at liquid nitrogen temperature (-197 °C); the temperature in the cryomill remains constant during grinding. Extracted teeth are placed in polycarbonate cylinders with metal caps, as shown in **Figure 2**. The stainless-steel end caps, impact rod, and polycarbonate vials must be disinfected before each use. One of the methods for such disinfection was described by Alves et al. in their study [19], describing the disinfection procedure protocol as follows: brushing and washing with a neutral detergent, rinsing with 70% ethanol, soaking in 2.5% NaClO for one minute, rinsing with milliQ water, drying at 70 °C, and sterilizing under ultraviolet light for 15 minutes. Another method of sterilizing the polycarbonate cylinders involves using an acidic environment with 0.1 M HCl. Under those conditions, depurination of DNA occurs through acid hydrolysis, leading to the gradual formation of shorter fragments and eventually complete hydrolysis of DNA. It is known that most bacteria cannot survive in the environment with pH lower than 4; thus, 0.1 M HCl, with a pH of 1, is ideal for sterilizing polycarbonate cylinders. After washing the cylinders in acid for one hour, they are thoroughly rinsed with milliQ water and dried at room temperature [34, 35].

Tooth samples are ground using a magnetically moving steel impact cylinder oscillating back and forth against two stationary stainless-steel end caps that enclose a cartridge. The cartridge is immersed in liquid nitrogen during grinding, maintaining a constant temperature even as the steel cylinder oscillates within the cartridge (the cartridge with the sample is pre-cooled for 15 minutes before the actual grinding). This is followed by grinding the sample in the cartridge for one minute and re-cooling for six seconds (the period between two working cycles when the cylinder's oscillation is paused to prevent friction-associated heating). Each sample typically undergoes two grinding cycles. The frequency of the impact rod is set to 10, resulting in 20 actual impacts per second (one on each side) [19]. The cryogenic grinding method is considered very simple and quick for obtaining a suitable matrix for subsequent DNA extraction from the tooth sample.

This method of grinding the tooth is capable of capturing bacterial taxa located in places such as the aforementioned accessory RC and the apical part of the root, which cannot be sampled using endodontic paper points. Analysis of the tooth using this method is thus considered much more accurate than sampling from the RC using endodontic instruments and paper points [17, 18]. The main disadvantage of the approach involving cryogenic grinding of the tooth is that it can only be applied to teeth indicated for periradicular surgery or extraction and cannot be used for case monitoring or to check for the elimination of bacteria after therapy. In most studies, careful disinfection of the external root surfaces before grinding is considered necessary to remove external contaminants and reduce the risk of false positive results. However, it is important to note that this approach can eliminate bacteria that potentially form extraradicular biofilm and may cause a post-treatment disease. Additionally, disinfectant solutions may enter the RC during their application, potentially compromising the analysis results [18]. In many studies, a swab from the surface of the disinfected tooth was taken to verify aseptic work compliance and to set proper methodological procedures [29, 32, 36].

Analysis of Bacteria Using Cultivation Methods

The literature review revealed that methods for analyzing and identifying bacteria in the RC of a tooth affected by AP can be highly variable. Cultivation methods have often been used to analyze bacteria in RC samples collected using endodontic paper points and other previously mentioned endodontic tools. Identification of bacteria after cultivation is typically performed using methods such as Gram staining, biochemical tests, microscopic observation, and colony-forming unit counting. The MALDI-TOF MS (e. g. Biotyper Microflex, Bruker Daltonics) method can also be utilized [36–38]. However, limitations of this method include its time-consuming nature and the fact that it can only identify culturable bacteria [39].

DNA EXTRACTION

The choice of DNA extraction procedure from a sample is a crucial step in microbiome analysis. It is important to note that without specific treatment, DNA is isolated from both living and dead cells. The vast majority of published studies uses commercial isolation kits intended for subsequent microbiome analysis for this purpose. These kits include the QIAamp DNA Mini Kit (Qiagen, Germany), QIAamp DNeasy Blood and Tissue Kit (Qiagen, Germany), QIAamp Fast DNA Kit (Qiagen, Germany), Direct DNA Isolation Kit (Bacteria) (Norgen, Canada), QIAamp Micro Kit (Qiagen, Germany), Mag Mini DNA Isolation Kit (AGOWA, LGC Genomics, Germany), and others. These extraction techniques are based on the DNA adsorption onto a solid substrate. While the Mag Mini DNA Isolation Kit uses magnetic beads for DNA binding, the other kits, which are based on column extraction, are based on selective binding of nucleic acids to a silica gel membrane, followed by washing the column with buffers, repeated centrifugation steps, and final elution of DNA with a specific buffer [40]. A significant advantage of this method, compared to other extraction techniques, is its high specificity and the yield of pure DNA without potential inhibitors of subsequent PCR reactions or Next Generation Sequencing (NGS) [41]. Before DNA extraction, the sample may be mechanically processed and/or incubated with lysozymes or other enzymes (or their mixtures), allowing the extraction of DNA from even gram-positive bacteria. According to our review, the QIAamp DNA Mini Kit (Qiagen, Germany) is most commonly used for DNA extraction intended for subsequent analysis of the microbiome of the RC of teeth with AP. Four of these studies used this kit in conjunction with lysozyme treatment of the sample [17, 22, 26, 31], which currently appears to be the optimal approach to ensure that gram-positive bacteria are not underestimated in the overall picture. Importantly, different kits contain different contaminants and, therefore, the method of extraction must be considered when comparing study results.

BACTERIOME ANALYSIS

Molecular biology techniques enabled studying the microbial colonization of the RC of teeth affected by AP, including the identification of microbial taxa that are difficult to culture. Studies employing molecular biological methods have revealed a higher complexity of endodontic microflora than previously reported in studies using culture approaches [16].

To determine the diversity and relative abundance of bacterial taxa, or the presence of their DNA in each sample, profiling using

PCR-based amplification of the gene for 16S rRNA is most commonly performed. By analyzing regions of this gene, it is possible to study the bacteriome without a prior cultivation of the sample. This gene contains hypervariable regions that can provide species-specific sequences useful for bacterial identification. According to our literature review, the Illumina platform is most commonly used for sequencing, with most studies analyzing the bacteriome of RCs in teeth with AP relying on sequencing of the hypervariable regions of the gene for 16S rRNA using Illumina kits on the MiSeq instrument. However, this method also has its technical limitations; in some cases, it can be challenging to distinguish closely related species based on the variability of this gene [42].

DNA-DNA hybridization is another common technique used for detecting different bacterial taxa in RC sample analysis. This rapid and sensitive molecular identification technique allows the detection of multiple bacterial species in a large number of samples containing complex mixtures of microorganisms. The limitations of this technique lie in the fact that it can only detect species for which DNA probes have been prepared, and relatively high detection limits of the method (10^3 to 10^4 cells) [30, 44, 45].

Studies focusing on the quantification of endodontic pathogens causing AP use also the qPCR method [27, 46, 47]. The advantage of qPCR is the ability to quantify selected bacterial taxa in real-time. This method is highly sensitive and provides high-quality results; however, it does not offer a comprehensive picture of bacterial DNA in the sample. Quantitative analysis of specific bacterial strains based on qPCR is simple, has high application potential in clinical practice, and is less costly compared to microbiome analysis using NGS. The quantitative profiling of the microbiome based on qPCR of the gene for 16S rRNA is recommended to be part of the standard microbiome analysis using NGS [47].

Less commonly used methods in studying bacteria colonizing the RC of a tooth affected by AP include PCR-DGGE (denaturing gradient gel electrophoresis) and AP-PCR (arbitrarily primed PCR, which uses primers that anneal to random sites on the template DNA under low stringency conditions, resulting in a characteristic set of amplicons for a given bacterial species). DGGE is a direct DNA diagnostic method, in which separated DNA fragments are analyzed through electrophoresis in a gradient denaturing gel. This highly sensitive technique is utilized in microbiology to evaluate bacterial diversity [48].

CONCLUSION

In this review article, we summarized the methods for analyzing the bacterial communities in the RC of teeth affected by AP. Extraction of the whole tooth without treating it with disinfecting solutions, followed by cryogenic grinding to obtain a homogeneous matrix for DNA extraction using a commercial kit and subsequent sequencing of the gene for 16S rRNA, appears to be the most suitable approach for studying the etiopathogenesis of AP and obtaining a comprehensive understanding of the bacterial DNA in the entire tooth. This procedure can be complemented with qPCR analysis to identify and quantify specific bacterial strains in the sample. Additionally, it is advisable to include bacterial DNA analysis from the tooth swabs, following the same protocol for DNA extraction and sequencing/qPCR. In the pre-analytical phase, it is crucial to adhere to proper sample storage methods to prevent degradation and avoid introducing errors into the analysis. When investigating the etiopathogenesis of this disease, it is essential to collect samples only from patients who did not undergo any antimicrobial therapy in the past month, as this could affect the results of the analyses.

Acknowledgments

Supported by the program project of the Ministry of Health of the Czech Republic, reg. no. NU-20-08-00205. All rights reserved under intellectual property protection regulations. This article was created with the support of the Ministry of Health of the Czech Republic – RVO (FNBr, 65269705). This work was carried out with the support of the RECETOX Research Infrastructure (ID LM2023069, Ministry of Education, Youth and Sports of the Czech Republic).

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Table S1. An overview of publications dealing with the analysis of bacterial DNA in root canals of teeth with apical periodontitis – analysis methods

Pretreatment				Sampling					Sample processing		Analysis after cultivation						DNA Extraction														Bacteriome analysis and data evaluation										
Mouth rinse	Tooth cleaning	Sterility check	Removal of surrounding tissues around the tooth	Paper points	Endodontic instruments	Root filling curettage	Resection	Extraction	Cultivation	Cryogenic tooth grinding	Gram staining	Microscopy	CFU	Biochemical analyses	MALDI-TOF	Isolation of bacteria, PCR and pulsed ELFO	QIAamp DNA Mini Kit (Qiagen, Germany)	QIAamp DNeasy Blood and Tissue Mini Kit (Qiagen, Germany)	QIAamp Fast DNA Kit (Qiagen, Germany)	Directed DNA Extraction Kit (Bacteria) (Norgen, Canada)	QIAamp Micro Kit (Qiagen, Germany)	Mag Mini DNA Isolation Kit (AGOWA, LGC Genomics, Germany)	CTAB	Commercial Kit (iNtRON, Kyunggi, Republic of Korea)	Easy DNA Kit (Invitrogen do Brasil Ltd, Sao Paulo, SP, Brazil)	Wizardâ Genomic DNA Purification Kit (Promega, A1120 Madison, WI, USA)	Boiling, centrifugation, removing the cell pellet	GeneClean kit for Ancient DNA (Bio 101 Inc., USA)	MasterPure Complete DNA and RNA Purification Kit (Epicentre Technologies, Madison, WI, USA)	MiniSpin DNA extraction Kit (Kasvi, Brazil)	16S rRNA gene sequencing	qPCR	DGGE	Reverse-capture Checkerboard Assay	cDNA synthesis	Multiple displacement amplification	RNA isolation, RT- PCR	Nested PCR	DNA Fingerprinting	Number of teeth	References
0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Hou et al., 2022)
1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	41	(Qian et al., 2019)
1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	41	(Keskin et al., 2017)
0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	26	(Persoon et al., 2017)
1	1	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Siqueira et al., 2016)
1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	27	(Antunes et al., 2015)
0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	25	(Özok et al., 2012)
0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	15	(Rôças et al., 2010)
0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Siqueira et al., 2011)
1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	43	(Bouillaguet et al., 2018)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	24	(Sánchez-Sanhueza et al., 2018)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	18	(Hong et al., 2013)
0	1	1	0	1	0	0	0	0	1	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20	(Barbosa-Ribeiro et al., 2021)
0	1	1	0	1	0	0	0	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	21	(Anderson et al., 2012)
0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	12	(Vengerfeldt et al., 2014)
1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Sun et al., 2022)
1	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	15	(Gomes et al., 2015)
1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	25	(Rôças & Siqueira, 2012)
0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Yun et al., 2017)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20	(Montagner et al., 2010)
0	1	1	0	1	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	5	(Karygianni et al., 2015)
1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	24	(Rôças & Siqueira, 2013)
1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	10	(Paiva et al., 2013)
0	1	1	1	1	1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20	(Nóbrega et al., 2016)
0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	15	(Rôças & Siqueira, 2010)
0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	6	(Y. Ito et al., 2012)
0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	5	(Nardello et al., 2020)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	39	(Nóbrega et al., 2013)
0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	8	(Santos et al., 2011)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	231	(Dumani et al., 2012)
0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	97	(Fouad et al., 2022)
1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	45	(Karataş et al., 2020)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	60	(Neves et al., 2020)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	10	(Zandi et al., 2018)
0	1	1	0	1	0	0	0	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	21	(Tennert et al., 2014)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	24	(Rôças & Siqueira, 2011b)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	41	(Rôças & Siqueira, 2011a)
0	1	1	0	1	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	15	(Endo et al., 2012)
0	1	0	1	1	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	62	(Lee et al., 2017)
1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	10	(Arruda-Vasconcelos et al., 2021)
1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	30	(Pereira et al., 2017)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	51	(Stojanovic et al., 2014)
0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	32	(de Miranda & Colombo, 2018)
1	1	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	20	(Ferreira et al., 2015)
1	1	0	1	1	0	0	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Cardoso et al., 2016)
0	1	0	0	1	0	0	0	0	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	(Vidana et al., 2011)
1	1	0	0	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	30	(I. Y. Ito et al., 2011)
0	1	1	0	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	47	(Delboni et al., 2017)
0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	26	(Chugal et al., 2011)
0	1	1	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	28	(Tandon, 2022)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	20	(Poptani et al., 2013)
0	1	0	0	1	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	33	(Mindere et al., 2010)
0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	36	(Martinho et al., 2022)
1	1	1	0	1	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	20	(Gabrielli et al., 2022)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	60	(Carvalho et al., 2020)
0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	28	(Amaral et al., 2020)
0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	17	(Takahama et al., 2018)
1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	19	(Vieira et al., 2018)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	59	(Neves et al., 2016)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	21	(Martinho et al., 2014)
0	1	1	0	1	0	0	0	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	(Endo et al., 2013)
0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	60	(Sassone et al., 2012)
0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	(Martinho et al., 2010)
1	1	0	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	58	(Wang et al., 2012)
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	10	(Zhang et al., 2022)
1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	10	(Lima et al., 2021)
1	1	0	0	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	45	(Korona-Glowniak et al., 2021)

0 = no; 1 = yes; * = incubation with lysozyme

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