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PHYSIOLOGICAL FACTORS MODULATING BACTERIAL SECOND MESSENGERS AND THE COMPARISON OF ANTIMICROBIAL EFFICACY OF ENDODONTIC IRRIGANTS

PhD thesis

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LIST OF ABBREVIATIONS

ΔCI − Delta cell index

ACs – Adenylyl cyclases

ASL – Airway surface liquid

ATR − Acid tolerance response

BHI – Brain-heart infusion

Cyclic AMP/ cAMP − 3',5'-cyclic adenosine monophosphate

C-di-AMP − Bis-(3′,5′)-cyclic diadenosine monophosphate

C-di-GMP − Bis-(3'-5')-cyclic dimeric guanosine monophosphate

CF – Cystic fibrosis

CFTR − Cystic fibrosis transmembrane conductance regulator

CFU – Colony forming unit

CHX − Chlorhexidine

CI – Confidence interval

COPD − Chronic obstructive pulmonary disease

Crp family – cAMP-receptor protein family

DACs – Diadenylate cyclases

DGCs – Diguanylate cyclases

DNA – Deoxyribonucleic acid

EPS − Extracellular polymeric substance

GRADE – Grading of Recommendations Assessment, Development and Evaluation

HPLC-MS – High-performance liquid chromatography with mass spectrometric detection

I^2 – Chi-squared test explains the percentage of variation among studies

MIB − Minimum bactericidal concentration

MIC − Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

NA – Not available

$NaHCO_3$ – Sodium bicarbonate

NaOCl − Sodium hypochlorite

PCL − Periciliary layer

PDEs – Phosphodiesterases

pH − Potential or power of hydrogen: a measure of acidity or alkalinity of the solution.

pGpG – P^1-(5'-Guanosyl)-P^2-(5'-guanosyl)-(3'→5')-diphosphate

ppGpp − Guanosine tetraphosphate

PPi − Inorganic pyrophosphate

RCTs − Randomized controlled trials

RR – Relative risk

RCT – Randomized controlled trial

RTCA − Real-Time Cell Analyzer

SCV − Small colony variant

SD – Standard deviation

SMD – Standardized mean difference

Vfr − Virulence factor regulator

WspR – Enzyme of Diguanylate cyclases

YiB (TbpB) – Enzyme of Diguanylate cyclases

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1. INTRODUCTION

1.1 Bacterial lifestyle transition

Microorganisms respond to environmental cues and hostile conditions by various biological activities as well as lifestyle transition which is a dynamic process between biofilm formation and planktonic lifestyle. Although bacteria represent two modes of lifestyle, biofilm is the preferred mode of life in most of bacterial species found in an environment {Flemming, 2019 #69}. In medical history, *Pseudomonas aeruginosa* biofilm was first observed by routine microscopic examination in cystic fibrosis (CF) sputum, as it was related to the etiology of persistent infection (2, 3). Consequently, the first microscopic image of biofilms was published in 1977 derived from the gram-stained smear of sputum from a CF patient chronically infected with mucoid *P. aeruginosa* (4). The first and well-defined model of biofilm development was also described in *P. aeruginosa* as following five phenotypically various stages including: 1) initial attachment reversible attachment, 2) irreversible attachment and maturation, 3) microcolony formation, 4) macrocolony formation, and 5) dispersion (5, 6).

Biofilm enables aggregation of microorganisms embedded in self-produced extracellular polymeric substance (EPS) matrix and adhered to each other and/or attached to surfaces (7, 8). EPS matrix consists of polysaccharides, lipids, proteins and extracellular DNA (9, 10); this protective structure encases bacterial communities providing high tolerance to immune response, antibiotics, and antimicrobial agents. Importantly, biofilm is up to 10-1000 times more resistant to various antibiotics than its planktonic counterpart (11, 12) because encapsulated bacterial cells in EPS are adjacent to each other, providing cell-cell communication and gene transfer resulting in phenotypic and genotypic changes (13, 14). Furthermore, host-derived components can be found in EPS depending on the site of the biofilms such as saliva glycoproteins, vaginal excretions and human serum (9, 15). Microorganisms have the ability to attach to a wide range of surfaces forming biofilm including living tissue, implantable medical devices, and industrial water system piping (16, 17). In marine industry, biofilm formation can colonize ships, sonar devices, boats, offshore structures and underwater instruments, and cause many issues including increased expense, reduced their functions, environmental concerns, corrosion and safety hazards (18). In addition, for food industry, biofilm harboring pathogens can be found in most water-pipe supplies which are susceptible to

biofilm formation. Fungi originating from biofilm can also generate poor taste or malodor (18).

Taken together, the biofilm formation causes serious problems not only in the medical field but also in other fields, e.g. the marine, food industry and several industrial fields. Therefore, it is worth to shed more light on the biofilm-related issues in order to provide a better understanding, efficient treatment and prevention.

1.2 Bacterial second messengers

Microorganisms choose to live the biofilm lifestyle for survival and adaptation since they experience the harsh environment in the human body and the natural world. The biofilm acts as "protective clothing" (8). Hostile environments such as extreme pH and temperature, ultraviolet radiation, inadequate nutrients, high pressure and antibiotic exposure are shown to provoke biofilm formation (19, 8). In order to respond to external stimuli, second messenger signal transduction is one of the biological processes that mediate between motile and sessile lifestyles (20). Cyclic nucleotide second messengers, namely cGMP, c-di-GMP, cAMP and c-di-AMP, have been found in all living organisms including bacteria, archaea and eukaryotes. (21, 22, 19). Moreover, they participate in various cellular activities such as biofilm formation, carbon catabolite repression, virulence expression and quorum sensing (QS) system (23-25).

1.2.1 C-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) was first discovered in *Komagataeibacter xylinus* (formerly known as *Gluconacetfobacter xylinus*) by Moshe Benziman in the mid-1980s as it is responsible for cellulose synthesis (26). Two major enzymes mediate intracellular c-di-GMP concentrations in the act of diguanylate cyclases (DGCs) for synthesis and c-di-GMP-specific phosphodiesterases (PDEs) for degradation (23) (Figure 1). These two enzymes related to c-di-GMP metabolism contain at least one of catalytic domains including GGDEF, EAL and HD-YGP domains, which are encrypted as their conserved central sequence pattern of the proteins (27). The DGCs contain a GGDEF domain (28), whereas PDEs contain either an EAL or HD-GYP domain (29). Additionally, both GGDEF and EAL domains can present in the same enzymatic protein, so-called "hybrid" proteins (30, 27). The condensation of two GTP molecules to c-di-GMP occurs at the GGDEF catalytic site requiring Mg^{2+} as a

cofactor (31). On the contrary, the EAL domain of PDEs hydrolyses c-di-GMP into linear 5′-phosphoguany- lyl-(3′-5′)-guanosine (pGpG) in the presence of Mg^{2+} and Mn^{2+} (32), whereas HD-GYP hydrolyses c-di-GMP into two molecules of GMP (27). The c-di-GMP has been universally recognized as a lifestyle switch regulator. Low intracellular levels of c-di-GMP are involved in the motility of planktonic cells. In contrast, high c-di-GMP levels support surface attachment and biofilm formations, as in Figure 2 (33).

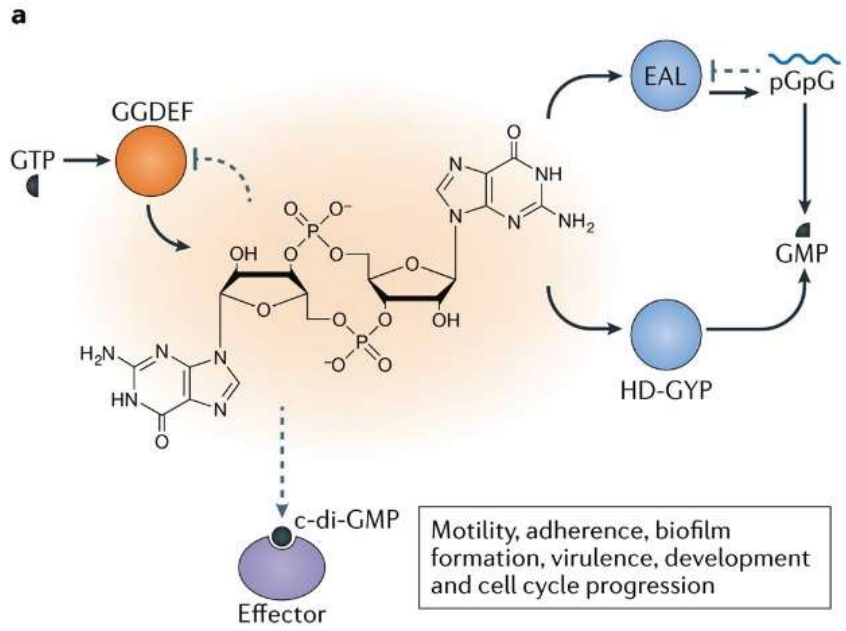

Figure 1. Diagram representing c-di-GMP metabolism, associated enzymes and effectors. C-di-GMP can bind to their specific effectors that are responsible for various bacterial activities. DGCs have feedback inhibition through the binding of c-di-GMP to an allosteric I site, while PDEs are subject to product inhibition via the binding of pGpG to the active site of its enzyme. Reprinted with permission from (23), Copyright 2021 Nature.

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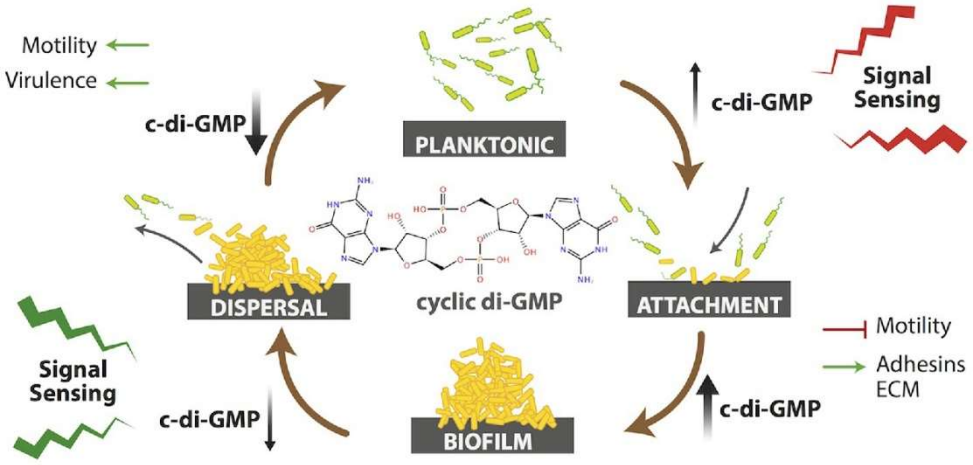

Figure 2. Schematic representation of the most common effects of c-di-GMP levels on biofilm dynamics. High levels of c-di-GMP promote the attachment of bacteria and development of biofilms, whereas a reduction in c-di-GMP intracellular concentration results in the increased virulence and motility, and dispersion of biofilms. ECM: extracellular matrix. Reprinted with permission from (33), Copyright 2021 Oxford Academic.

1.2.2 Cyclic AMP

Cyclic AMP or 3',5'-cyclic adenosine monophosphate (cAMP) is the first characterized second messenger discovered in eukaryotic cells responsible for mammalian hormonal regulation. It was subsequently detected in a prokaryote, *Escherichia coli* since it promotes catabolite repression, motile lifestyle and virulence expression. Cyclic AMP is synthesized by adenylyl cyclases (ACs), and degraded by cAMP-specific PDEs. In *P. aeruginosa*, three ACs have been identified as cytosolic enzymes: CyaA and CyaB, and as the exoenzyme or host-activated AC toxin: ExoY (34, 35). Most of the intracellular cAMP production is responsible by CyaB, which belongs to membrane sensor protein that is sensible for low environmental Ca^{2+} levels and/or contacts with host cells (Figure 3) (24, 36). Cyclic AMP is an allosteric activator of a virulence factor regulator (Vfr), referred to as the cAMP-Vfr complex in *P. aeruginosa*. High cAMP production vastly increases the expression of many acute virulence factors such as type II and III secretion systems and type IV pili (37, 38), as well as enhances twitching motility of bacterial cells via transcription factor Vfr. In addition, cAMP-Vfr is

involved in activation of the QS system that leads to secretion of autoinducer of surface contact process (39).

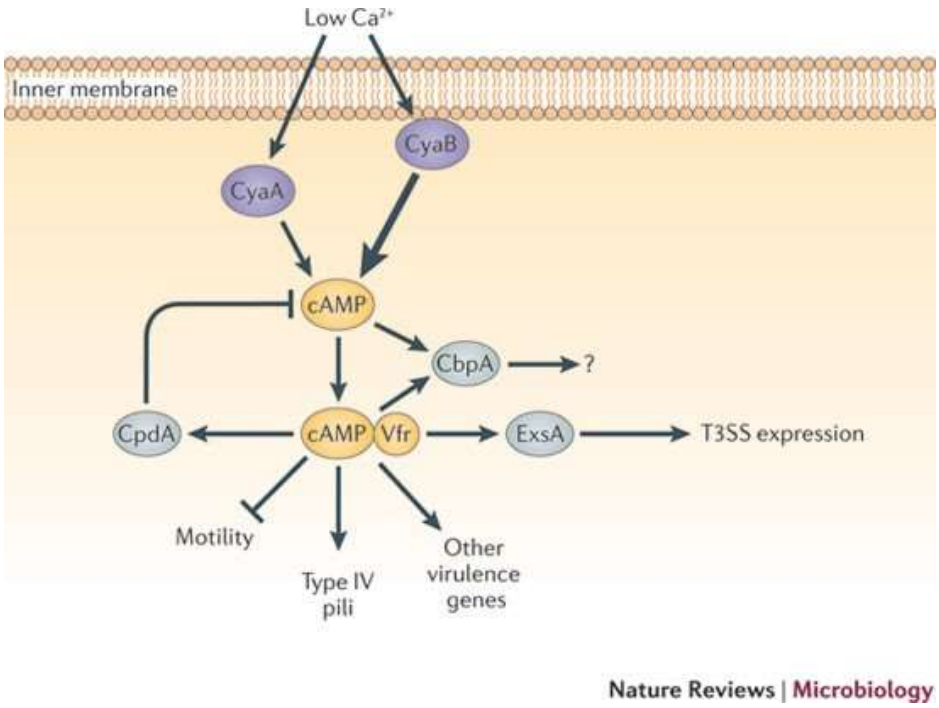

Figure 3. Schematic illustration of cAMP metabolism and signal transduction in *P. aeruginosa*. Low Ca^{2+} levels activate ACs including CyaA and CyaB; however, CyaB is the most prevalent ACs of cAMP. Cyclic AMP can bind to cAMP receptor protein, cAMP-receptor protein family (Crp family) and virulence factor regulator (Vfr). Cyclic AMP–Vfr complex upregulate the activity of PDEs (CpdA), responsible for cAMP degradation. Intermediate regulatory factors are shown, including exoenzyme S synthesis regulatory protein (ExsA), which control the expression of the T3SS components, and CbpA, the downstream functions which are unknown. Reprinted with permission from (24), Copyright 2021 Nature.

1.2.3 C-di-AMP

Cyclic di-adenosine monophosphate (c-di-AMP) is first discovered in the crystal structure of the DNA integrity scanning protein A in *Thermomotoga maritima* and *Bacillus subtilis* as a sporulation checkpoint in 2008 (40) which scans for normal DNA integrity and synthesizes c-di-AMP allowing the sporulation process (21, 41). It was subsequently identified in the cytosol of host cells infected by a bacterial pathogen

Listeria monocytogenes in 2010 as it activates levels of interferon β (42). Diadenylate cyclases (DACs) synthesize c-di-AMP from molecules of ATP. C-di-AMP is subsequently degraded into pApA by c-di-AMP specific phosphodiesterases (PDEs) (Figure 4). C-di-AMP is involved in diverse cellular activities such as cell wall and membrane homeostasis, biofilm formation, regulation of osmotic pressure, acid stress response, DNA damage repair, and sporulation (21, 43). It mainly presents in several gram-positive bacteria, a few of gram-negative species and in some archaea (44). *Staphylococcus aureus* utilizes c-di-AMP for maintaining cell size under the extreme membrane and cell wall stress (45). Notably, c-di-AMP has also been implicated in the regulation of biofilm formation in *S. aureus*, *Streptococcus suis* and *Streptococcus mutans* by mediating the expression of biofilm matrix regulator proteins (45-47). However, a lot of studies about second messengers have been evidently researched in gram-negative bacteria, particularly *P. aeruginous* biofilm model regulated by c-di-GMP. In recent years, considerable research attention has been devoted to c-di-GMP in gram-positive bacteria (48). Still, relatively little is understood about c-di-AMP, especially in oral bacteria (49). Therefore, c-di-AMP is in focus in the present day.

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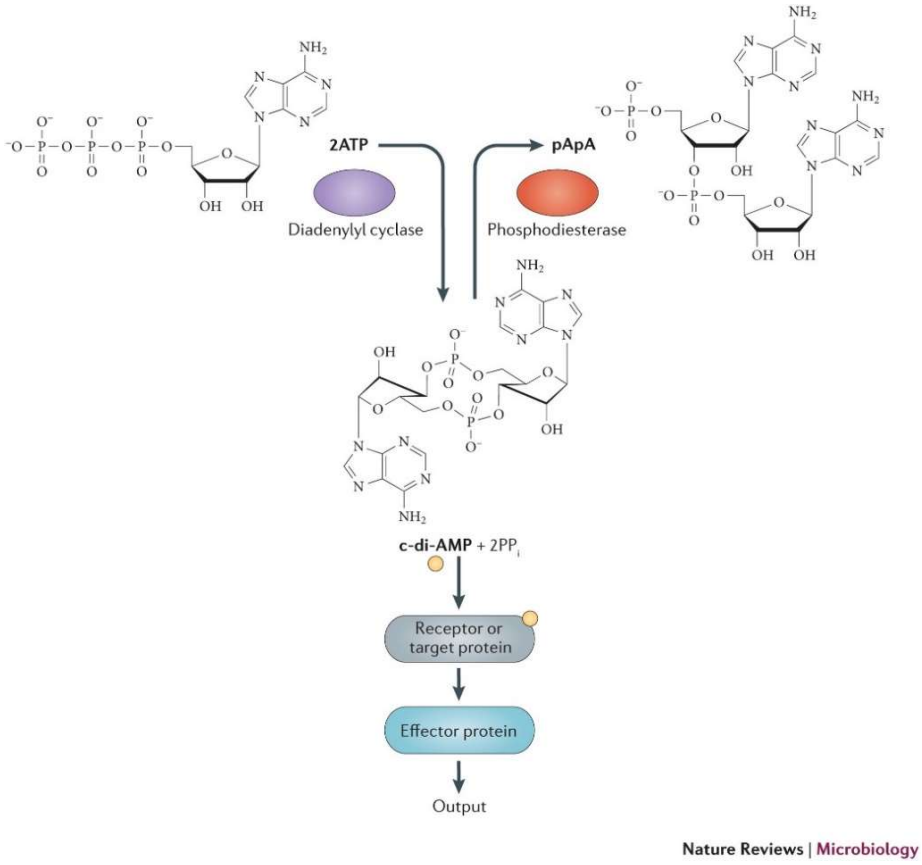

Figure 4. Schematic representation of c-di-AMP metabolism, associated enzymes and target proteins. DAC enzymes including DacA, DNA integrity scanning protein and DacB are involved in the c-di-AMP synthesis. GGDEF is currently the only characterized domain on PDE of c-di-AMP degradation. C-di-AMP can bind to specific receptors or target proteins, which contribute to the controlling of specific cellular pathways. Reprinted with permission from (44), Copyright 2021 Nature.

1.3 External stimuli affect second messengers

Many studies reveal that nucleotide-based second messengers systematically crosstalk to each other and other second messengers such as linear nucleotides: guanosine tetraphosphate (ppGpp) (50, 51). They orchestrate bacterial cellular processes by binding to specific effectors and various targets at several levels including transcriptional, translational and post-translation levels (23). The cAMP controls biofilm formation via the c-di-GMP signaling pathways in *Vibrio cholerae* and *P.aeruginosa* (37, 52, 53). Based on available studies, c-di-GMP and cAMP levels inversely change when the bacteria alter their mode of life. However, the relationship of recently discovered c-di-AMP (40) with other second messengers and their effectors is still poorly understood.

Numerous external stimuli and small molecules have been recognized as an efficient approach to inhibit or eradicate biofilm-related infection. For example, nitric oxide is found to trigger biofilm dispersal in *P. aeruginosa* by decreased c-di-GMP levels via phosphodiesterase activity (54). Small molecules including Sildenafil, Apremilast, Enoximone have practically been used in the clinic as PDEs inhibitors through cAMP/cGMP signaling. In addition, Bromophenol-TH was the first discovered DAC inhibitor (19). Nevertheless, many unknown substances and their mechanism are yet to be explored. For instance, HCO_3^-, an absence molecule in CF airway and a buffering agent in the oral cavity, is shown to decrease the growth and biofilm formation of pathogenic bacteria (55, 56), but their molecular mechanisms via the second messengers in response to HCO_3^- has not yet been investigated. Therefore, our study focused on this topic.

1.4 Bicarbonate and pH homeostasis in the lung

Acid-base homeostasis and pH regulation are essential systems to maintain normal physiological function and cell metabolism. The acid-base balance is generally preserved with arterial pH ranging from 7.36 to 7.44, and intracellular pH is typically about 7.2 (57). The most crucial buffer system is an HCO_3^-/CO_2 buffer which takes part in several organs such as the lung, the oral cavity and the kidney. In addition to normal homeostasis, HCO_3^- provide antimicrobial function against infection (58). Since hygiene is the utmost importance of the respiratory tract, HCO_3^- also serves this function, especially in airway surface liquid (ASL) (59, 60). Normal ASL maintains the pH around 7.0 and contains 10-20 mM HCO_3^- (61). The ASL sustains ciliary function, mucociliary clearance and antimicrobial properties of the airway. However, the CF airway or mucoviscidosis is an autosomal recessive genetic disease involving gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) protein. The normal homeostasis in ASL is disrupted as it is primarily caused by the dysfunction of CFTR. CFTR is unable to transport apical Cl^- and HCO_3^- into the lumen (62). Sodium and water subsequently move out from the lumen or mucus layer of the ASL, which lead to dehydrated ASL as well as increased acidic and viscous mucus production. Impaired HCO_3^- transport by CFTR results in acidic ASL which provides favorable conditions for bacterial colonization. In addition to maintaining ion balance, HCO_3^- is responsible for conserving mucus properties by enhancing mucin expansion, but in CF, the lack of HCO_3^- secretion

creates thick mucus which leads to mucus blockage and impaired mucociliary transport (63). The depletion of volume, the acidic pH, impaired mucociliary clearance of the ASL, contribute to an unfavorable environment for antimicrobial function in the airway as well as accelerated bacterial colonization causing persistent infections (64). Although many studies have paid attention to the homeostasis and antimicrobial effect of HCO_3^- in the airway (65, 63, 60), no study has examined the effect of HCO_3^- on c-di-GMP and cAMP levels in *P. aeruginosa* related infection in CF.

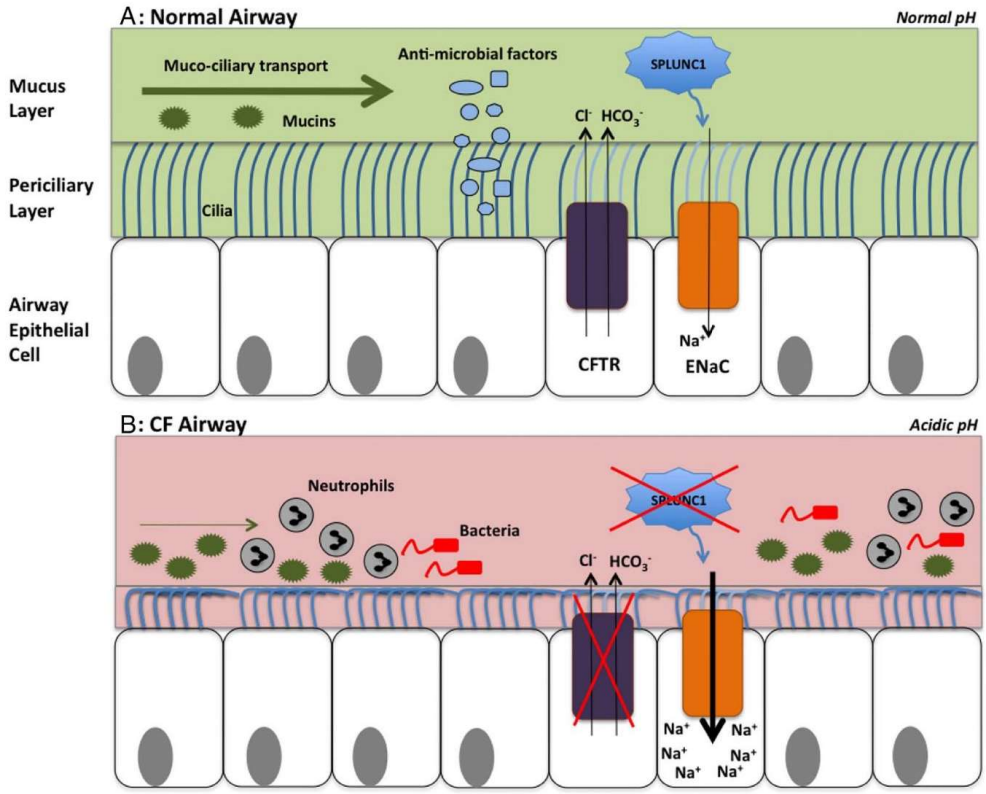

Figure 5. Diagram illustration of the airway surface liquid in normal and cystic fibrosis condition. The airway surface liquid composes of a mucus layer containing mucins and the pericilliary layer (PCL). (A) In the normal airway, mucins capture inhaled particles, and they are subsequently eliminated by mucociliary clearance. In contrast, the PCL forms a tight macromolecular mesh that maintains PCL volume in the hydrated airway. The CFTR protein usually transports Cl^- and HCO_3^- into the airway. (B) In contrast, CFTR is unable to transport Cl^- and HCO_3^- resulting in dehydrated airway and acidic

ASL in the CF airway. Therefore, the antimicrobial function is impaired that leads to bacterial colonization and persistent infection of pathogens. Reprinted with permission from (64), Copyright 2021 BMJ.

1.4.1 Bacterial infection in cystic fibrosis

P. aeruginosa is one of the most common pathogens in the CF lung that worsen pulmonary function and is identified as the pathogen causing nosocomial infections. Almost 30% of infants under one year of age can acquire initial *P. aeruginosa* strains from the environment resulting in acute infections (66). *P. aeruginosa* adapts itself over time as a mucoid phenotype causing chronic infections, subsequently becomes persistent and challenging to eliminate until the end of the patients' life in almost 60-80% of adults (66, 67). After an initial stage of CF lung infection, *P. aeruginosa* experiences variable stress factors such as inflammatory response, competition from other colonizers, nutritional inadequacy, antibiotics, etc. These environmental stress induce genotypic and phenotypic changes associated with enhanced antibiotic resistance, decreased metabolism, retard growth rate, lower motility, deficient quorum sensing, and overproduction of polysaccharide alginate which is primarily found in *P. aeruginosa* (68, 66). As a result, small colony variants (SCV) of *P. aeruginosa* are frequently detected in chronically infected respiratory tracts of CF patients, chronic obstructive pulmonary disease (COPD) and mechanically ventilated patients (69-71)(70–72). Therefore, chronic *P. aeruginosa* infections are challenging to treat and prevent in CF and related-respiratory diseases (72).

Another most commonly found pathogen in CF lung is *S. aureus* which is early detected in the first decades of CF patients, whereas *P. aeruginosa* becomes predominant in adult patients (68). *S. aureus* is a gram-positive coccus typically found on human skin in both CF and non-CF patients. Furthermore, methicillin-resistant *S. aureus* (MRSA) has been in focus, especially in CF for the last ten years. MRSA infection is considered a risk factor in the failure to recover lung function after an acute pulmonary exacerbation. Dasenbrook and colleagues found that CF patients without MRSA infection have greater lung function and survival rates than those with MRSA infection (73). Chronic MRSA infection was considered to be involved in enhanced mortality in CF as well (74). Although *S. aureus* and *P. aeruginosa* are found to be abundant of different

lifetimes (75), coinfection is significant as it becomes more prevalent among CF patients (76) leading to poor clinical outcomes (77, 78).

Besides chronic respiratory infection, CF affects oral health status as well. Oral manifestation related to cystic fibrosis is xerostomia because of the dysfunction of salivary glands. The composition of stimulated saliva of CF patients also changes resulting in higher pH and buffering capacity than non-CF patients. Increased calculus deposition was also reported in CF patients leading to periodontal attachment loss. Furthermore, enamel defects caused by hypomineralization are commonly reported, leading to weakening enamel integrity and unprotected dentin (79-81).

1.5 Bicarbonate and pH homeostasis in the oral cavity

Bicarbonate ions are also part of the carbonic acid-bicarbonate system in the oral cavity that maintains pH balance. Bicarbonate ions derived from saliva does not only served as a buffering agent but also provide an antimicrobial effect against pathogens causing dental caries (82, 83). Since acid production from bacteria lowering environmental pH and dissolving dental hard tissue is the etiological factor of dental caries (84), HCO_3^- generally preserves natural pH balance preventing the progression of dental caries and erosion, and promoting remineralization process (85). The normal pH of saliva is approximately 6.2 to 7.6 (6.7 being the average) whenever bacteria utilize carbohydrates, and subsequently release lactic acid, butyric acid and aspartic acid; thereby, reducing environmental pH. Once the pH goes below 5.5 (critical pH value), enamel on teeth starts to be dissolved by the acids, that is likely to develop dental caries if this phenomenon repeatedly occurs (86). Bicarbonate is one of the several inorganic components secreted by the salivary glands, which its concentration depends on the salivary flow rate. Stimulated saliva can increase the salivary flow rate which leads to washing out acid and enhancing the concentration of HCO_3^- accompanied by raising pH and substantially increasing buffering power (85, 87). Typically, HCO_3^- concentration in stimulated saliva ranges from 15 to 80 mM/L and in resting saliva is around 5 mM/L based on different studies, as it depends on several factors including the type of stimulation, method of measurement and source of salivary gland (88, 89).

The dynamic environment in the oral cavity is typically influenced by various factors including pH, ion, nutrients, temperature and microbial communities. The

etiological of dental caries can be explained by the disruption of well-balanced equilibrium between the oral microorganisms and the host. Microbial consortium shifts to high cariogenicity, namely acidophil bacteria such as *Streptococcus, Actinomyces and Lactobacillus* (90). Since the required factors including excess fermentable carbohydrates, acidogenic-aciduric bacterial species and host susceptibility, present for a period of time, the progression of dental caries finally occurs. *S. mutans* is prominently found in the development of early childhood caries, enamel carious lesions, cavitated lesions, or carious dentin (90). It has high cariogenic potential because of its ability to produce a large amount of EPS containing glucan from sucrose, its ability to metabolize various carbohydrates into organic acid and to survive under extreme environments especially, low pH (84). The common mechanism of *S. mutans* is referred as acid tolerance response (ATR) (91). High consumption of dietary carbohydrates plays a vital role in the shift of microbial consortium related to more cariogenic species. Sucrose is recognized as one of the most cariogenic sugar since it changes biochemical and physiological characteristics of the biofilm (92, 93). Moreover, sucrose is a substrate for polysaccharide synthesis, which is part of the EPS in dental biofilm (94).

For decades, $NaHCO_3$ has been supplemented in oral care products including dentifrice and mouth rinse since HCO_3^- is recognized to have an essential role in the oral cavity. Baking soda-containing dentifrice shows an antibacterial effect on single and mixed cultures, and is not due to its alkaline pH (95, 82). Notably, mature dental biofilm is also disrupted by $NaHCO_3$ (96). Newbrun and colleagues reported that HCO_3^- inhibits selected periodontal pathogens; the higher the concentration, the better the killing ability (97). In a clinical study, $NaHCO_3$ application improves clinical parameters of patients with chronic periodontitis after three months of follow-up (98). $NaHCO_3$ combining sodium metaperiodate and sodium dodecyl sulfate is identified to inhibit and disperse biofilm formation associated with bacteria and yeast in the dental unit waterline as well (99).

Besides the sporadic usage of HCO_3^- in the oral cavity, other commercially available antimicrobial agents and antibiotics has been applied to supplement the substances produced by the body to accomplish effective disinfection. Bacterial infections may cause dental caries and consequent endodontic infection, or periodontal disease which may result in not only tooth loss as the ultimate endpoint but also infections

of head and neck such as osteomyelitis or may play an important role in the pathogenesis of systemic diseases for example cardiovascular disease, diabetes mellitus, rheumatoid arthritis, inflammatory bowel diseases and others. Therefore, researchers focus on developing strategies, substances, irrigants and devices to achieve effective disinfection at present. Although there are many available substances for endodontic disinfection, none of them has all ideal properties of root canal irrigants, even the most frequently used irrigant: sodium hypochlorite (NaOCl) or chlorhexidine (CHX).

1.6 Endodontic antimicrobial agents

Root canal irrigants should have broad antibacterial spectrum, endotoxin inactivation ability, ability to dissolve necrotic tissue, ability to prevent and remove smear layer, non cariogenic and non toxic to periapical tissue, and other ideal properties (100, 101). The NaOCl and CHX, synthetic agents, assemble some mentioned properties. They are commonly used as endodontic irrigants in root canal therapy. In adjunct to mechanical debridement, chemical irrigation is also introduced for effective disinfection as a part of the chemomechanical preparation. Because the complexity of root canal system (102, 103) hampers the accessibility of mechanical instrumentation and provides a shelter for bacteria (104), chemomechanical preparation is one of the available techniques to achieve the goal of endodontic treatment.

1.6.1 Sodium hypochlorite

NaOCl has been recognized as the most widely used irrigant (105) since it is effective against pathogenic microorganisms and provides tissue dissolving properties (106). NaOCl irrigant can be found in the range of 0.5–5.25% were, and the most generally applied concentration is 2.5% (107). Higher concentrations of NaOCl possess better antimicrobial activity and tissue dissolving capacity (108). On the other hand, increased cytotoxicity (109) and irritation of periapical tissue occurs in higher concentration (110). Furthermore, its high concentrions reduce mechanical properties of root canal dentin (111, 112). The literature regarding the efficacy of different concentrations remains arguable (100) since higher concentrations of NaOCl have higher antimicrobial effect against *Enterococcus faecalis* and *Candida albicans* in some *in vitro* studies (108, 113, 114). On the contrary, clinical studies have shown equal antibacterial

effect between high and low concentrations of NaOCl (115, 116). However, its effect also depends on bacterial species and susceptibility.

1.6.2 Chlorhexidine

An alternatively used antimicrobial irrigant is CHX which has a broad-spectrum (117, 118) and substantially lower toxicity than NaOCl (119). CHX at 2% is the most used concentration in endodontic treatment. Whereas NaOCl seems to have concentration dependent effect on its efficacy, CHX exhibits only bactericidal effect in high concentration and a bacteriostatic effect in a low concentration (120). Substantivity effect is the recognizable feature of CHX because its positive charges can attach to negative charges of dental surfaces, causing prolonged adhesion which leads to long-term antimicrobial effect (121, 120). Nonetheless, the noticeable disadvantage of CHX irrigation is the absence of tissue dissolving capacity (122).

To the best of our knowledge, none of the available irrigants has all the ideal properties of endodontic irrigants such as board spectrum antimicrobial effect, ability to dissolve necrotic pulp tissue, long-term antimicrobial effect (substantivity), nontoxic to vital tissue, ability to remove smear layer, etc (123).

Despite NaOCl and CHX being the most widely used endo irrigants all over the world, clinical studies showed no agreement on the antimicrobial efficacy of CHX versus NaOCl as their results were contradictory (124-126). In addition, a recent systematic review comparing the antimicrobial efficacy of these two irrigants summarized that studies published were insufficient and inconsistent to draw a conclusion, and suggested that further well-designed randomized controlled trials (RCTs) should be performed (127). A meta-analysis is also required in order to provide practical and reliable evidence that could help improve the clinical outcome of endodontic treatment.

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2. OBJECTIVES

2.1 We aimed to investigate the effects of HCO_3^- on second messenger concentrations and biofilm formation of *Pseudomonas aeruginosa*.

Our study aimed to explain the antimicrobial effect of HCO_3^- on the most prevalent bacteria in cystic fibrosis airway, especially *P. aeruginosa*. Furthermore, we aimed to develop a protocol to simultaneously investigate c-di-GMP and cAMP levels related to their biofilm formation and to investigate the biofilm formation during HCO_3^- supplementation in real-time.

2.2 Our aim was to investigate the effects of different pH values on second messenger concentrations and biofilm formation of *Streptococcus mutans*.

We aimed to study how *S. mutans* responds to extracellular alkaline and acidic pH by changing its second messengers, including the c-di-GMP, cAMP and recently discovered c-di-AMP, related to the growth and biofilm formation.

2.3 We aimed to compare the clinical antimicrobial efficacy of the most frequently used root canal irrigants, NaOCl and CHX.

Our systematic review supplemented with meta-analysis aimed to study the antimicrobial efficacy of NaOCl and CHX during root canal therapy, as well as other related factors such as type of irrigants, concentration and exposure time from the available RTCs.

3. RESULTS

3.1 Antimicrobial effect of bicarbonate on *P. aeruginosa*

3.1.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) measurement by broth microdilution

MIC and MBC of *P. aeruginosa* (ATCC 27853 and a clinical isolate) and *S. aureus* (ATCC 29213 and a clinical isolate) were determined after 24 h incubation. The MIC of $NaHCO_3$ was 125 mM/ml on all four tested bacteria. The MBCs in two *P. aeruginosa* strains were 500 mM/ml, whereas the MBCs of the two *S. aureus* strains remain higher than the highest $NaHCO_3$ concentration (MBC > 1000 mM/ml). Brief methods can be found in the appendix (page 70).

3.1.2 Bicarbonate and alkaline pH increased intracellular cAMP levels based on an ELISA-based method

Intracellular cAMP levels after 16 h incubation are presented in a figure 6. Brain-heart infusion (BHI) medium with 100 mM $NaHCO_3$ significantly increased cAMP concentrations in *P. aeruginosa* and *S. aureus* compared to the absolute control (BHI medium alone) and BHI media with the same osmolarity (100 mM NaCl). In *S. aureus*, the effect of both 25 and 100 mM $NaHCO_3$ was detected compared to the BHI medium alone. Notably, the supplementation of 100 mM NaCl in BHI media did not affect cAMP concentrations in both *P. aeruginosa* and *S. aureus* compared to the absolute control.

In addition, various pH values ranging as 6.0, 6.8, 7.4 and 9.0 were tested on cAMP concentrations *P. aeruginosa*. Alkaline pH values slightly increased cAMP levels stepwise from lower pH to higher pH values (Figure 7). Interestingly, even the cAMP concentrations induced by the highest pH value (9.0) were significantly lower than those caused by 100 mM $NaHCO_3$. Taken together, increased cAMP levels are mainly due to HCO_3^-*per se*.

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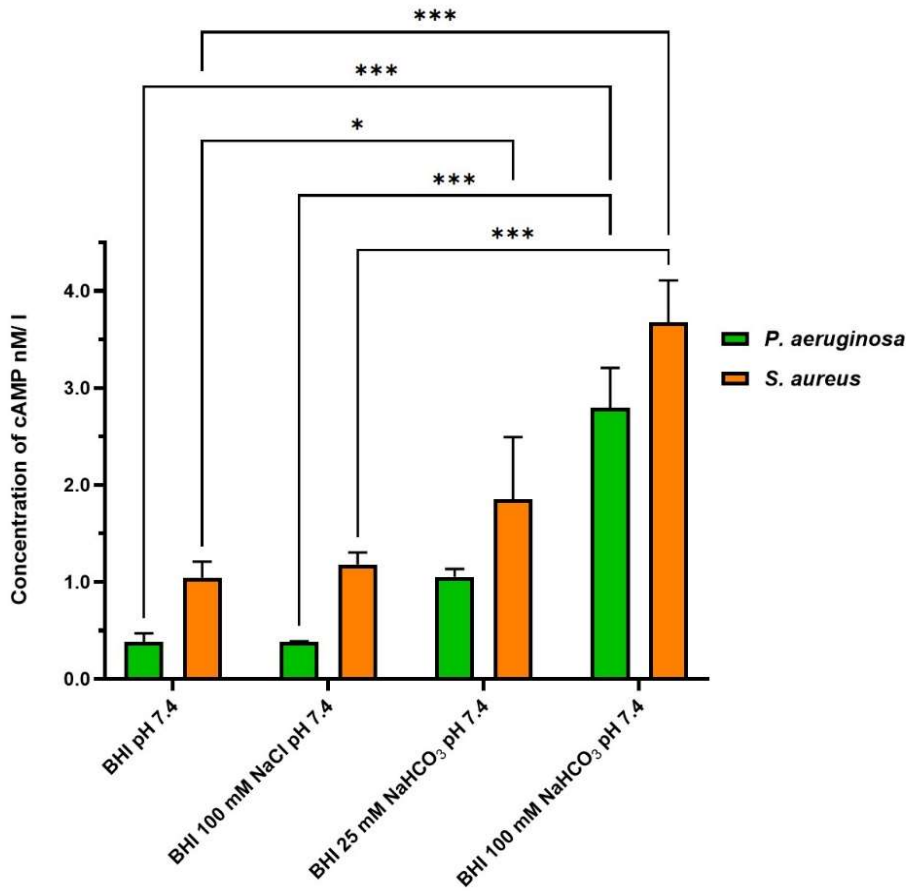

Figure 6. Intracellular cAMP concentrations of *P. aeruginosa* and *S. aureus* clinical isolates influenced by different concentrations of $NaHCO_3$ or NaCl compared to absolute control BHI medium after 16 h incubation; Data were presented as means ± standard deviation (SD) obtained from 3 independent experiments: two-way ANOVA followed by Tukey comparison test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Modified with permission from ref. (55), Copyright 2021, Frontier.

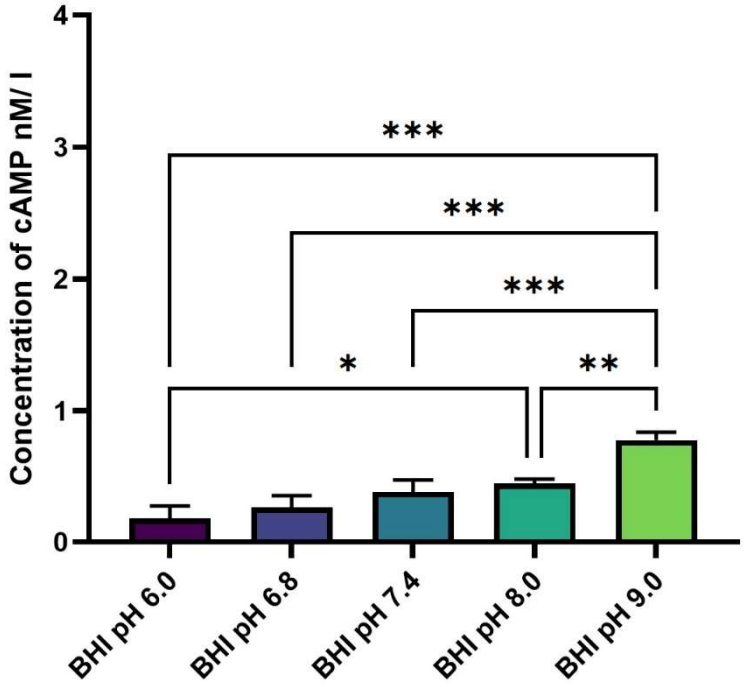

Figure 7. Intracellular cAMP production of *P. aeruginosa* clinical isolate influenced by different pH values after 16 h incubation; Data were present as means ± SD obtained from 3 independent experiments. One-way ANOVA followed by Tukey comparison test: *$p <$ 0.05 and **$p < 0.01$. Modified with permission from (55), Copyright 2021 Frontier.

3.1.3 Bicarbonate decreased intracellular c-di-GMP and increased cAMP Levels based on an HPLC-MS method

Intracellular c-di-GMP and cAMP concentrations after exposure to designated media for 16 h are presented in a figure 8. The control groups (NaCl) were compared to treatment groups ($NaHCO_3$) at the equivalent osmolarity and pH. In *P. aeruginosa* ATCC 27853, both 25 and 50 mM $NaHCO_3$ significantly reduced c-di-GMP concentrations compared to the 25 and 50 mM NaCl with the same pH, respectively (Figure 8a). In *P. aeruginosa* clinical isolate, only 50 mM $NaHCO_3$ depressed c-di-GMP levels compared to 50 mM NaCl at pH 7.7 (Figure 8c). These results also suggested that osmolarity and pH had no considerable effect on c-di-GMP levels. Furthermore, intracellular c-di-GMP concentrations further decreased from 25 mM to 50 mM $NaHCO_3$ indicating a dose-dependent effect of HCO_3^- in both ATCC and clinical isolate (Figure 8 a,c). Therefore, these results show that HCO_3^- *per se* inhibits the intracellular c-di-GMP levels.

Regarding cAMP levels in both *P. aeruginosa* ATCC and clinical isolate, 25 and 50 mM $NaHCO_3$ significantly increased cAMP levels compared to NaCl groups with equal concentrations and pH values (Figure 8 b,d). We also detected the does-dependent effect of HCO_3^- on cAMP levels in ATCC strain and clinical isolate because the cAMP levels increased in BHI media from 25 mM to 50 mM $NaHCO_3$. In addition, neither the administration of BHI medium with NaCl nor pH changes the intracellular cAMP levels indicating that the only HCO_3^- enhanced cAMP levels.

Taken all the above together, HCO_3^- increases cAMP, while decreases c-di-GMP concentrations in a dose-dependent manner. These changes were not significantly caused by the alterations in osmolarity or pH of HCO_3^-.

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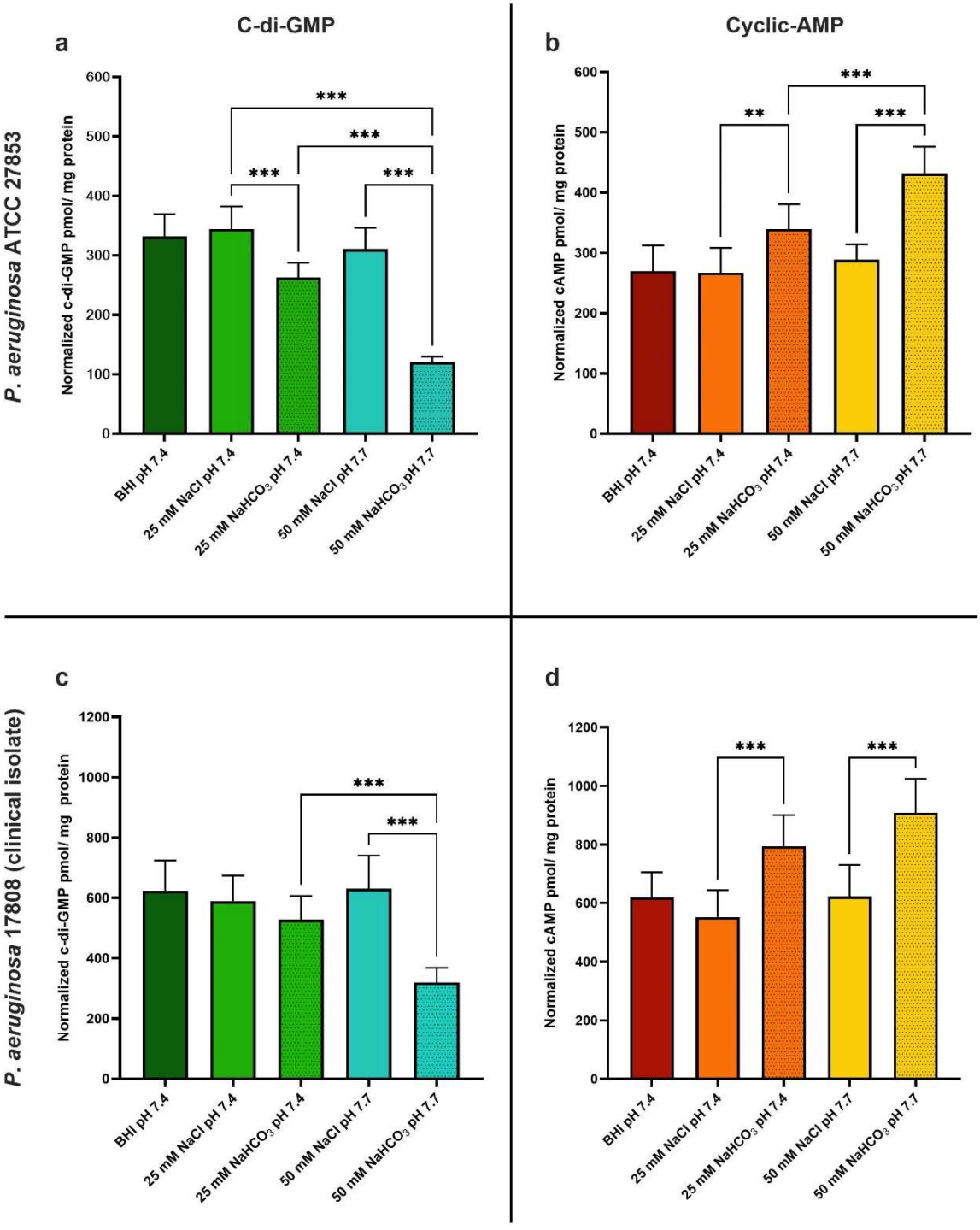

Figure 8. Intracellular second messenger concentrations after 16 h incubation: (a) c-di-GMP levels and (b) cAMP levels in *P. aeruginosa* ATCC 27853; (c) c-di-GMP levels, and (d) cAMP levels in *P. aeruginosa* 17808 (clinical isolate). Values are presented as normalized c-di-GMP or cAMP means ± SD obtained from 3 independent experiments. One-way ANOVA and Tukey's multiple comparisons test: ** $p < 0.01$ and *** $p < 0.001$

when comparing second messengers concentrations in BHI medium supplemented with $NaHCO_3$ to BHI medium with equal concentrations of NaCl at the same pH values. Reprinted by permission from (128), Copyright 2021 MDPI.

3.1.4 Effects of bicarbonate on biofilm formation based on a cell-impedance based method

A Real-Time Cell Analyzer (RTCA) was used to measure biofilm formation. Delta cell indices (ΔCI) representing the attachment of bacterial cells and biofilm formation of *P. aeruginosa* ATCC and clinical strain are shown in figure 9 at specified time points following 6, 12, 24 and 48 h. Parallel measurements were repeated 4-5 times. Generally, *P. aeruginosa* requires at least 24 h to form biofilm (129). Of note, ΔCI in clinical isolate largely increased at 48 h suggesting significantly strong biofilm formation (Figure 9).

The effect of HCO_3^- was investigated in BHI medium supplemented with either $NaHCO_3$ (25 or 50 mM) compared to control media (NaCl 25 or 50 mM) (Figure 9). Despite whether the BHI medium was supplemented with $NaHCO_3$ or NaCl, ΔCI enhanced with longer incubation time in both ATCC strain and clinical isolate. The ΔCI of clinical isolate in BHI medium alone and NaCl groups at 48 h dramatically increased compared to previous time points (Figure 9b,d). However, both 25 and 50 mM $NaHCO_3$ significantly reduced ΔCI compared to the equal concentrations of NaCl following 48 h incubation.

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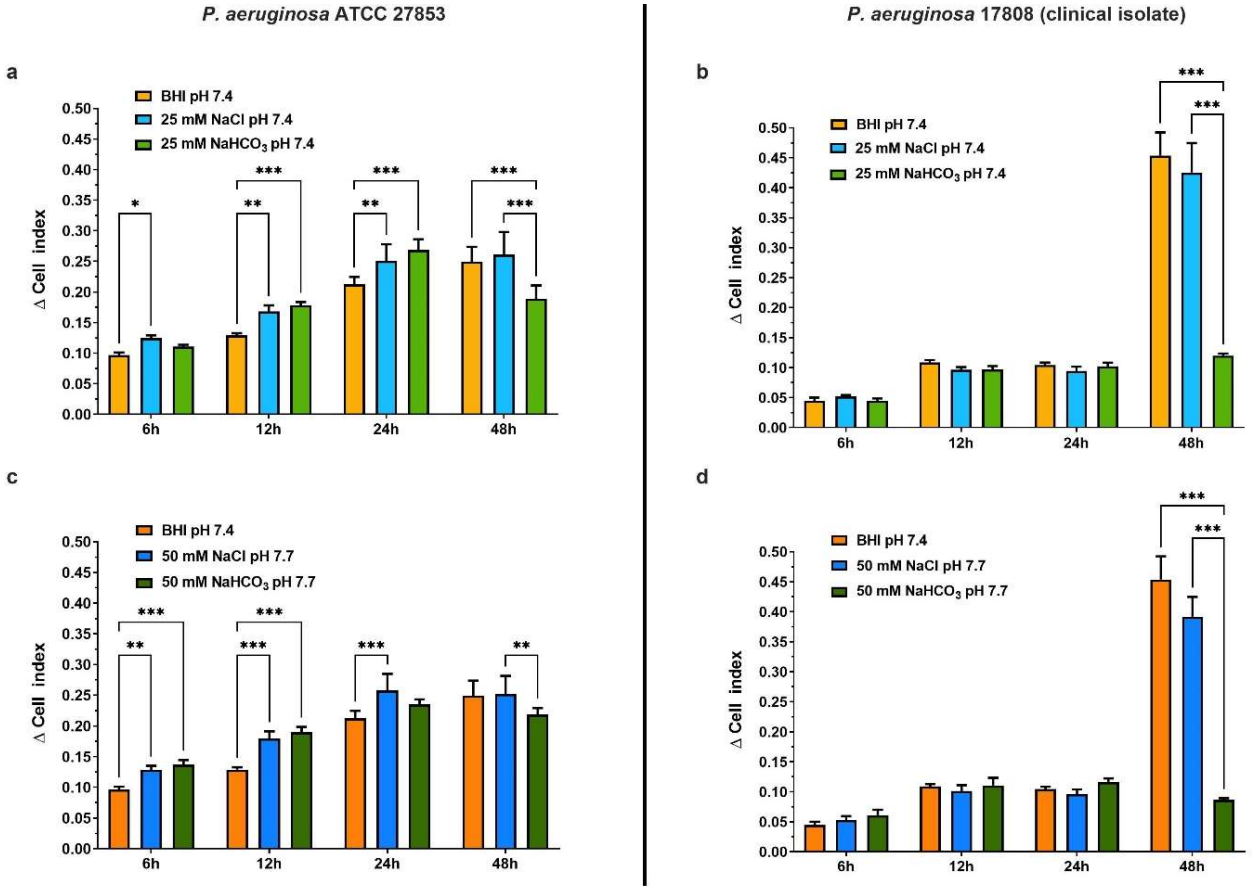

Figure 9. Biofilm formation based on ΔCI of *P. aeruginosa* ATCC 27853 (a,c) and *P. aeruginosa* 17808 (clinical isolate) (b,d) in media supplemented with 25 mM (a,b) or 50 mM (c,d) NaCl or $NaHCO_3$ using an impedance-based method at specified time points. Values are presented as means of Δ cell index ± SD from 4–5 parallel measurements. Two-way ANOVA and Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when comparing ΔCI in pure BHI medium, BHI medium supplemented with $NaHCO_3$ and BHI medium with an equal concentration of NaCl. Reprinted with permission from (128), Copyright 2021 MDPI.

3.1.5 Effects of sodium bicarbonate on biofilm formation assessed by a crystal violet assay

To confirm the biofilm formation result by the RTCA method, a conventional crystal violet assay was performed after 48 h incubation representing OD_{595} values of both *P. aeruginosa* strains (Figure 10). In *P. aeruginosa* ATCC 27853, both 25 and 50 mM $NaHCO_3$ did not decrease biofilm formation as judged by the crystal violet assay

(Figure 10a). Nonetheless, 50 mM $NaHCO_3$ significantly reduced biofilm formation compared to the NaCl group with an equal concentration and pH in *P. aeruginosa* clinical isolate (Figure 10b) indicating that the osmotic or pH changes did not play a significant role in the inhibitory effect of $NaHCO_3$ on biofilm formation. Interestingly, 25 mM NaCl induced higher biofilm formation compared to the BHI medium alone in clinical isolate. However, 25 mM $NaHCO_3$ did not have this effect suggesting that this is not related to osmotic alteration.

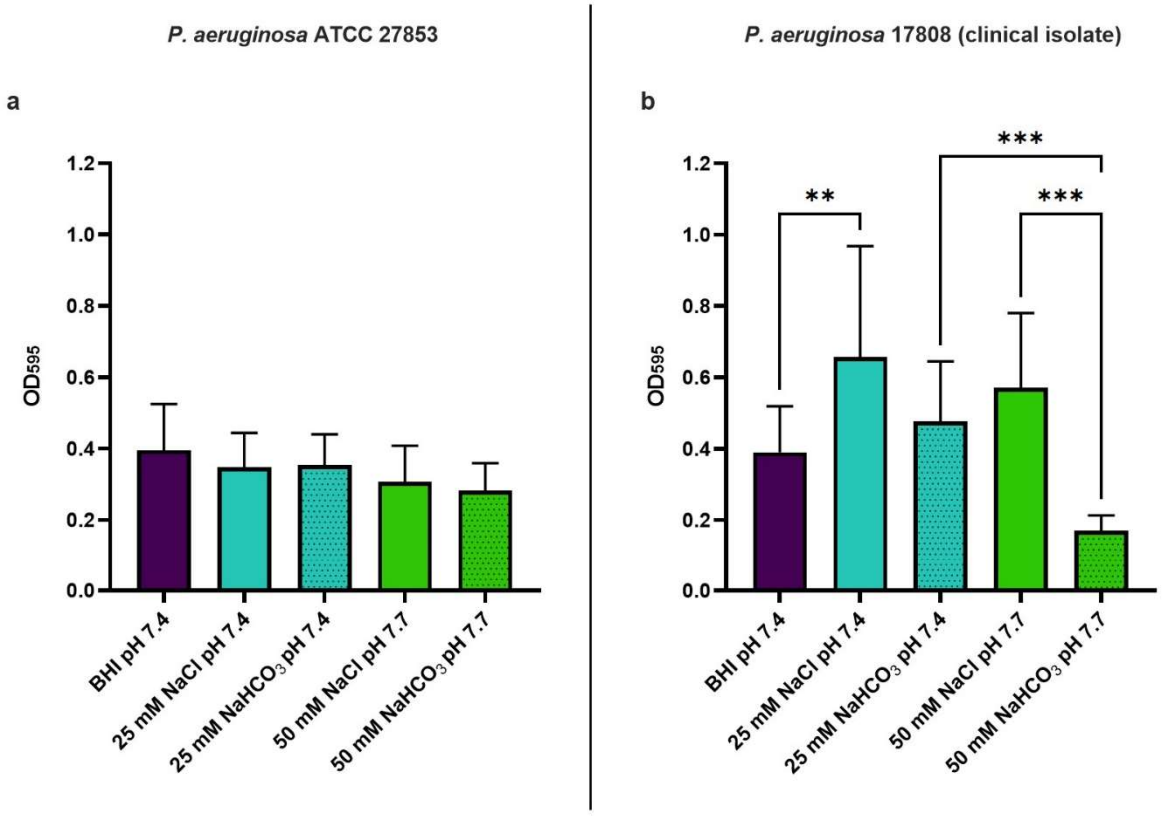

Figure 10. Biofilm formation based on OD_{595} at 48 h using crystal violet assay: (a) *P. aeruginosa* ATCC 27853 and (b) *P. aeruginosa* 17808 (clinical isolate). Values are presented as means of OD_{595} ± SD from 3 independent experiments. One-way ANOVA and Tukey's multiple comparisons test: ** $p < 0.01$ and *** $p < 0.001$ when comparing BHI medium supplemented with $NaHCO_3$ and BHI medium supplemented with the equal concentration of NaCl. Reprinted with permission from (128), Copyright 2021 MDPI.

3.2 Effect of pH on second messengers and biofilm formation in *S. mutans*

3.2.1 Sucrose concentrations affected the growth of *S. mutans*

Figure 11 shows that 1% sucrose supplementation induced the highest growth of *S. mutans* ATCC 35668 compared to other concentrations at 8 h incubation. Although the bacterial growth was generally more robust according to the length of the incubation period, no significant difference among sucrose concentrations was detected following 16 h. Based on these results, 1% sucrose would be used for further experiments.

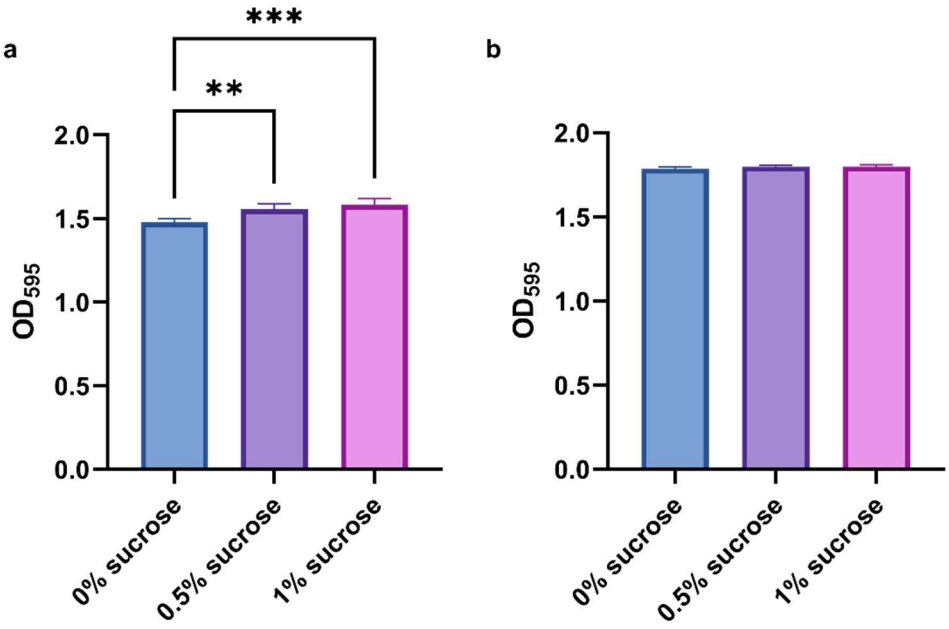

Figure 11. Growth of *S. mutans* ATCC 35668 based on OD_{595} under different sucrose concentrations at (a) 8 h and (b) 16 h. Values are presented as means of OD_{595} ± SD from 6 measurements. One-way ANOVA and Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when comparing BHI medium supplemented with different sucrose concentrations to each other.

3.2.2 Effect of different pH values on second messengers in *S. mutans*

We extracted and measured c-di-GMP, cAMP and c-di-AMP levels after exposure to pH 4.5, 5.5, 7.5 and 8.0 for 0.5 or 3 h. All normalized second messengers showed no significant difference between 0.5 and 3 h exposure time (Figure 12), except the c-di-AMP levels at pH 8.0 which were significantly different between two-time points ($p <$

0.05) (Figure 12c: statistic is not shown on the graph). These data indicate that these incubation times do not influence these second messenger levels under different pH exposure.

At both incubation periods, BHI pH 8.0 significantly reduced c-di-GMP concentrations compared to pH 7.5. On the other hand, acidic pH values did not alter c-di-GMP levels.

In case of cAMP, no significant difference was observed at 0.5 h due to various pH values exposure, but it decreased in a stepwise manner from pH 5.5 to the more alkaline pH values at 3 h incubation time (Figure 12b). Although there was no significant difference of cAMP levels at 0.5 h, cAMP levels under pH 8.0 at 3 h were significantly lower than cAMP levels under acidic pH 4.5 and 5.5.

C-di-AMP levels similarly changed as c-di-GMP under pH alterations (Figure 12c). However, overall concentrations of c-di-AMP are essentially lower than c-di-GMP. C-di-AMP levels decreased both in the acidic and in the basic directions compared to pH 7.5 following 0.5 and 3 h.

3.2.3 Effect of different pH values on biofilm formation of *S. mutans*

Biofilm formation of *S. mutans* ATCC was assessed by crystal violet assay after 48 h incubation. The data showed that alkaline pH 8.0 increased biofilm formation, while more acidic pH values (pH 4.5 and 5.5) decreased biofilm formation following 48 h compared to pH 7.5 (Figure 13). However, there was no significant difference between pH 4.5 and 5.5.

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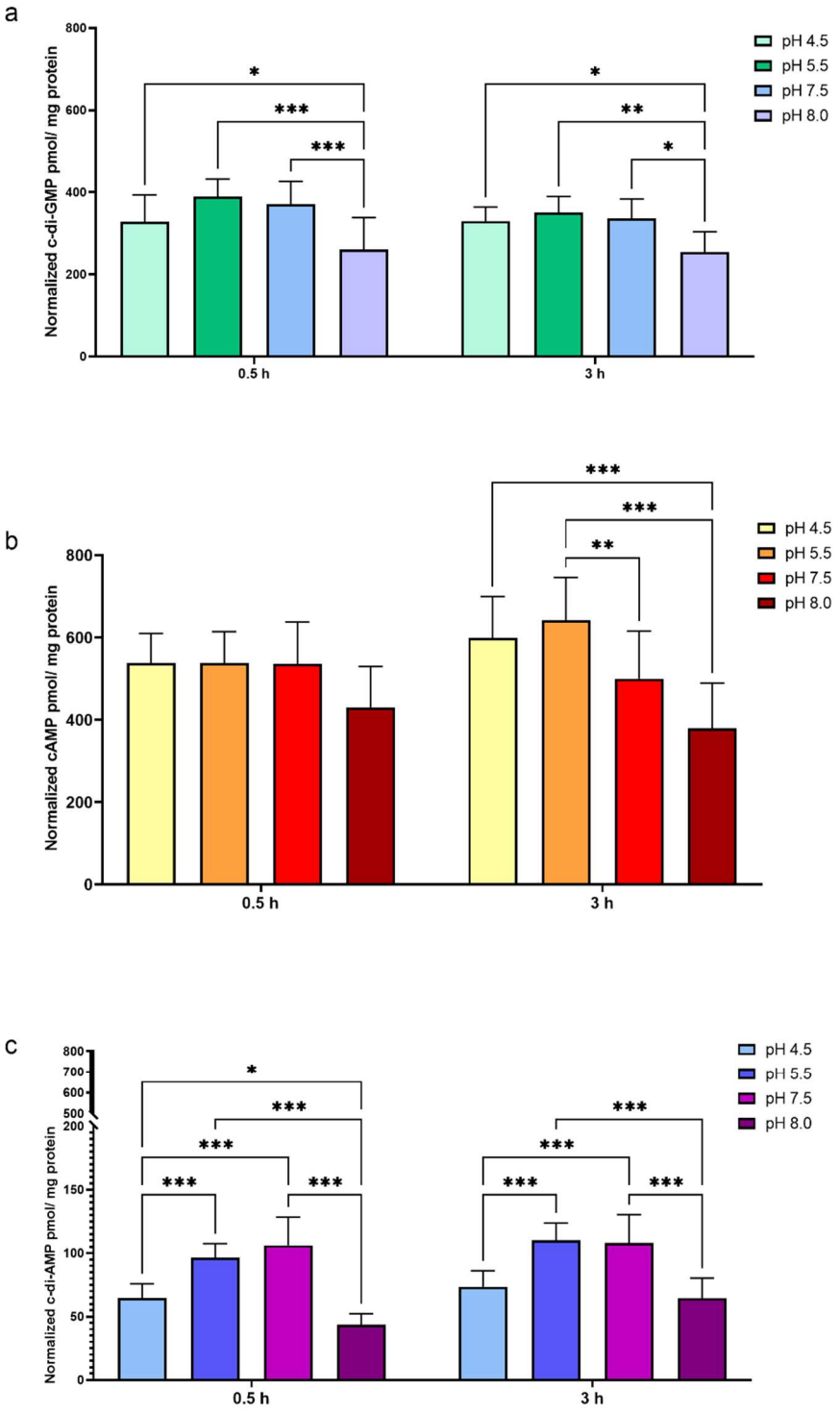

Figure 12. Effect of different pH values on *S. mutans* second messengers including (a) c-di-GMP (b) cAMP and (c) C-di-AMP at 0.5 and 3 h exposure time; second messenger levels are presented as means ± SD from 4 independent experiments: two-way ANOVA

and Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p <$ 0.001 when comparing all groups to each other.

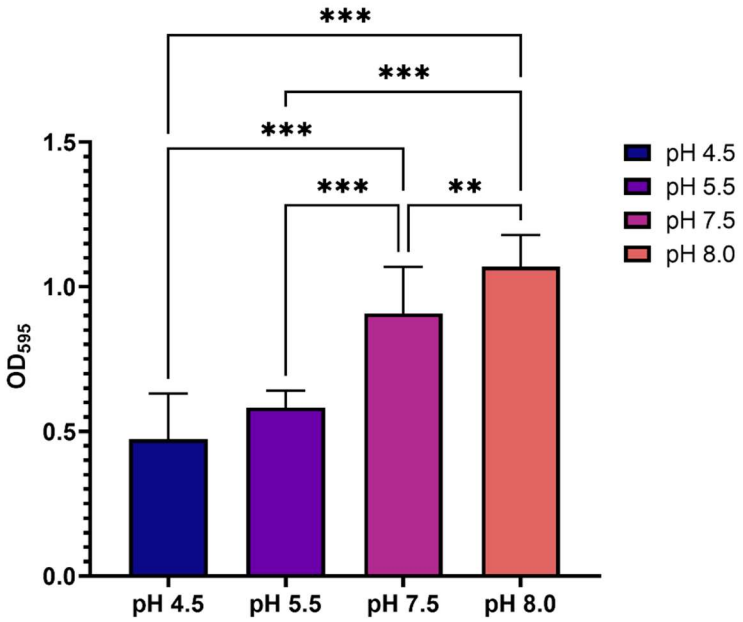

Figure 13. Effect of different pH values on Biofilm formation of *S. mutans* at 48 h. Data are presented as means ± SD from 3 independent experiments: two-way ANOVA and Tukey's multiple comparisons test ** $p < 0.01$, *** $p < 0.001$ when comparing all groups to each other.

3.3 Comparison of the effectiveness of NaOCl and CHX in root canal irrigation

3.3.1 Search and selection of studies

A systematic search from 4 databases until March 2020, including PubMed (via MEDLINE), EMBASE, Web of Knowledge, and Cochrane Library (CENTRAL), yielded 2,763 records as figure 14 presents the flow diagram of the search and selection processes. Another record (130) was found in the reference list of a review article (131). After removing duplicated records, the remaining 2,110 records were screened for eligibility by titles and abstracts. A total of 2,099 records had to be excluded since they were irrelevant. Finally, the full-text review of 11 articles was performed for eligibility. At this step, 3 articles were also removed because of the following reasons: one study investigated only endotoxin levels as an outcome (132); another study was a non-RCT (133); and the last one investigated only the effect of gaseous ozone in combination with

NaOCl and CHX (134). Consequently, 8 studies were included for systematic review (124, 135, 130, 125, 126, 136-138).

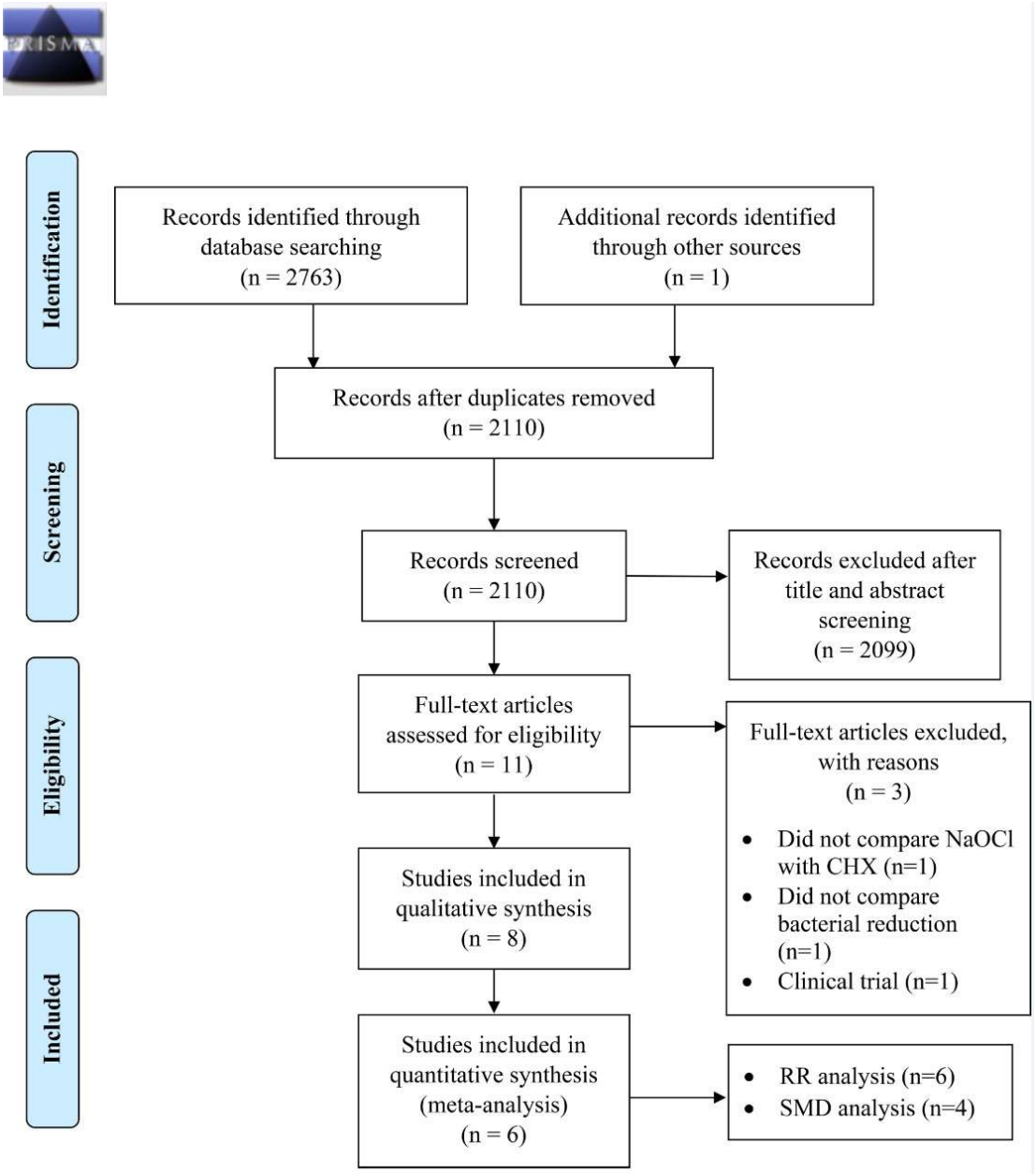

Figure 14. A Preferred Reporting Items for Systematic Reviews and Meta-analysis Statement flowchart of the search and selection strategy. Reprinted with permission from (139), Copyright 2021 Elsevier.

According to our search strategy and selection criteria, 6 of 8 studies provided the number of samples with positive and negative bacterial growth (124, 135, 125, 126, 136,

138) that could be included for relative risk (RR) analysis as an incidence of positive bacterial samples after irrigation (see appendix for more information). Two of these 8 studies were excluded because one reported only the number of visits yielded negative culture (130) and another one reported only secondary outcome (137) (clinical parameters). As a result, these 2 studies are only suitable for systematic review but not a meta-analysis. Furthermore, one of the 6 studies reported only specific bacterial strains (124) and another one provided only the range of bacterial amounts (135) which was categorized data and cannot be calculated for meta-analysis. As these 2 studies provided inappropriate data types, they could not be analyzed for standardized mean difference (SMD) analysis as it was calculated from bacterial means before and after irrigation. Finally, 4 studies were eligible for SMD analysis since they presented the number of bacteria before and after irrigation (125, 126, 136, 138).

3.3.2 Characteristics of the included studies

The characteristics of included studies are presented in table 1. All studies were single-center RCTs. They described the forms and concentrations of irrigants. Four studies indicated volume of each instrumentation (135, 125, 137, 138), whereas only 3 studies reported the total amount used of irrigant (124, 126, 136). Another study did not define any particular volume but it obscurely mentioned: “a copious amount of irrigant” (130).

Every study used sterile paper points with/without transporting media for sample collection. The data taken from first visit samples were extracted and subsequently analyzed. One of included 8 studies used both culture and molecular bacterial detection methods (126). Three studies used molecular methods (125, 137, 138), while the remaining 4 studies used culture methods (124, 135, 130, 136). In addition, fungi were not investigated by any studies. Only one study investigated the clinical and radiographic-related outcome following 1 and 4 years (137); this was the ongoing study (138) by Zandi et al.

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Table 1. Characteristics of the included studies (continued). Reprinted with permission from (139), Copyright 2021 Elsevier.

Authors/publication year	Location and country of the study	Systemic antibiotic treatment	Tooth			Irrigation protocol			
			Number	Type	Infectious status	Type/concentration	Volume with instrumentation	Total volume	Exposure time
Ercan et al, 2004	NA	NA	30	Single-rooted teeth	Primary endodontic infection	2% CHX/5.25% NaOCl	2 mL CHX/2 mL NaOCl	NA	NA
Kuruvilla and Kamath, 1998	NA	Excluded	40	Single-rooted teeth	Primary endodontic infection	0.2% CHX/2.5% NaOCl		3 mL CHX/3 mL NaOCl	NA
Ringel et al, 1982	NA	NA	60	Single-rooted teeth	Primary endodontic infection	0.2% CHX/2.5% NaOCl	NA	Copious amount	Minimum 30 minutes
Rôças et al, 2016	Dental school, Brazil	Excluded	50	Single-rooted teeth	Primary endodontic infection	2% CHX/2.5% NaOCl	NA	15 mL CHX/15 mL NaOCl	NA
Vianna et al, 2006	Dental school, Brazil	Excluded	32	Single-rooted teeth	Primary endodontic infection	2% CHX gel/2.5% NaOCl	1 mL CHX/5 mL NaOCl	NA	NA
Xavier et al, 2013	Dental school, Brazil	Excluded	48	Single-rooted teeth	Primary endodontic infection	2% CHX gel/1% NaOCl	1 mL CHX/5 mL NaOCl	NA	NA
Zandi et al, 2016, 2019*	Dental school, Norway	Excluded	49	Single-rooted and multiple-rooted teeth	Persistent endodontic infection	2% CHX/1% NaOCl	NA	10 mL CHX/10 mL NaOCl	NA

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Table 1. Characteristics of the included studies (continued). Reprinted with permission from (139), Copyright 2021 Elsevier.

Authors/year	Sampling technique	Primary outcome		Secondary outcome
		Culture method	Molecular method	
Ercan et al, 2004	Sterile paper point	Yes: *Enterococcus faecalis, Staphylococcus aureus, Streptococcus salivarius, Actinomyces israelii*	No	No
Kuruvilla and Kamath, 1998	Sterile paper point with distilled water	Yes: total bacteria	No	No
Ringel et al, 1982	Sterile paper point with reduced transport fluid	Yes: number of visits to yield negative culture	No	No
Rôças et al, 2016	Sterile paper point	No	Yes: total bacteria, *Streptococcus* species	No
Vianna et al, 2006	Sterile paper point	Yes: total bacteria	Yes: total bacteria	No
Xavier et al, 2013	Sterile paper point	Yes: total bacteria	No	No
Zandi et al, 2016, 2019*	Sterile paper point with sterile saline	No	Yes: total bacteria, *E. faecalis, Streptococcus* species	Yes: clinical and radiographic outcome (periapical status) after 1- and 4-year follow-ups

CHX, chlorhexidine; NA, not available; NaOCl, sodium hypochlorite.
*The study published in 2019 by Zandi et al. is the continuation of the study in 2016.

3.3.3 Risk of bias assessment

Cochrane risk of Bias Tool (140) was used to assess the quality of included RCTs. All 6 domains, and "other bias" domain which was defined in our systematic review as a unit of randomization and irrigation protocol, were scored as "high," "unclear," and "low" risk of bias.

The risk of bias of eight RCTs is assessed and therefore summarized in Figure 15. One pair of studies was evaluated together since they are continuous studies (137, 138). Although 2 of 4 studies presented a "high" risk of bias in the allocation concealment domain (137, 138), all four studies remain overall "low" risk of bias (125, 126, 137, 138). The remaining 4 studies (124, 135, 130, 136) had overall "unclear" risk of bias because they did not show their randomization methods, allocation concealment, and irrigation in sufficient detail. Moreover, one of these four was scored as "high" risk of bias on the "other bias" domain (124).

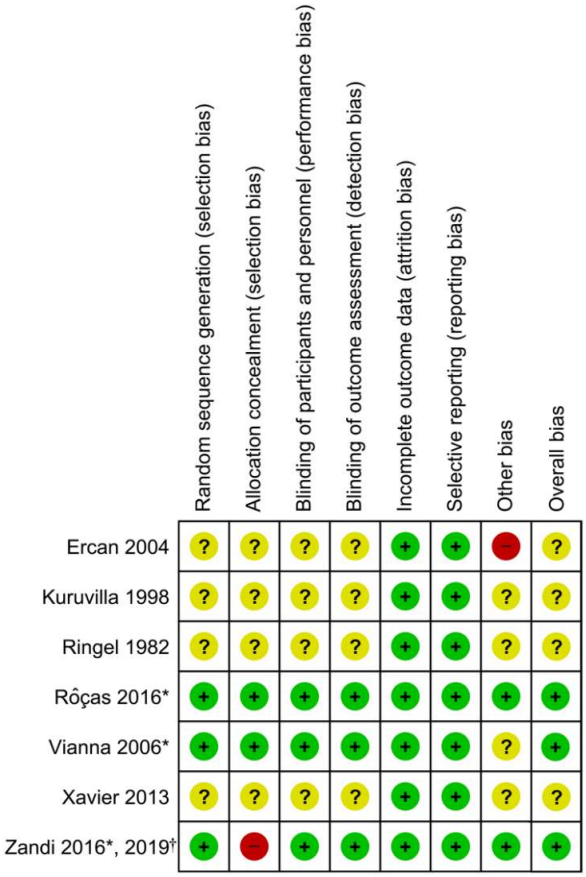

Figure 15. A summary of the risk of bias of the included studies. *The authors provided further information requested by personal e-mail to evaluate the risk of bias. †The study

published in 2019 by Zandi et al. is the continuous study from 2016. Reprinted with permission from (139), Copyright 2021 Elsevier.

3.3.4 Antimicrobial efficacy of NaOCl and CHX irrigants

The pooled RR of samples with positive bacterial growth is presented in the forest plot (Figure 16). The data showed no significant difference in the incidence of positive samples in patients irrigated with CHX or NaOCl (RR=1.003, 95% CI: 0.729–1.380, p=0.987; heterogeneity: I^2=0.000%, p=0.673). Subgroup analysis based on detection method was performed including culture subgroup (RR=0.990, 95% CI: 0.649–1.509, p=0.962; heterogeneity: I^2=0.000%, p=0.408) and molecular subgroups (RR=1.020, 95% CI: 0.626–1.663, p=0.936, I^2=0.000, p=0.601; heterogeneity: I^2=0.000%, p=0.601), this suggested no significant differences between CHX and NaOCl treatments. The heterogeneities among studies were considered as low.

The forest plot of SMD analysis is shown in Figure 17, which represents the difference of mean bacterial number between CHX and NaOCl treatment. The SMD of CHX and NaOCl treatment indicated no significant difference (SMD=0.311, 95% CI: -0.368–0.991, p=0.3. However, these data were considerably heterogeneous (I^2=76.336%, p=0.005). Furthermore, subgroup analysis based on the bacterial detection method was performed (Figure 18), but the overall result was not calculated because one study used the same patient population for both methods in its analysis (126). Subgroup analysis did not show any significant difference between 2 methods (culture method: SMD=0.275, 95% CI: -0.765–1.315; molecular method: SMD=0.173, 95% CI: -0.636–0.982; p=0.880). The culture method revealed substantial heterogeneity (I^2=69.449%, p=0.070) whereas molecular method was considerable (I^2=81.463%, p=0.005).

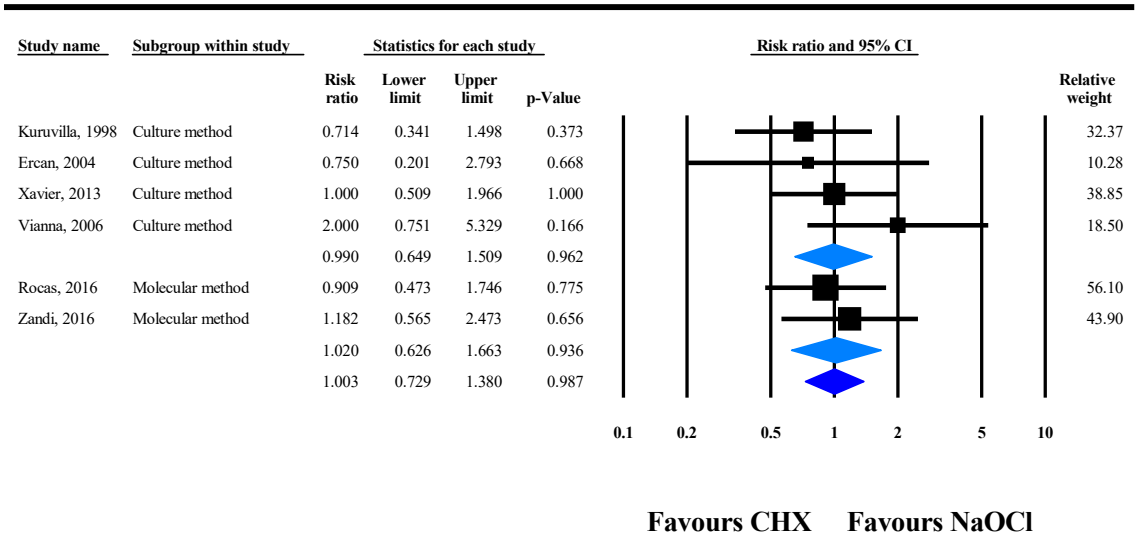

Figure 16. Relative Risk (RR) of samples with positive bacterial growth after irrigation with CHX versus NaOCl. Reprinted with permission from (139), Copyright 2021 Elsevier.

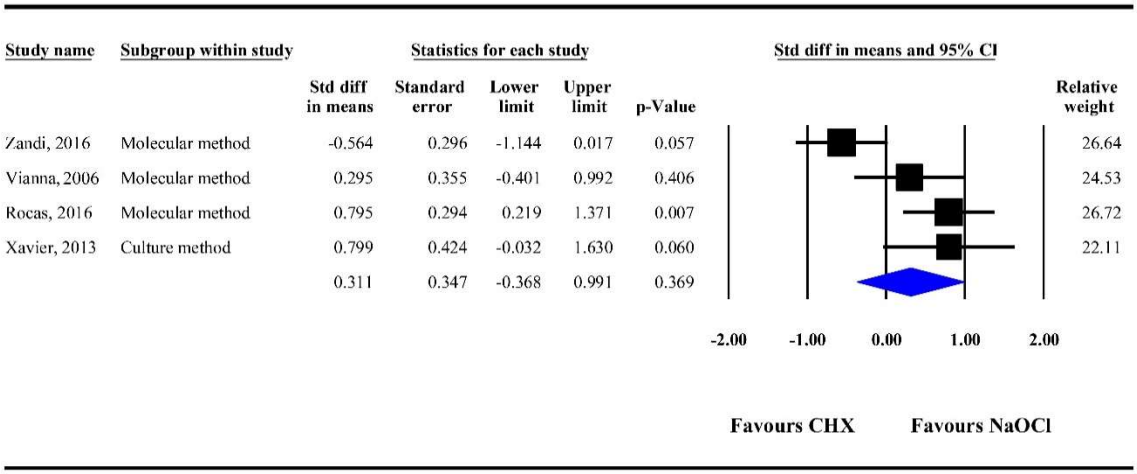

Figure 17. Forest plot of standardized mean difference of bacterial numbers after irrigation with CHX versus NaOCl. Reprinted with permission from (139), Copyright 2021 Elsevier.

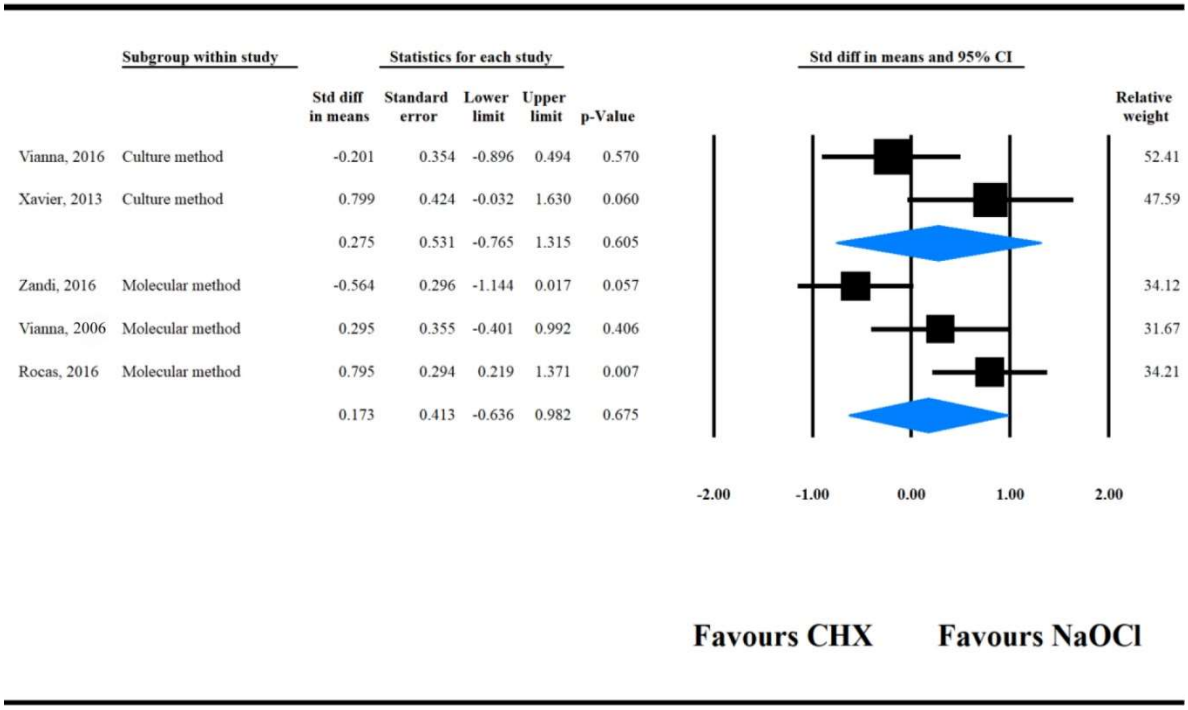

Figure 18. Forest plot of subgroup analysis based on bacterial detection methods of standardized mean difference (SMD) of bacterial numbers after irrigation with CHX versus NaOCl. Reprinted with permission from (139), Copyright 2021 Elsevier.

3.3.5 Certainty of Evidence (GRADE approach)

The confidence of evidence obtained from this meta-analysis was determined by the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) approach. The evidence-based comparison of the antimicrobial efficacy between CHX and NaOCl treatment on antibacterial parameters is shown in a summary of findings (SoF) table (Table 2) constructed with the software GRADEpro GDT: GRADEpro Guideline Development Tool; McMaster University, 2015 (developed by Evidence Prime, Inc.). Total bacterial number changes before and after irrigation were rated as very low evidence according to the serious inconsistency, indirectness, and imprecision domains. Incidence of positive bacterial growth in the samples after irrigation was graded as low evidence because of serious indirectness and imprecision domains.

Table 2. Summary of Findings (SoF) based for certainty assessment

Outcome	Number of teeth (studies)	Certainty assessment		Level of evidence
		Domain	Rate	
Total bacterial number reduction after irrigation during the first visit	105 teeth (4 RCTs)	Study design	Randomized controlled trial	⊕○○○ Very low
		Risk of bias	Not serious	
		Inconsistency	Serious[a]	
		Indirectness	Serious[b,c]	
		Imprecision	Serious[d]	
		Publication bias	Probably present*	
Incidence of samples with positive bacterial growth after irrigation during the first visit	205 teeth (6 RCTs)	Study design	Randomized controlled trial	⊕⊕○○ low
		Risk of bias	Not serious	
		Inconsistency	Not serious	
		Indirectness	Serious[b,c]	
		Imprecision	Serious[d]	
		Publication bias	Probably present*	

a. Considerable heterogeneity (I^2=76.336%) was presented in the standardized mean difference (SMD) analysis

b. The patients having teeth with either primary or persistent endodontic infection were included in the analysis.

c. The different concentrations of NaOCl solution and CHX in solution and gel forms were used during the root canal therapy.

d. Low information size resulted in the uncertainty of the result

*The publication bias was not performed. It was manually assigned to “Probably presented”.

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4. DISCUSSION

4.1 Effect of bicarbonate on second messengers and biofilm formation in *P. aeruginosa*

Acid-base transportation in ASL is crucial to the homeostasis of the respiratory system. The CFTR plays an essential role in HCO_3^- secretion from the epithelia to the luminal airway (64); however, defective HCO_3^- transport in CF creates dehydrated and acidic environment which provides preferable conditions for *P. aeruginosa* colonization (68, 62). Enzymes regulating the second messengers turnover are known to be susceptible to changes in HCO_3^- concentrations. The alteration of c-di-GMP and cAMP could contribute to the *P. aeruginosa* infection in CF airways since these second messengers may affect bacterial lifestyle transition.

To investigate the effect of HCO_3^- on second messenger levels, we selected 25, 50 and 50 mM $NaHCO_3$ because 25 mM is the estimated physiological concentration of HCO_3^-in ASL (61), and 50 or 100 mM are the pharmacological concentrations. Because we required a sufficient amount of bacteria for second messenger extraction, we could not use MIC or MBC. Although 25-50 mM $NaHCO_3$ did not reach MIC or MBC, these concentrations may decrease the growth and biofilm formation of *P. aeruginosa* (56) and, at the same time, they importantly provide a sufficient amount of bacterial cell extracts for further second messenger measurement.

Cyclic AMP plays a crucial role in lifestyle transition, particularly the motility of bacteria. The effect of HCO_3^- on cAMP concentrations was first investigated by using Cyclic AMP XP® Assay Kit, the ELISA-based method. Although this assay kit is initially designed for eukaryotic cells, the measurement of bacterial cAMP is applicable (141, 142). The subsequent measurement was carefully designed to distinguish the effect of $NaHCO_3$ from the osmolarity and pH changes, and the HPLC-MS method was introduced to this experiment for parallel measurement of c-di-GMP and cAMP. Notably, HPLC-MS was applied because it allows the detection of cAMP, c-di-GMP, c-di-AMP and other related compounds concomitantly, such as GTP, GMP, and pGpG (143). The HPLC has been widely used to determine the presence and amount of bacterial second messengers, particularly c-di-GMP (144-146). Mass spectrometry (MS) used in the second experiment is recognized as a highly sensitive approach to quantify the second messenger in picomolar or even in femtomolar range based on the detection method (44, 143, 147).

By using Cyclic AMP XP® Assay Kit, the results indicated that 100 mM $NaHCO_3$ significantly enhanced intracellular cAMP in both *P. aeruginosa* and *S. aureus* (Figure 6). It was subsequently confirmed by the HPLC-MS method that 25 and 50 mM $NaHCO_3$ increased intracellular cAMP concentrations from bacterial cell extracts, but not by the equivalent concentrations of NaCl (Figure 8), suggesting the effect of HCO_3^- *per se*. The results from the two methods corresponded to each other and were in accordance with the previous studies revealing that $NaHCO_3$ range from 5 to 25 mM stimulates soluble AC in both mammalian and bacterial cells (148, 149). In addition, HCO_3^- shows the stimulatory effect on soluble AC inducing cAMP production in corals (150). However, the likelihood of HCO_3^- mediated PDEs activities cannot be excluded, but no current evidence existents to support this hypothesis.

In addition to HCO_3^-, the effect of pH on cAMP concentrations was also conducted using Cyclic AMP XP® Assay Kit because the physiological pH change depends on the equilibrium between HCO_3^- and CO_2, and it has been well demonstrated that ACs activity is sensible to vary environmental stimuli such as calcium ion, HCO_3^-, CO_2 and pH (151, 24). The result indicated that pH range from 6.0 to 9.0 slightly increased cAMP concentrations, especially alkaline pH 8.0 and 9.0 (Figure 7). This result is in line with the previous literature showing the activation of soluble AC is modulated by external pH (152). However, the highest cAMP concentrations induced by the tested highest alkaline pH (9.0) (Figure 7) were even lower than those caused by tested highest $NaHCO_3$ concentration (100 mM) (Figure 6), suggesting that increasing of cAMP levels are mainly due to HCO_3^- but not by increased pH values.

Besides cAMP, the c-di-GMP is well characterized as the lifestyle switch regulator (23). It mainly contributes to the sessility of bacteria. Generally, high intracellular c-di-GMP concentrations provoke biofilm formation, whereas lower concentrations promote planktonic lifestyle and biofilm dispersion (5, 23, 27). Our results showed that c-di-GMP levels in *P. aeruginosa* ATCC were inhibited by both 25 and 50 mM $NaHCO_3$, but only 50 mM $NaHCO_3$ reduced c-di-GMP in clinical isolate. These data suggest that these bacterial strains have diverse sensitivity to external HCO_3^- (Figure 8).

According to the literature, the modulation of intracellular c-di-GMP concentration can be explained by either activating PDEs and/or inhibiting DGCs activity (27). In *V. cholerae*, HCO_3^- and bile acids can inhibit DGCs activity and concurrently

stimulate PDEs, which both of these actions lead to decreased c-di-GMP concentrations (153). Further studies are definitely required to identify the molecular mechanisms in which HCO_3^- control c-di-GMP levels in *P. aeruginosa*.

The persistence of *P. aeruginosa* infection in CF is related to its genotypic and phenotypic changes (63). *P. aeruginosa* isolated from CF sputum of chronic CF patients are commonly derived from the same strain but show broad phenotypic heterogeneity (154). One of these variants, SCVs, develop an enhanced ability to form biofilms and are repeatedly resistant to antibiotics (155). The principal mechanisms for the origination of SCVs are still ambiguous. However, the final common mechanism seems to be related to increased c-di-GMP levels (156, 157). The SCVs also produce large quantities of exopolysaccharides which play a pivotal role in the progression of antibiotic resistance (155, 70). An increase in c-di-GMP is acknowledged to be essential for promoting SCV phenotypic change due to overexpression and or activation of DGCs such as WspR or YiB (TbpB) (27). This finding supports the idea that inhalation of aerosolized $NaHCO_3$ might affect the phenotypic change of *P. aeruginosa* in CF infection. The HCO_3^- could effectively reduce c-di-GMP concentrations and therefore inhibit the possibility of biofilm formation in the airways.

Assessment of the alteration of c-di-GMP and cAMP simultaneously indicated that HCO_3^- reciprocally modulates c-di-GMP and cAMP concentrations. The mechanism behind these second messenger synthesis, degradation and their coupling actions are still unclear. Previous studies have revealed that cAMP accumulation suppresses irreversible attachment that leads to decreased biofilm formation in *P. aeruginosa* (37, 158). Furthermore, Almblad and colleagues recently showed that a subset of PDEs is associated with cAMP-Vfr mediated inhibition of c-di-GMP levels (37). As a result, increased cAMP concentrations can reduce c-di-GMP concentrations. On the contrary, enhanced c-di-GMP can reduce cAMP levels and inhibit multiple acute virulence factors, but the detailed mechanisms are not identified up to the present (37).

The effect of HCO_3^- on biofilm formation was investigated by the impedance-based method using RTCA. For microbiology research, this method has been applied to determine the ability of biofilm formation between biofilm and non-biofilm-producing strains (159), to evaluate antiseptic (160) and antibiotic efficacy (161). The results from this method can be presented as either normalized cell index and Δ cell index (162). In

this study, ΔCI values were selected for the analysis because of the noticeable drop in the initial cell index leading to negative cell index under HCO_3^- supplementation. It should take into consideration that the current experiment was performed in the presence of 5% CO_2; thereby, BHI without supplementation or BHI medium with NaCl also contained small quantities of HCO_3^- that could have affected the growth and biofilm formation which was not determined in these results. Our result highlighted that the inhibitory effect of HCO_3^- on biofilm formation of the clinical isolate is more noticeable than ATCC strain because both 25 and 50 mM $NaHCO_3$ stronger decreased ΔCI of clinical isolate more effectively following 48 h incubation (Figure 9b,d).

The detected decrease in ΔCI by RTCA showed that both 25 and 50 mM $NaHCO_3$ significantly reduced biofilm formation in both strains of *P. aeruginosa* after 48 h compared to the control media (Figure 9). Notably, ΔCI progressively increased up to 24 h in the ATCC strain (Figure 9a,b), and the values of media supplemented with NaCl or $NaHCO_3$ were significantly higher than the control medium. These results suggest that increased ionic strength may initially influence bacterial attachment.

The biofilm formation in *P. aeruginosa* can be detected by crystal violet assay after 24 h incubation because visible and physical biofilm can not be captured by this assay in a shorter of time period less than 20 h (129). By using crystal violet assay in our study, the inhibitory effect of 50 mM $NaHCO_3$ on biofilm formation was identified in the clinical isolate but not in the ATCC strain (Figure 10a,b). Although similar changes of HCO_3^- mediated-cyclic nucleotide levels were detected in both *P. aeruginosa* strains, other factors might contribute to their biofilm-forming ability. The controversial data concerning the biofilm-forming ability of *P. aeruginosa* ATCC has been shown in the literature (163, 164). Moreover, *P. aeruginosa* typically forms biofilm at an air-liquid interface referred to floating biofilms (165-167) which produce viscous matrix containing extensive amounts of water. Consequently, the crystal violet assay may not be a reliable method for the measurement of *P. aeruginosa* biofilm as it was formerly suggested (168).

Taken together, these findings point out that HCO_3^- *per se* reduces intracellular c-di-GMP, whereas it enhances cAMP concentrations. The bicarbonate-mediated reciprocal changes in second messenger levels suppress biofilm formation in *P. aeruginosa*. This evidence also indicates that the effects of HCO_3^- are independent of its pH action, but the alkalinization of the ASL that may occur following administration of

HCO_3^- providing an additional advantage in reducing the viscosity of CF sputum (169, 170) and therefore facilitate mucociliary clearance. Both the *in vitro* study (65) and clinical trial (171) show that the administration of bicarbonate is safe for CF. Furthermore, nebulized sodium bicarbonate has no adverse effect on smooth muscle in the airway (172). Thereby, this evidence recommends that aerosolized $NaHCO_3$ is possibly a supportive treatment in CF and could be in other pulmonary diseases related to persistent bacterial infections and viscous mucus production such as COPD.

4.2 Role of pH on second messenger concentrations and biofilm formation in *S. mutans*

Sucrose has been well-known as the most cariogenic dietary sugar compared to glucose, galactose and fructose (93, 173). Gutiérrez and colleagues demonstrated that *S. mutans* incubated in the BHI medium supplemented with 1% sucrose could induce more biofilm formation 2% or 0.2% glucose (159). Thereby, we did not compare other sugar to sucrose in our study (Figure 11). However, the concentrations of sucrose-mediated biofilm formation were still investigated to find proper conditions for the following second messenger experiment. The results indicated that 1% significantly increased biofilm formation at 16 h, but not at 24 h.

We showed the effect of different pH exposures on the second messengers and biofilm formation in *S. mutans*. The biofilm formation of *S. mutans* decreased from alkaline pH to acidic pH values (Figure 13). A previous study indicated that most of the Streptococci exposed to pH range between 3.6-8.6 which can survive at the lowest pH 3.6 (174). Another study shows that *S. mutans* can tolerate acid pH as low as pH 3.0 (175). Castillo and colleagues also showed that noticeable reduction of cell viability count (CFU) could be detected from pH lower than 5.0 in all tested streptococci (174). In addition, our previous study found that pH 8.0 increased CFU in *S. aureus* compared to pH 7.4 (56). In our *S. mutans* experiment, acidic pH also has more negative effect on initial survival cells number that may lead to lower biofilm formation than alkaline pH. We speculated that *S. mutans* under alkaline pH slowly adapted themselves better for 48 h by increasing cell density and thus developing a high amount of EPS which protected biofilm cells (176) in opposition to the cells in acid conditions. In acid conditions, bacterial cells are experienced an acidic shock after a single exposure to modified pH

media resulting in substantial low viable cells during the early exponential phase. Notably, bacteria were transferred from neutral pH 7.5 to variable pH, and they were subsequently measured for biofilm formation and second messenger extraction. *S. mutans* in both biofilm and second messengers extraction less tolerate acid shock because their ATR mechanisms were probably not priorly induced. It has been demonstrated that *S. mutans* cells or biofilms without initial ATR induction significantly reduced biofilm formation and survival rate after exposure to pH 3.5 compared to those with previous ATR induction by pH 5.5 (177, 178, 175).

The pH-induced changes in second messengers were investigated after 0.5 and 3 h incubation. Our result showed that no significant difference between 0.5 and 3 h was detected except c-di-AMP for pH 8.0 (Figure 12). As we designed our exposure times based on a previous study, pH exposure at different time points (0.5, 1, 2 h) affects the acid adaptation of *S. mutans* by changing the expression of related small RNAs with corresponding target genes (179). Therefore, we tested the pH exposure affecting second messenger levels at two broader time points, but no significant difference was found excluding c-di-AMP levels at pH 8.0. Although the small RNAs expression is not directly related to second messenger levels, both small RNAs and second messengers somehow relate to ATR mechanisms in *S. mutans* (179, 180). Accordingly, further experiments are required to find proper incubation time replicating the situation of pH fluctuation in the oral cavity. Our second messenger results at our 2 considered time points may be explained by the stability and degradation of these second messengers during the extraction and pH changes in the culture during the incubation leading to no difference in our experiment.

Second messengers were extracted after a single exposure to different pH values for lesser incubation time (16 h) than in the biofilm experiment (48 h) because the detectable second messenger alterations might occur earlier than the biofilm formation. We tested the effect of both alkaline and acid exposure. Regarding the alkaline challenge condition, pH 8.0 potentially reduced all second messengers compared to pH 5.5 and 7.5, even better than the inhibitory effect of pH 4.5 (Figure 12). This phenomenon indicates that alkaline pH has more substantial impact than acidic pH on second messengers in *S. mutans* supported by the idea that alkali inhibits the growth of acidophilic bacteria (181). Most of the bacteria in dental biofilm are not necessary to adapt themselves to alkaline

environment from approximately pH 8.0, especially *S. mutans* (181). Mutans streptococci do not adequately function in alkaline conditions because of their insufficient Na+/H+ antiporter systems which are responsible for alkaline pH homeostasis and are commonly found in alkaliphile bacteria (181, 182). *S. mutans* rather frequently colonizes in various acidic locations in the oral cavity: dental plaque and dental caries of enamel and dentin, and infected root canal system (183-185). In addition, caries-resistant populations showed more significant ammonia levels with higher resting pH in their plaque (186). It has been demonstrated that the alkali pH and pH generation by some oral bacteria such as *Streptococcus salivarius, Actinomyces naeslundii* and oral haemophili, play an essential role in inhibiting dental caries, and controlling pH drop in the oral cavity dynamically as a response of acid production by aciduric bacteria (181).

In this experiment, we also investigated c-di-AMP predominantly found in gram-positive bacteria including *S. mutans* (44). Because environmental pH in the oral cavity changes between acid and alkaline, bacteria need to respond to the fluctuation of pH ranges through second messengers by forming biofilm for their survival. Apart from c-di-AMP mediating biofilm formation, it is responsible for acid stress tolerance (50, 45, 187, 188). Under acidic conditions, we found that the c-di-AMP concentrations after pH 4.5 exposure were significantly lower than after pH 5.5 and 7.5 exposure (Figure 12c). The same tendency was also found in slightly decreased c-di-GMP concentrations at pH 4.5 (Figure 12a). One possible explanation of our experiment is the lack of ATR in *S. mutans* under acid shock may reduce cell viability and growth, and subsequently cause the lower c-di-AMP concentrations because bacterial growth and viability of cells are associated with second messenger levels from cell extracts (21, 147). In addition, the consequences of low pH are the dysfunction of the acid-sensitive glycolytic enzyme, cell membrane, DNA and protein damages (189).

To date, a limited number of studies addressed the precise roles of pH on c-di-AMP in relationship to the biofilm formation of *S. mutans*. Moreover, the results from previous studies remain controversial regarding the c-di-AMP concentrations and their ability to regulate biofilm formation (190, 191, 47, 192). In contrast, c-di-GMP has been widely accepted as the biofilm regulator in gram-negative bacteria. The interplay between second messengers has been generally described in gram-negative bacteria that increased c-di-GMP levels, whereas decreased cAMP levels support biofilm formation (22, 49).

These relations are also reinforced by our result of *P. aeruginosa* as described above (Figure 8). Previous studies have shown that both c-di-GMP and c-di-AMP are found to regulate biofilm formation and EPS production in *S. mutans* (190, 191, 193), but none of them mentioned cAMP. Based on our result, acidic pH negatively regulated biofilm formation (Figure 13). We show the relationship between tested second messengers and biofilm formation that decreased c-di-AMP in pH 4.5 compared to pH 7.5 (Figure 12c) may relate to the reduced biofilm formation in acidified media (Figure 13). Our result is in accordance with a recent study that a mutant of *Streptococcus mitis* with decreased c-di-AMP production diminishes the adhesion to the abiotic surface of assay plates (180). Surprisingly, pH 8.0 increased biofilm formation with decreased levels of all tested second messengers, contrasting with our assumption. Reduced cAMP levels might be associated with enhanced biofilm formation at pH 8.0. In summary, acidic exposure reduced c-di-AMP and alkaline decreased all tested second messenger concentrations in *S. mutans*.

It is important to note that the distinct experimental designs may cause contradiction between the hypothetic second messenger levels and biofilm formation. For second messenger determination, the bacteria were first incubated in BHI medium pH 7.5 for 16 h because *S. mutans* can reach exponential growth during 8-16 h (194). Furthermore, the second messenger is generally from the early exponential phase (143). Then they were exposed to different pH values for 0.5 and 3 h when the bacteria were still in the planktonic stage, and the second messengers were afterward extracted. Nevertheless, biofilm formation was measured at 48 h since *S. mutans* usually require at least 24 h to form biofilms (194, 195). Therefore, different incubation times between these two parts may cause contradictory results, but proper incubation time awaits further investigations. Typically, the culture conditions dynamically change depending on the type of cultures (static or dynamic conditions) which are determined by available nutrients, oxygen, temperature, pH or surfaces (196). For the biofilm measurement, *S. mutans* cultures were inoculated in 96 wells-plate containing 200 μL of BHI medium, whereas the measurement of second messengers required approximately 10 ml of bacterial cultures. Consequently, the volume and containers are different. In the second messenger extraction experiment, the same amount of *S. mutans* was immediately exposed to the higher volume of media with acid or alkaline conditions for short periods

(single exposure) resulting in both alkaline or acidic stress, which led to reduced second messenger concentrations.

Further experiments are required to clarify these results and the association between tested second messengers and pH because other confounding factors may contribute to external pH response through second messenger-induced biofilm formation as well as controversial outcomes among previous studies. For example, cell density, regulatory system in acid tolerance phenotypes, sessility and motility of *S. mutans* may affect acid tolerance mechanisms (197). The alkaline resistance mechanisms and its related factors also need to be addressed in detail since they are less understood than the acid tolerance mechanisms (198). In addition, the experimental design replicating the fluctuation of pH values clinically in the oral cavity is required.

After a single exposure of *S. mutans* to acid, the c-di-AMP concentrations decreased. On the contrary, a single alkaline exposure decreased all second messengers including c-di-GMP, cAMP and c-di-AMP levels. However, only acidic conditions suppress biofilm formation. These changes conflict with our hypothesis based on the *P. aeruginosa* result. Therefore, further investigations are needed.

4.3 Antimicrobial efficacy of NaOCl and CHX endodontic irrigants

This systematic review with meta-analysis aimed to compare the antimicrobial efficacy of CHX and NaOCl irrigants in permanent teeth during endodontic treatment. The findings indicate that no significant difference is found in their antimicrobial efficacy.

Previous studies have demonstrated that effective chemomechanical preparation using chemical irrigation can improve the clinical outcome and long-term success of root canal therapy (199, 200). This study included only RCTs, which are recognized as the highest level in the hierarchy of evidence. However, the search strategies yielded numerous records from 4 databases, but only 8 RCTs were eligible for a systematic review with meta-analysis. Heterogenous data, namely tooth type, instrumentation protocol, irrigation protocol and bacterial detection method, infection type, were indicated among included RCTs (Table 1). A single teated root canal with persistent infections contains similar quantities of bacteria as in the untreated root canals with

primary infections; nonetheless, Siqueira and Rôças revealed that bacterial diversity decreases after the treatment of persistent infection (201).

Several irrigation parameters of CHX and NaOCl were applied in the included RCTs e.g. concentrations and volume of irrigation. Their antimicrobial effect relies on frequency (202) and exposure time (203) during irrrigation. Longer exposure time or greater volume, and repeated change of irrigants, could offset the lesser volume. However, these parameters were not clearly mentioned in the included trials. Additionally, formulations of the irrigants (108), activation techniques and devices (204, 205), and multivariable ratios of all parameters are definitely associated with antimicrobial efficacy. Due to the limited data, this meta-analysis was unable to explain how these confounding factors influence the obtained results and evidence. Additional RCTs should provide the mentioned parameters in detail since they play an essential role in the effectiveness of irrigants.

Considering sample collection methods, sterile paper points can only obtain bacteria from the main root canal that have insufficient information of bacteria hidden in the other parts of the complex root canal (206, 207). Thereby, bacteria acquired by this method might not be a perfect representative of all bacterial populations in the entire root canal system. However, no better sampling method is presently available. Included studies described two bacterial detection methods including culture and molecular methods. The culture method provides estimate bacterial load and information of virulence factors or antibiotic susceptibility (208, 209). Nonetheless, this method is unable to characterize various microorganisms in parallel or detect uncultivatable bacteria. A molecular method is an alternative approach that aims to overcome the limitation of the previous culture method (201). Most of the included trials applied either the culture or molecular methods except a study by Vianna and colleagues that used both methods (126). According to the molecular method, Taqman and SYBRGreen assays were compared. Taqman assay can precisely detect low target samples (210, 126). In Vianna's study, Taqman assay was selected for RR and SMD analysis, respectively. Regarding the diverse sensitivity of different methods, subgroup analysis of SMD was analyzed separately (Figure 18). The results showed that no significant differences between these two methods of bacterial counting can be detected following the chemomechanical cleaning procedure, which is in accordance with a recent *in vitro* study

(211). Thereby, both approaches are proper for bacterial detection in endodontic treatment.

The controversial results among the included studies might be affected by sample size since inadequate sample size will not provide strong power to indicate any differences between the investigated interventions (212). Only 2 included studies defined sample size calculation (125, 138).

In this meta-analysis, RR and SMD parameters were used to determine statistical differences in antimicrobial efficacy between CHX and NaOCl but no significant differences were found among them. This RR result extended the evidence from a previous meta-analysis reported 8 years ago (131) which was based only on 2 RCTs (124, 126). The finding of a recent meta-analysis that NaOCl could effectively reduce gram-negative endotoxin levels better than CHX but none of the gram-positive parameters were explored (213).

Of note, both CHX and NaOCl could induce the inhibition of bacterial growth in more than half of the samples after application, but neither of them could totally eradicate the bacterial population from the root canal system. Although CHX and NaOCl have equal antimicrobial efficacy, their molecular mechanisms of action were distinct. A clinician should consider other properties of root canal irrigants during endodontic treatment such as the necrotic pulp tissue dissolving ability of NaOCl (122) as well as the substantivity effect of CHX (121). To achieve the most effective treatment, the combination of CHX and NaOCl may be advocated during root canal therapy. In case of their simultaneous application, there is precipitate–formation, which might occlude the dentinal tubules (214); hence, intermediate flushing between these two irrigants is recommended. Furthermore, activation during irrigation, such as manual dynamic agitation, sonic (Endo Activator), passive ultrasonic activation or laser activation is required to enhance root canal disinfection (215, 111).

The included studies showed underlying discrepancies which introduce certain limitations. However, 4 studies still retained an overall low risk of bias (125, 126, 137, 138). Two of 4 studies did not perform allocation concealment (137, 138), and therefore the overall risk of bias remained low because of the prevalence of other low-risk key domains. The remaining 4 studies were assigned to unclear (124, 135, 130, 136). One of these studies exhibits a high risk of other bias (124) because of the unclear randomization

method in a patient with multiple teeth, whether individual teeth or patients themselves were a unit of randomization. There is also a possibility that this investigation is dependent, and the outcome may be susceptible to a clustering effect. The examined teeth may be subjected to cross-contamination as well.

None of the included studies reported any patient-relevant outcomes such as clinical symptoms with their remission, which could also be directly correlated to the effectiveness of root canal irrigant. Nonetheless, only one study revealed the success rate based on radiographic appearance after endodontic treatment (137). Taking all of these limitations into consideration, this meta-analysis is confined by insufficient and inconsistent data obtained from included studies which lower the confidence of evidence. Consequently, additional well-designed RCTs applying various types of teeth, containing proper sample size as well as investigating relevant clinical outcomes, are needed.

In conclusion, the evidence from this meta-analysis recommended that both CHX and NaOCl were equally effective in reducing bacterial infection, but not totally during the root canal treatment. Although their efficacy is comparable, their molecular mechanisms are still different. Since the precipitation of these irrigants can occur during their simultaneous application, consecutive irrigation with intermediate flushes between them is suggested. Finally, the development of a more potent possessing the ideal property of irrigant is desired.

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5. CONCLUSION

1. **Investigating the effects of bicarbonate on second messengers and biofilm formation of *Pseudomonas aeruginosa***
   - In *P. aeruginosa*, which plays a crucial role in cystic fibrosis-related lung infections, HCO_3^- alone enhances cAMP and decreases c-di-GMP concentrations, thereby inducing a biofilm to planktonic state transition. These reciprocal changes in second messenger concentrations were not influenced by the medium pH or osmolarity. Our data demonstrate the antimicrobial properties of HCO_3^-, encouraging the use of aerosolized $NaHCO_3$ inhalation as a supportive treatment in CF patients.
2. **Investigating the effects of different pH values on second messengers and biofilm formation of *Streptococcus mutans***
   - In *S. mutans*, a notable cariogenic bacterium, environmental pH regulates its second messenger concentrations. Biofilm formations decreased with decreasing pH related to reduced c-di-AMP concentrations. On the other hand, alkaline pH reduced all second messengers, but increased biofilm formation. The environmental pH has an influence on all tested second messenger. However, further studies are required to find explicit association between pH alterations and related second messenger and biofilm changes.
3. **Comparing the clinical antimicrobial efficacy of the most frequently used root canal irrigants, NaOCl and CHX**
   - Our findings suggest that both NaOCl and CHX significantly, but not completely, reduce endodontic infections during root canal therapy. Based on our meta-analysis, no significant difference was found between the molecular and culture bacterial detection methods; therefore, both approaches are appropriate for detecting bacterial infections during root canal treatment.
   - Both CHX and NaOCl were equally effective, despite their differing molecular mechanisms. Therefore, each of them can be used as the main antibacterial root canal irrigants. However, because a mixture of these two chemical substances can cause precipitate formation, we suggest a consecutive application with intermediate flushes between each irrigant and propose the development of more potent antibacterial agents.

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6. SUMMARY

Bicarbonate has been recognized to provide antimicrobial function in the normal airway. However, it is absent in cystic fibrosis which leads to enhanced pathogen colonization. We aimed to investigate the effect of extracellular HCO_3^- on intracellular c-di-GMP and cAMP responsible for motility and sessility in *P. aeruginosa*. By using the ELISA-based and HPLC-MS methods to quantify these second messengers. The supplementation of BHI media with HCO_3^- increased c-di-GMP and decreased cAMP levels concurrently, which were not due to osmolarity and pH changes. Our findings indicate that HCO_3^- *per se* modulates the concentrations of both c-di-GMP and cAMP; therefore, decreasing biofilm formation and supporting planktonic lifestyle of the bacteria. These findings would contribute to better understanding and developing effective treatments against biofilm-related infections.

Dental caries is primarily associated with *S. mutans* within biofilms. Although *S. mutans* can survive under acid stress because of its acid tolerance, the second messengers related to this mechanism are poorly understood. Hence, our study aimed to investigate the effects of pH on c-di-GMP, cAMP, and c-di-AMP. Our results showed that acid has an inhibitory effect on c-di-AMP and alkaline exposure on all tested second messengers. However, only acidic pH suppressed biofilm formation. We concluded pH affects all tested second messengers, but additional studies are required to find their clear associations to biofilm formation as these findings supplement information on bacterial acid adaptation in the oral cavity. Therefore, they may advocate the development of treatment by targeting second messengers to inhibit biofilm formation and dental caries.

The primary goal of endodontic treatment is to decrease microbial infections and prevent reinfections. Chemical irrigation is a potential approach for disinfecting root canal systems. However, the present literature shows no consensus regarding the antimicrobial effect of the commonly used irrigants, chlorhexidine and NaOCl. Therefore, we aimed to compare the clinical antimicrobial efficacy of chlorhexidine and NaOCl irrigants in a meta-analysis. The equal effect of these two irrigants suggests that both chlorhexidine and NaOCl can be used as the main endodontic irrigants, despite their different molecular mechanisms. We recommend consecutive irrigation with intermediate flushes between them. Finally, the development of a more potent irrigant with ideal properties is recommended.

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9. LIST OF OWN PUBLICATIONS

The publications related to the PhD thesis (peer-review paper):

1) Ruksakiet K, Stercz B, Tóth G, Jaikumpun P, Gróf I, Tengölics R, Lohinai ZM, Horváth P, Deli MA, Steward MC, Dobay O, Zsembery Á. Bicarbonate evokes reciprocal changes in intracellular cyclic di-GMP and cyclic AMP Levels in *Pseudomonas aeruginosa*. Biology. 2021; 10(6):519. doi10.3390/biology10060519

SJR Scopus - Agricultural and Biological Sciences (miscellaneous): D1, IF: 5.079 (Expected IF value)

2) Dobay O, Laub K, Stercz B, Kéri A, Balázs B, Tóthpál A, Kardos S, Jaikumpun P, **Ruksakiet K**, Quinton PM, Zsembery Á. Bicarbonate inhibits bacterial growth and biofilm formation of prevalent cystic fibrosis pathogens. Front Microbiol. 2018 Sep 19;9:2245. doi: 10.3389/fmicb.2018.02245. PMID: 30283433; PMCID: PMC6157313.

SJR Scopus - Microbiology (medical): Q1, IF: 4.259

3) Ruksakiet K, Hanák L, Farkas N, Hegyi P, Sadaeng W, Czumbel LM, Sang-Ngoen T, Garami A, Mikó A, Varga G, Lohinai Z. Antimicrobial efficacy of chlorhexidine and sodium hypochlorite in root canal disinfection: A systematic review and meta-analysis of randomized controlled trials. J Endod. 2020 Aug;46(8):1032-1041.e7. doi: 10.1016/j.joen.2020.05.002. Epub 2020 May 12. PMID: 32413440.

SJR Scopus - Dentistry (miscellaneous): D1, IF: 4.171

The publications not related to PhD thesis (peer-review paper):

1) Jaikumpun P, **Ruksakiet K**, Stercz B, Pállinger É, Steward M, Lohinai Z, Dobay O, Zsembery Á. Antibacterial effects of bicarbonate in media modified to mimic cystic fibrosis sputum. Int J Mol Sci. 2020 Nov 16;21(22):8614. doi: 10.3390/ijms21228614. PMID: 33207565; PMCID: PMC7696793.

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SJR Scopus - Multidisciplinary: D1, IF: 4.380

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Levine M, Ruksakiet K, Földes A, Dinya E and Lohinai ZM. Genotypes detect strong and weak innate immunity phenotypes to oral bacteria in humans. Journal of Clinical Medicine by MDPI

Conference proceeding abstracts:

1) **Ruksakiet K**, Stercz B, Jaikumpun P, Dobay O, Zsembery Á, Tóth G, Horváth P, Lohinai Z. The effects of pH on second messengers in *S. mutans*. In: Proceeding of the CED-IADR/NOF Oral Health Research Congress. Brussels, Belgium (2021)
   Journal Article/Abstract (Journal Article)/Scientific
2) **Ruksakiet K**, Stercz B, Tóth G, Jaikumpun P, Dobay O, Horváth P, Zsembery Á, Lohinai Z. External pH regulates intracellular second messengers in *Streptococcus mutans*. In: Proceeding of the 68th ORCA Congress. Zagreb, Croatia (2021)
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3) Levine M, **Ruksakiet K**, Földes A, Dinya E, Lohinai Z. Genes Control Gingival Crevicular fluid responsiveness to bacterial lysine decarboxylase. In: Proceeding of the 99th IADR General session, USA (2021)
   Journal Article/Abstract (Journal Article)/Scientific
4) **Ruksakiet K**, Stercz B, Tóth G, Jaikumpun P, Lohinai Z, Horváth P, Dobay O, Zsembery Á. Bicarbonate oppositely regulates cyclic di-GMP and cyclic AMP levels in *Pseudomonas aeruginosa.* In: Rakonczay, Zoltán; Kiss, Lóránd (eds.) Proceedings of the EFOP-3.6.2-16-2017-00006 (LIVE LONGER) project. Szeged, Hungary: University of Szeged (2020) 99 page 38
   Chapter in Book/Abstract (Chapter in Book)/Scientific
5) Jaikumpun P, **Ruksakiet K**, Stercz B, Pállinger É, Steward MC, Lohinai Z, Dobay O, Zsembery Á. Sodium bicarbonate inhibits bacterial growth in artificial sputum medium. In: Rakonczay, Zoltán; Kiss, Lóránd (eds.) Proceedings of the EFOP-3.6.2-16-2017-00006 (LIVE LONGER) project
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   Chapter in Book/Abstract (Chapter in Book)/Scientific
6) **Ruksakiet K**, Hanák L, Farkas N, Hegyi P, Sadaeng W, Czumbel L, Sang-ngoen T, Garami A, Mikó A, Varga G, Lohinai Z. Antimicrobial effectiveness of sodium hypochlorite and chlorhexidine irrigation: A meta-analysis Journal of dental research 98: B Paper: 0506 (2019)
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7) Jaikumpun P, **Ruksakiet K**, Stercz B, Lohinai Z, Dobay O, Zsembery A. Effects of Bicarbonate on members of periodontal microbiota causing Chronic lung disease. Journal of dental research 98: B Paper: 507 (2019)
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9) Zsembery Á, Jaikumpun P, **Ruksakiet K**, Stercz B, Lohinai Z, Dobay O. A bikarbonát és a pH szerepe a légutak védelmében - mire tanít minket a CF?
   In: Bagdy, György (eds.) FAMÉ 2019 Magyar Kísérletes és Klinikai Farmakológiai Társaság; Magyar Anatómus Társaság; Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság; Magyar Élettani Társaság

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11. APPENDIX

Brief methods for better understanding the results (ordering of titles in this section is in accordance with the result section)

1. Antimicrobial effect of bicarbonate on *P. aeruginosa*

1.1 Minimum inhibitory concentration (MIC) and minimum bactericidal (MBC)

To determine the MIC and MBC of HCO_3^-, *P. aeruginosa* (ATCC 27853 and a clinical isolate) and *S. aureus* (ATCC 29213 and a clinical isolate) were used. Suspension of diluted bacteria in Mueller–Hinton (MH) Broth were prepared as standardized inoculum. The concentrations of $NaHCO_3$ were diluted serially two-fold in 10 steps and subsequently ranged from 1000 to 1.95 mM/ml. After 24 h incubation, MIC and MBC were determined.

1.2 Determination of intracellular cAMP levels based on an ELISA-based method

We first investigated the effect of HCO_3^- on cAMP levels in both *P. aeruginosa* and *S. aureus*. The bacteria were incubated in BHI medium (Mast Group Ltd., Merseyside, UK) supplemented with $NaHCO_3$ or NaCl for 16 h. The pH of BHI medium alone and BHI media with NaCl were set to 7.4 by HCl or NaOH, whereas the NaHCO3 groups were equilibrated with CO_2 incubation to maintain the desired pH. The cAMP levels were subsequently measured by Cyclic AMP XP® Assay Kit (Cell Signaling Technology, Leiden, Netherlands). Bacteria were first grown in the desired medium for 15–16 h, then diluted in the same fresh medium and allowed to grow for a few hours until reaching a log phase at OD_{595} = 0.4–0.5. The cultures were centrifuged, and the bacteria were re-suspended in the kit lysis buffer. Finally, the supernatant was transferred to the assay plate and subsequently measured at 450 nm in a PR2100 microplate reader according to the kit protocol.

1.3 Determination of intracellular c-di-GMP and cAMP Levels based on an HPLC-MS method

We further investigated the effect of HCO_3^- on c-di-GMP parallel with cAMP. Thereby, we carefully designed new conditions to exclude the possible effect of pH and osmolarity from HCO_3^- as well as introduce an HPLC-MS method for second messenger determination. The BHI medium was supplemented with either NaHCO3 or NaCl as follows: 1) BHI alone pH 7.4; 2) BHI + 25 mM NaCl pH 7.4; 3) BHI + 25 mM NaHCO3

pH 7.4; 4) BHI + 50 mM NaCl, pH 7.7; and 5) BHI + 50 mM NaHCO3 pH 7.7. The pH of the BHI medium alone and BHI media supplemented with NaCl was adjusted with NaOH or HCl. BHI medium supplemented with $NaHCO_3$ was equilibrated with 5% CO_2 to maintain the pH values described above. The cultures in BHI media with NaCl were incubated at 37 ◦C in ambient air, while cultures in added $NaHCO_3$ media were incubated in the presence of 5% CO_2.

The bacteria were grown in the designated media in the condition described above. After 16 h incubation, the extraction was performed immediately. The extraction method was modified from Petrova and Sauer protocol (138). Briefly, the OD_{595} values of the bacterial cultures were determined to obtain the same number of bacteria for extraction. Bacterial cells were harvested by centrifugation, while the media were discarded. The cell pellets were resuspended in ice-cold PBS and the extraction of cyclic nucleotides was induced by 100 ˚C incubation and followed by the addition of absolute ice-cold ethanol. The solution was centrifuged, and the supernatant containing the extracted cyclic nucleotides was removed and kept. The extraction was repeated twice from the retained cell pellets. The supernatant was dried in a centrifugal evaporator (Labconco Centrivap Concentrator, Kansas City, MO, USA) and the second messengers in the samples were subsequently determined by the HPLC-MS method. The remaining cell pellets were used for protein measurement. Total cellular protein was measured by using a Quant-iT™ protein assay (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Finally, the total protein content was used to normalize the c-di-GMP and cAMP levels obtained from the HPLC-MS method. Three independent bacterial cultures were performed in all cases.

1.4 Measurement of biofilm formation assessed by cell-impedance based method

To measure the biofilm formation, we inoculated the diluted *P. aeruginosa* in 96-well E-plates equipped with an xCELLigence Real-time cell analyzer (RTCA) (Bioscience Inc., San Diego, CA, USA) in 5% CO_2 incubator. The RTCA can detect the attachment of bacterial cells by the modification of cell impedance signals. The bacteria were inoculated in different designated media including BHI medium alone, BHI supplemented with either 25 or 50 mM NaCl, and 25 or 50 mM $NaHCO_3$. The pH of media supplemented with NaCl (25 or 50 mM) was adjusted with NaOH to pH 8.0 or pH 8.4, respectively. The pH values were adjusted 0.6–0.7 units higher than the desired values because the plates

were only incubated in the presence of 5% CO_2 which reduced the pH of the media during the measurements. Similarly, the pH of the BHI medium alone was adjusted to either 8.0 or 8.4 as the control medium. The E-plate was incubated at 37 ◦C with 5% CO_2 for 48 h. After the inoculation, cell impedance signals were recorded every 10 min for 48 h. The signals obtained at 6, 12, 24 and 48 h were converted by the xCELLigence software to delta cell indices (ΔCI). The measurements were parallelly performed 4-5 times.

1.5 Measurement of biofilm formation assessed by a crystal violet assay

To confirm the cell-impedance measurement, we grew *P. aeruginosa* ATCC 27853 and 17808 (clinical isolate) in 96-well polystyrene flat-bottom microtiter plates (Eppendorf®, 0030730119, Hamburg, Germany). BHI media were prepared as described in the cell impedance-based method. The diluted overnight cultures at OD_{595} = 0.1 were inoculated in each well containing designated medium in 5 parallels for each group. The plate was incubated for 48 h at 37 ◦C in 5% CO2. The media was carefully aspirated from each well, which was then washed by PBS and discarded. This washing step was repeated twice. The plate was then dried at 42.5 ◦C for 90 min. We added 0.1% crystal violet to each well for staining the adherent cells and biofilms. After 15 min, the excess crystal violet was removed and washed. Finally, 30% acetic acid was added to each well to dissolve crystal violet and transferred to a new plate for absorbance measurement. Three independent biological cultures were performed.

2. Effect of pH on second messengers and biofilm formation in *S. mutans*

2.1 Measurement of sucrose concentrations on the growth of *S. mutans*

S. mutans ATCC 35668 were grown in BHI media supplemented with 0%, 0.5 and 1% (w/v) sucrose for 8 and 16 h. The OD_{595} was measured to compare the growth of bacteria under different concentrations of sucrose.

2.2 Investigation of the effect of different pH values on second messengers in *S. mutans*

To test the effect of different pH values on the second messenger in *S. mutans*, we grow *S. mutans* ATCC in BHI media at pH to 4.5, 5.5, 7.5 and 8.0. Bacteria were grown until they reached the stationary phase for 16 h and then transferred to expose designated media with different pH values for 0.5 or 3 h. The extraction was immediately performed after

pH exposure as the same protocol as in *P. aeruginosa*. In *S. mutans*, we include c-di-AMP in the HPLC-MS determination method in addition to c-di-GMP and cAMP.

2.3 Investigation of the effect of different pH values on biofilm formation of *S. mutans*

S. mutans ATCC were inoculated in 96 wells-plate with BHI media at pH 4.5, 5.5, 7.5 and 8.0. The biofilm formation was assessed by crystal violet assay after 48 h incubation as described protocol above.

2.4 Statistical analysis

Normalized c-di-GMP, cAMP and c-di-AMP concentrations were calculated by Microsoft Excel 2019 using a previously described formula (138). All data are presented as mean ± SD. One-way ANOVA or TWO-way ANOVA was used for statistical analysis followed by a Tukey's multiple comparison test. GraphPad Prism version 8.0.0 was used for calculation and analysis. Significance was accepted at $P < 0.05$.

3. Comparison of the effectiveness of NaOCl and CHX in root canal irrigation

3.1 Search and selection of studies

A systematic search was performed until March 2020 from 4 databases including PubMed (via MEDLINE), EMBASE, Web of Knowledge, and Cochrane Library (CENTRAL). Reference lists from the identified studies were also searched for additional studies in the English language. The search strategy complied with PICO components and selection criteria as follows:

P - Population: participants with pulpal and/or periapical disease receiving endodontic treatment in permanent teeth; Intervention, CHX irrigant

C - Comparison: NaOCl irrigant

O – Outcome: the primary outcome was reduced bacterial amount and incidence of positive bacterial samples after irrigation, whereas the secondary outcome was an improvement of clinical symptoms, radiographic parameters, and postoperative pain;

S - Study type: Randomized clinical trials (RCTs).

Our inclusion criteria were randomized controlled trial studies (RCTs) that applied irrigants in permanent teeth with pulpal and/or periapical disease during endodontic treatment. These studies compared the antimicrobial effects between CHX

and NaOCl irrigants and reported the outcome as bacterial reduction by using bacterial culture and/or molecular microbiological methods. The studies did not compare the individual effects of NaOCl and CHX, and those performed in primary teeth or open apex teeth were excluded.

3.2 Data extraction

Two reviewers independently extracted data from full-text studies eligible with the inclusion criteria. If multiple treatment groups were presented in a study, the data exclusively conforming with PICOS were collected. Additional information for a better risk of bias evaluation and the mean with standard deviation values were obtained through personal e-mail communication.

3.3 Quality assessment

The quality of each RCT was assessed according to the Cochrane Risk of Bias Tool. All 6 domains and the "other bias" domain, which was defined as a unit of randomization and irrigation protocol, were rated as "high," "unclear," and "low" risk of bias. To conclude the overall risk of bias, only 4 domains were considered as key domains:

(1) randomization processes,

(2) allocation concealment,

(3) incomplete outcome data, and

(4) other bias.

The decision was made based on key domains and categorized as a "low" risk of bias when more than half of all the key domains were low. A study was characterized as a "high" risk of bias when there were at least 2 "high" key domains. Besides these criteria, the overall result was considered "unclear."

3.4 Statistical analysis of meta-analysis

Relative risk (RR) was calculated for studies that reported samples showing positive and negative bacterial growth after irrigation. Standardized mean difference (SMD) was calculated for studies that reported the means of bacteria before and after irrigation. The 95% confidence intervals (CIs) were calculated for RR and SMD. In case of sufficient data, subgroup analysis was conducted. The significance of any variation and degree of heterogeneity was determined by I^2 and chi-square statistics, respectively. Pooled estimates were calculated with a random effects model using the DerSimonian-Laird

method. We used a comprehensive Meta-Analysis Software Version 3 to compute the RR and SMD.

3.5 Certainty of evidence assessment

The confidence of evidence was evaluated according to the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) approach using a summary of findings table constructed with GRADEpro Guideline Development Tool software (Evidence Prime, Inc, Seattle, WA). Each GRADE criterion was assessed individually and then computed for the certainty of the evidence. Classification of the certainty of evidence consists of the following 4 grades: high, moderate, low, or very low.