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Physiological role of bicarbonate in microbes: A double-edged sword?

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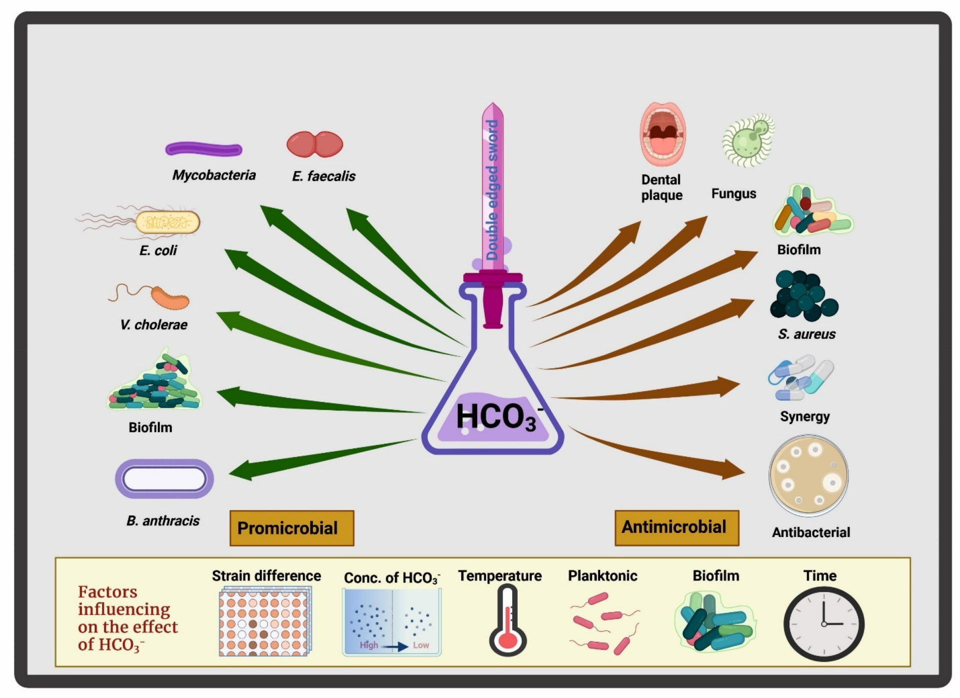
ABSTRACT
HCO_3^- is involved in pH homoeostasis and plays a multifaceted role in human health. HCO_3^- has been recognized for its antimicrobial properties and is pivotal in bacterial antibiotic susceptibility. Notably, the interconversion between CO_2 and HCO_3^-, facilitated by the enzyme carbonic anhydrase (CA), is crucial in tissues infected by pathogens. Studies have highlighted the antimicrobial potency of CA inhibitors, emphasizing the importance of this enzyme in this area. The potential of HCO_3^- as an antibiotic adjuvant is evident; its ability to increase virulence in pathogens such as *Enterococcus faecalis* and *Mycobacterium tuberculosis* requires meticulous scrutiny. HCO_3^- modulates bacterial behaviours in diverse manners: it promotes *Escherichia coli* O157:H7 colonization in the human gut by altering specific gene expression and, with *Pseudomonas aeruginosa*, amplifies the effect of tobramycin on planktonic cells while promoting biofilm formation. These multifaceted effects necessitate profound mechanistic exploration before HCO_3^- can be considered a promising clinical adjuvant.



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Introduction

HCO_3^- plays a multifaceted role in both human health and disease. Serving as the primary pH buffer in mammals, it maintains the acid-base balance, which is essential for normal cellular function [1]. The stability of the body's pH is critical, and even minor deviations can lead to severe metabolic dysfunction [1]. HCO_3^- production is a fascinating biochemical process. Carbon dioxide (CO_2), a byproduct of various metabolic processes, is present in the body and combines with water (H_2O). This reaction is catalysed by an enzyme known as carbonic anhydrase (CA), resulting in the formation of HCO_3^- and a proton (H^+). Given the importance of this reaction in pH regulation, the

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widespread use of CA enzymes is unsurprising. Interestingly, bacteria that have evolved various physiological mechanisms in eggs possess multiple CA genes, emphasizing their importance [1]. Thus, the role of HCO_3^- extends beyond human physiology.

The pathogenic bacteria responsible for various infectious diseases have evolved to thrive in HCO_3^--rich environments. This underlines the importance of HCO_3^- in understanding host-pathogen interactions [2]. The antibacterial and antifungal properties of HCO_3^- have been recognized for years [3]. Historically, research since the 1980s has highlighted its efficacy against periodontal pathogens, leading to its incorporation into dental hygiene products [4]. Over the years, its antimicrobial effects against a plethora of pathogens, such as *E. coli*, *Lactobacillus plantarum (L. plantarum)*, *Staphylococcus aureus* (*S. aureus*), and *P. aeruginosa*, and even fungi, such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Hansenula wingei* (*H. wingei*), have been documented [5]. However, the exact molecular mechanisms remain the subject of intensive research.

HCO_3^- modulates the pH gradient across bacterial membranes, affecting both gram-positive and gram-negative bacteria. Notably, HCO_3^- hinders biofilm formation by *P. aeruginosa*, a notorious pathogen, thereby hampering its virulence [6]. The enhancement of antibiotic potency by HCO_3^- is truly remarkable. In addition to enhancing the effects of traditional antibiotics, HCO_3^- increases the efficacy of antimicrobial peptides. Thus, it could be an adjunct to antibiotic treatment and should be considered when new antibacterial drugs are developed [7]. Although HCO_3^- enhances the efficacy of antibiotics, it also promotes the growth of certain pathogenic bacteria. For example, *M. tuberculosis*, the causative agent of tuberculosis, flourishes in macrophage compartments at various pH values. HCO_3^- contributes to the virulence of this pathogen [8]. Moreover, HCO_3^- positively influences the export of extracellular DNA in several non-tuberculous mycobacteria, an effect that is pH-independent [8,9]. Hence, the dual role of HCO_3^- as both a supporter and an inhibitor of bacterial growth necessitates a cautious approach when considering its therapeutic applications [9,10].

HCO_3^- has a complex role in bacterial interactions, enhancing antibiotic effectiveness and promoting virulence gene expression in various bacteria, including *B. anthracis* and *V. cholerae* [11]. Interestingly, CA inhibitors can inhibit this HCO_3^--induced virulence. Furthermore, although HCO_3^-synergizes with tobramycin against *P. aeruginosa* in one context, it also promotes biofilm growth [12]. Given these contradictory findings, a nuanced understanding of the molecular role of HCO_3^- is essential before its use as an antibiotic adjuvant. In conclusion, the roles of HCO_3^- in health, disease, and therapeutic applications are multifaceted. With a surge in HCO_3^--related studies, the future promises more insights into its potential benefits and challenges.

Physiological role of HCO_3^- in health and diseases in humans

HCO_3^- is produced by the reversible hydration of CO_2 by CAs ($CO_2 + h_2O \rightleftharpoons HCO_3^- + h^+$); it is one of the major anions in humans, and the main function of HCO_3^- is pH homoeostasis [1,13]. In addition to intracellular fluid, body secretions such as saliva, tears, aqueous humour, pancreatic juice, intestinal fluid, airway surface liquid (ASL), and cerebrospinal fluid contain different concentrations of HCO_3^-, which then controls the activity and stability of the proteins dissolved in these fluids [14]. Many epithelia specialize in HCO_3^--rich fluid secretion, which generates flow, alters viscosity, controls pH, and potentially protects luminal and intracellular structures from chemical stress and infections. In hypertensive adults, a higher concentration of HCO_3^- is associated with better cognitive and executive performance than a lower concentration of HCO_3^-, suggesting that a low concentration of HCO_3^- is harmful to neuronal activity [15]. HCO_3^- plays an important role in the capacitation of sperm by alkalizing the sperm cytosol, which is needed for plasma membrane hyperpolarization and hyperactivation of motility and acts as a second messenger, triggering sperm hypermobility and the acrosomal reaction [16]. The transport of HCO_3^- through anion channels influences the membrane potential of epithelial cells. In the nervous system, HCO_3^- transport through ion channels, including glycine and GABA receptors, is needed for the regulation of neuronal excitability [14]. In mucosal epithelia, HCO_3^- promotes the solubilization and expansion of mucin molecules [14]. HCO_3^- has been shown to play a role in cardiac function in isolated cardiomyocytes, which show increased contractility [17].

Studies have shown that rhinoviruses and coronaviruses enter host cells via fusion with cellular membranes at low pH [18]. In SARS-CoV-2-infected patients, nasal irrigation and oral rinsing with 59.5 mm HCO_3^- solution cleared the virus [18]. It is believed that HCO_3^- increases the pH in vesicles/endosomes and inhibits the nucleocapsid release of SARS-CoV-2, which requires an acidic endosomal environment for uncoating. In addition, in people with flu, there was a decrease in the levels of HCO_3^- both in blood plasma and in tissues, suggesting that proper regulation of HCO_3^- protects humans from infections.

Defects in either the production or transport of HCO_3^- lead to various diseases, including systemic acidosis, brain dysfunction, kidney stones, hyperammonaemia, hypertension, respiratory, gastrointestinal, and genitourinary system diseases, cystic fibrosis (CF), xerostomia, pancreatitis, and infertility [1,13,14]. HCO_3^- has been used for the treatment of sepsis, which is a life-threatening medical emergency, and CF [19]. HCO_3^- has been shown to improve the outcome of sepsis patients with acidosis [19]. Patients with renal tubular acidosis and diarrhoea are given HCO_3^- replacement therapy. When HCO_3^- is administered, it primarily increases plasma HCO_3^- levels, thus countering acidaemia and helping restore the physiological pH balance [20]. The therapeutic effects of HCO_3^- administration are especially important in conditions such as metabolic acidosis, where there is an imbalance in the acid-base equilibrium [20]. However, HCO_3^- administration has several possible adverse effects. Some of these effects include an increased risk of hypokalemia, in which blood potassium levels become dangerously low [20]. There is also the possibility of inducing metabolic alkalosis, a condition in which there is a primary increase in serum HCO_3^- [20].

The potential physiological consequences of administered HCO_3^- might also include a sudden increase in haemoglobin-oxygen affinity through the Bohr effect, hypercapnia, and other related effects [21]. It has been shown that 89.3–100 mm HCO_3^- is safe for the human body and has no considerable side effects. Treatment with intravenous infusion of HCO_3^- leads to significant inhibition of lower respiratory tract pathogens such as bacteria, fungi, and mycobacteria. A cohort study regarding the oral rinse of sodium bicarbonate revealed a significant increase in salivary pH and prevented overgrowth of acid uric bacteria [22].

HCO_3^- and bacteria as foes: antimicrobial potential of HCO_3^- and its influence on other antimicrobial agents

Antifungal and antibacterial activity of HCO_3^-

HCO_3^- exhibits significant antibacterial and antifungal properties and can effectively inhibit the growth of *Streptococcus mutans*, especially when combined with sodium dodecyl sulphate [23]. A study on the antifungal activity of HCO_3^- involving 70 fungal strains isolated from skin and nail infections revealed that HCO_3^- (119.05 mm) could curb the growth of 80% of these pathogens. The effectiveness varies based on the type of fungi, with a minimum inhibitory concentration (MIC_{90}) against yeast of 59.52 mm, dermatophytes requiring 238.10 mm, and moulds needing up to 476.19 mm [24]. Furthermore, when tested against clinical isolates, HCO_3^- (119.05 mm) completely stopped the growth of 19 out of 24 samples and notably reduced the growth of the remaining samples within a week compared with samples without HCO_3^- [24]. These findings suggest that HCO_3^- can be used in combination with other antifungal agents, particularly for skin fungal infections and onychomycosis.

A recent study on the antibacterial properties of HCO_3^- against *S. aureus*, *P. aeruginosa*, and *E. coli* has indicated its potential for the treatment of concomitant sepsis [25]. They reported a significant decrease in bacterial colonies within 24 h of HCO_3^- exposure. Given that HCO_3^- is already used to manage metabolic acidosis in intensive care units, these findings suggest that it may also offer antimicrobial benefits for patients with sepsis [25].

Recent studies have expanded our understanding of the antimicrobial and antibiofilm activities of HCO_3^-. It inhibits the growth and biofilm formation of various microbes, including *P. aeruginosa*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Actinomyces naeslundii* (*A. naeslundii*), and *Candida albicans* (*C. albicans*). It also has a lethal effect on *Aspergillus parasiticus*, unrelated to pH or aflatoxin distribution [26]. HCO_3^- drastically reduced aerobic plate counts in tests against aerobic and anaerobic bacteria such as *E. coli*, *S. aureus*, and *P. aeruginosa*. A 1,000-fold reduction was observed at a 120 mm concentration. Even common yeasts such as *S. cerevisiae* and *H. wingei* are highly sensitive, with counts reduced 100,000-fold by 60 mm HCO_3^- [27].

Another study revealed that 1 M HCO_3^- significantly inhibited bacterial, fungal, and mycobacterial growth both *in vitro* and *in vivo*. In both experiments, compared with saline (negative control), HCO_3^- substantially reduced the number of colony-forming units for bacteria and fungi. Moreover, HCO_3^- was particularly effective against *M. tuberculosis*, resulting in a lower prevalence of acid-fast bacilli than saline in both settings [28]. These findings highlight the potential of HCO_3^- as an antimicrobial agent against a range of bacterial and fungal pathogens. Table 1 summarizes the antibacterial and antifungal activities of HCO_3^-.

HCO_3^- enhances antibacterial peptide activity

In human systems, the role of antimicrobial peptides (AMPs) as natural antibiotics, which often have no effect on microbes under culture conditions, remains largely elusive [7]. HCO_3^- plays a key role in enhancing the effectiveness of AMPs, such as cathelicidins and

Table 1. Antibacterial and antifungal activities of HCO_3^-.

Pathogen	Inhibition	Biofilm	Reference
S. aureus	+	+	[6,25,27],
P. aeruginosa	+	+	[6,25,27,29]
E. coli	+	–	[25,27]
H. influenzae	+	–	[29]
B. cepacia	+	–	[29]
Prevotella intermedia	+	–	[30]
Streptococcus sanguinis	+	–	[30]
Aggregatibacter actinomycetemcomitans	+	–	[4,30]
Actinomyces viscosus	+	–	[30]
K. pneumoniae	+	+	[26]
A. naeslundii	+	+	[26]
Haemophilus aphrophilus (*H. aphrophilus*)	+	–	[4]
Eikenella corrodens (*E. corrodens*)	+	–	[4]
Capnocytophaga gingivalis (*C. gingivalis*)	+	–	[4]
M. tuberculosis	+	–	[28]
H. wingei	+	–	[27]
C. albicans	+	+	[26]
A. parasiticus	+	–	[31]
S. cerevisiae	+	–	[24,27]

"+" indicates growth inhibition of the organism and inhibition of biofilm, whereas "–" indicates no growth inhibition or biofilm.

defensins [32,33]. To test the hypothesis that HCO_3^- enhances the effectiveness of AMPs, an *in vitro* study was conducted using animal cell cultures with HCO_3^- in the medium. A study revealed that pathogenic bacteria, such as *S. aureus* and *E. coli*, changed cell wall thickness and sigma factor B expression, increasing their susceptibility to the AMP LL-37. These findings suggest that HCO_3^- could act as a cofactor to increase the antimicrobial potency of AMPs, such as LL-37 [7]. Moreover, HCO_3^- enhances the antimicrobial activity of various structurally different AMPs, including murine cathelicidins (mCRAM), linear porcine cathelicidin (PR-39), and both β-murine and human defensins (Cryptdin-4 and HBD-2) [7]. These findings shed light on the interplay between HCO_3^- and AMPs and suggest that HCO_3^- can act as a cofactor to increase the antimicrobial potency of AMPs.

HCO_3^- is a crucial component of the buffering system in the human body and has a broad-spectrum antimicrobial effect. It also amplifies the antibacterial activity of other innate immune elements. A previous study investigated the effects of physiological concentrations of HCO_3^- (25 mm) on various innate immunity mediators, including defensins and cathelicidins [34]. A previous study revealed that HCO_3^- enhanced the antimicrobial activities of α-defensin, LL-37, indolicidin, bactenesin, and leukocyte protegrin against *E. coli* and *S. aureus* [34].

Moreover, many innate immune system components can disrupt the bacterial membrane potential. This suggests that the host uses a coordinated approach to target bacterial PMF in an HCO_3^--rich environment [35]. Thus, HCO_3^- possesses intrinsic antibacterial properties and is vital for boosting the immune response. It synergizes with the body's physical and chemical defences to effectively eliminate infection-causing pathogens [35].

Effect of HCO_3^- on other antimicrobial compounds

Innovative antibacterial drug screening methods that mimic the host environment where bacteria reside continue to emerge [36]. Recent advances involve chemicals specifically designed to inhibit bacterial growth under conditions that closely mimic those within the host [35]. HCO_3^- is a chemical that is gaining attention for its ability to augment the activity of antimicrobial agents [37]. For example, a previous study demonstrated a significant synergistic effect when HCO_3^- (5 mm) was combined with kanamycin (3.12 µg/mL), resulting in an 80% (w/v) reduction in *E. coli* growth. In contrast, when used alone, HCO_3^- and kanamycin reduce *E. coli* growth by only 5% (w/v) and 15% (w/v), respectively [37]. Interestingly, HCO_3^- also displayed paradoxical eagle-like behaviour at concentrations greater than 20 mm. Further studies are needed to understand its variable interactions with antibiotics, as antibiotic activity is enhanced and suppressed depending on antibiotic concentration [37].

Studies with 25 mm HCO_3^- revealed its ability to increase the efficacy of eight antibiotic classes against both gram-positive and gram-negative bacteria [35]. Specific antibiotics, such as fluoroquinolones, tetracyclines, fosfomycin, and novobiocin, showed variable responses depending on the HCO_3^- concentration. This synergistic activity was not pH dependent but was rooted in the chemical properties of HCO_3^-.

One study reported that HCO_3^- disrupts the proton motive force (PMF) in bacterial cells, thereby affecting antibiotic effectiveness. The PMF, which is crucial for bacterial energy production, consists of an electrical potential (Δψ) and a proton gradient (ΔpH) [38]. The influence of HCO_3^- varies depending on the antibiotic and its reliance on the PMF components. For example, it suppresses the use of tetracyclines, which rely on the ΔpH, while enhancing the use of aminoglycosides, which depend on the Δψ [39,40]. This suggests that HCO_3^- modulates antibiotic uptake by altering PMF components, with effects extending to fluoroquinolones, depending on the specific conditions (Table 2) [35].

Figure 1 illustrates a hypothetical model that shows the influence of HCO_3^- on antibiotics with different physicochemical properties. HCO_3^- is a constituent of the medium ΔpH, representing the acidic extracellular environment and contributing to the PMF across the cytoplasmic membrane in gram-negative and gram-positive bacterial species. Aminoglycosides with a positive charge utilize the negative interior component of the membrane potential (Δψ) for transportation [35].

One study explored through transcriptional analysis how HCO_3^- enhances the efficacy of aminoglycosides in *E. coli* [37,43]. In this study, HCO_3^- (40 mm) treatment for 40 min during the logarithmic growth phase reduced the growth of viable *E. coli*. Using RNA-seq analysis, the study revealed that HCO_3^- significantly altered gene expression, resulting in a tenfold increase in the expression of *tnaA*, which encodes tryptophanase, an enzyme crucial for tryptophan degradation. These results imply that HCO_3^- could influence bacterial metabolism and susceptibility to antibiotics, although the specific mechanism remains unclear. This study highlights the complex interactions between HCO_3^- and bacterial growth. Elevated expression of *tnaA*, which leads to increased indole production, was found to work synergistically with HCO_3^- to inhibit the growth of *E. coli*. These findings suggest a potential role for indoles in growth inhibition. Moreover, HCO_3^- led to significant changes in iron metabolism, as indicated by the upregulation of genes related to iron acquisition and the downregulation of genes related to iron – sulphur proteins. These findings suggest that HCO_3^--induced growth inhibition may be partially due to iron deficiency [37,43]. In another study, HCO_3^- was found to have varying effects on the effectiveness of fluoroquinolone antibiotics (Table 2) [41]. Specifically, it prevents the intracellular accumulation of delafloxacin, reducing its efficacy against multidrug-resistant *S. aureus* and *P. aeruginosa*.

Interestingly, HCO_3^- also exhibited dual behaviour with tobramycin, first showing increased antibiotic efficacy against planktonic *P. aeruginosa* but having an antagonistic effect that encouraged biofilm growth. These findings highlight the nuanced roles that HCO_3^- can play in modulating antibiotic activity and underscore the importance of understanding these interactions for more effective treatment strategies [12].

Table 2. Effect of HCO_3^- on antibiotic activity.

Antibiotic	Activity of HCO_3^-	Reference
Gentamicin, kanamycin, fluoroquinolones, macrolides aminoglycosides, polymyxin B, tobramycin ß-lactam, cefazolin (CFZ), and oxacillin (OXA)	Synergistic	[12,37,41,42]
Tetracyclines, fosfomycin, novobiocin, some fluoroquinolones such as delafloxacin, kanamycin[a], gentamicin[a], nigericin, tobramycin[a]	Antagonistic	[12,35,41]

[a] HCO_3^- concentration >20 mm had an antagonistic effect on kanamycin.

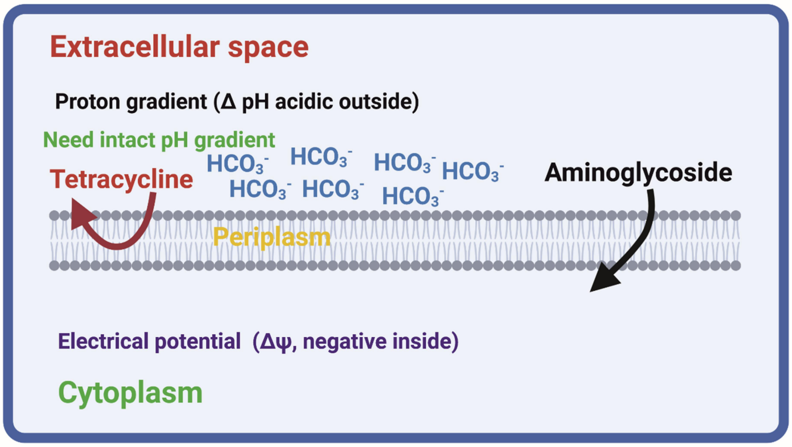

Figure 1. A model depicts the effect of HCO_3^- on various classes of antibiotics. Credit: created via BioRender.com.

The increasing threat of antibiotic-resistant bacteria necessitates a comprehensive and globally coordinated response to ensure the continued efficacy of antibiotic treatments. The reliance of the healthcare industry on a single type of laboratory test for antibiotic susceptibility is a significant shortcoming, especially given that this test often does not replicate the complex interplay between hosts and pathogens in a living system. Emerging research has highlighted the limitations of standard antimicrobial susceptibility testing (AST) conducted in laboratory-specific media, which can produce misleading results. Some antibiotics that have proven ineffective in standard tests are highly effective in media that mimic host conditions. Conversely, certain antibiotics that pass conventional AST have demonstrated poor performance in living organisms.

A promising avenue for improving AST predictive accuracy involves incorporating HCO_3^- into the test medium. HCO_3^-, a common molecule in biological systems, triggers far-reaching changes in bacterial physiology and gene expression. By better simulating *in vivo* conditions, this modification enhances the ability of the test to identify effective treatments, thereby streamlining the development and prescription of antibiotics. The incorporation of HCO_3^- and other host-specific conditions into AST protocols could revolutionize the approach to combat antibiotic-resistant bacteria. By moving towards a more nuanced and biologically accurate model of infection, these advancements could lead to more targeted and effective therapies, reducing the risk of antibiotic resistance and improving patient outcomes [44].

HCO_3^- sensitizes MRSA to β-lactams by regulating gene expression

There are two types of methicillin-resistant *S. aureus* (MRSA): one is susceptible to β-lactams, such as oxacillin and cefazolin, in the presence of HCO_3^-, whereas the other is not [45]. The effect of HCO_3^- on susceptibility is multifaceted: it alters PMF and downregulates key resistance genes, *mecA*, and *sarA*, in responsive strains [35,45,46]. Studies indicate that HCO_3^- in culture media enhances the susceptibility of specific MRSA strains to β-lactams, such as cefazolin and oxacillin. Enhanced susceptibility was also observed in *ex vivo* endocarditis models, where HCO_3^-sensitizes MRSA strains to β-lactams. This effect varies depending on the genetic background of the strain [42,45–47].

HCO_3^- exposure modulates gene expression in MRSA strains, affecting key genes, such as *mecA*, *blaZ, pbp4, vraSR, prsA, sigB*, and *floA*, which are critical for alternative penicillin-binding protein (PBP2a) production and maturation and membrane PBP2a and PrsA protein content. Specifically, it significantly downregulated *mecA, blaZ, the vraSR-prsA gene axis, pbp4*, and carotenoids (Figure 2) while upregulating *floA* across all MRSA strains [46].

These findings revealed that HCO_3^- modulates a set of genes essential for the HCO_3^--responsive MRSA

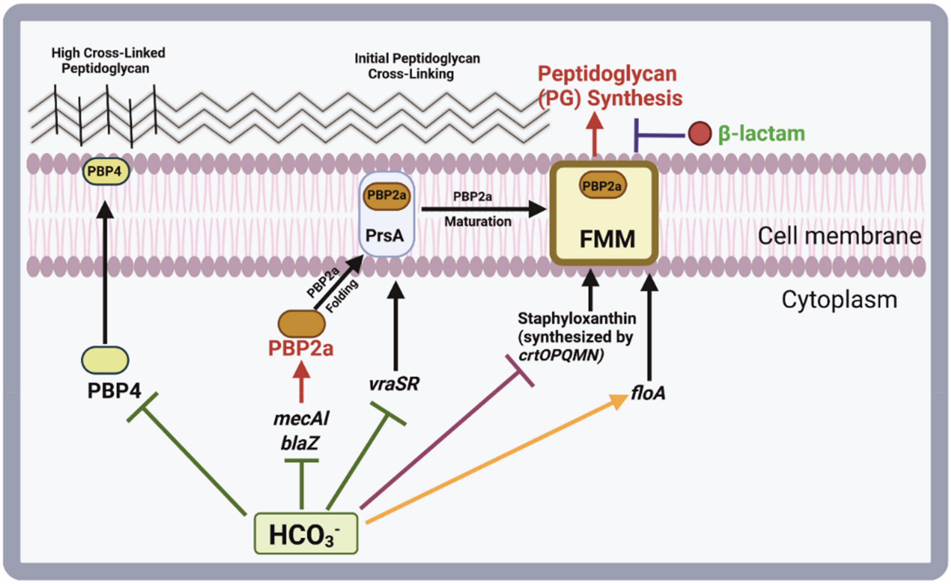

Figure 2. The model illustrates the effects of HCO_3^- on PBP2 and peptidoglycan biosynthesis. HCO_3^-responsive strains display effects such as decreased production of the PBP2a protein, reduced expression of *pbp4* and *vraSR*, and lower levels of *PrsA*. Moreover, HCO_3^- inhibits carotenoid production and increases *floA* expression, leading to unstable functional membrane microdomains (FMMs) [46]. Credit: created via BioRender.com.

phenotype via PBP2a function and maturation [46]. Another study revealed that HCO_3^--responsive MRSA isolates are susceptible to CFZ and OXA, and genotypic markers such as clonal complex 8 [CC8], agr I, and spa t008 are associated with $^{\tau\eta\varepsilon}$ responsiveness of $HCO_3^-$to OXA [42]. RNA-Seq identified key genes that were differentially expressed in HCO_3^--responsive strains, including those in the sigB-sarA-agr axis, cell wall-associated genes, and those related to autolysis (Table 3) [46]. Studies with a fluorescent penicillin probe (bocillin-FL) suggested that HCO_3^- affects β-lactam binding to both the cell surface and PBP2a, indicating the responsiveness of MRSA to β-lactams [47].

Recent research revealed that HCO_3^- decreases wall teichoic acid (WTA) levels and molecular weights in HCO_3^--responsive MRSA strains. It also induces increased autolysis and irregular cell division, both of which are associated with the disruption of WTA synthesis. These data suggest that HCO_3^- inhibits WTA biosynthesis via a posttranslational mechanism involving specific genes such as *tarO*, *tarG*, *dltA*, and *fmtA* (Figure 3). This study revealed that HCO_3^- directly influences WTA biosynthesis in HCO_3^--responsive MRSA strains [50].

Figure 3 shows the pathways involved in the synthesis and maturation of PG and WTA, underscoring the role of HCO_3^- in affecting these processes and contributing to the β-lactam- and HCO_3^--responsive phenotype. This study confirms the inhibitory effect of HCO_3^- on WTA production, which is linked to the increased susceptibility of MRSA to β-lactam

Table 3. Regulation of genes by HCO_3^- and susceptibility to β-lactams.

Regulated genes	Pathogen	Pathway effected	Reference
mecA and sarA	MRSA HCO_3^--responsive	Production/maturation of PBP2a and PrsA protein	[42]
crtM, sigB, sarA, agrA, hla, fnbA, and icaA	*S. aureus*	Regulation of virulence factors	[48]
mecA and blaZ, vraSR-prsA gene axis, and pbp4.	MRSA HCO_3^--responsive	Production/maturation of PBP2a,	[49]
sigB-sarA-agr regulon cap8, clpL, sasD, aaa, vra X,kdpABCDF, betAB, icaR, rsp, clfA, clfB, agr, sdrH,fnbA, fnbB, atl, sceD, isaA, fmtA, ddh, pbp2, bccT,usp	MRSA HCO_3^--responsive and nonresponsive	Virulence autolysins cell wall synthesis, osmotic stress response,	[46]

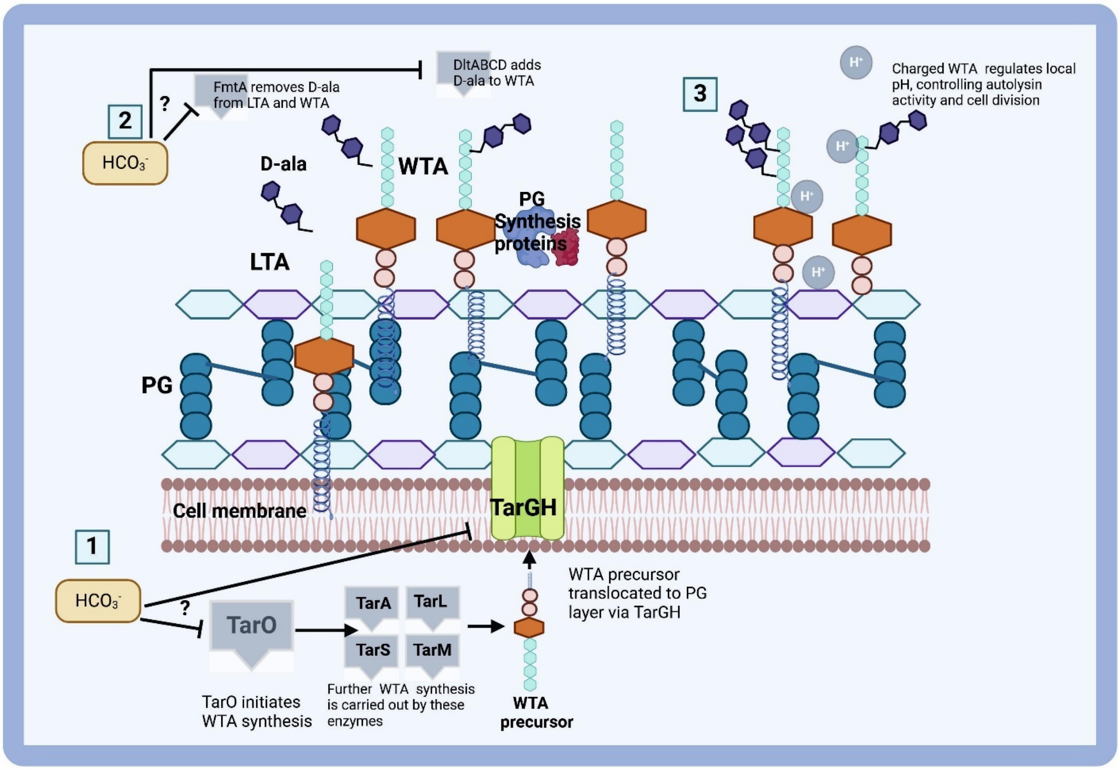

Figure 3. The model elucidates the impact of HCO_3^- on the synthesis of WTA and the susceptibility of strains responsive to β-lactams [50]. Credit: created via BioRender.com.

antibiotics [50]. Another study revealed that HCO_3^- enhances the efficacy of antibacterial drugs against *S. aureus* both *in vitro* and *in vivo*, reducing the production of virulence factors and increasing susceptibility to oxidative stress [48].

HCO_3^- and bacteria as friends: regulation of virulence genes in pathogenic strains

Effect of HCO_3^- on virulence expression in E. coli

Enterohemorrhagic *E. coli* (EHEC), known for causing bloody diarrhoea and haemolytic uraemic syndrome, adheres to the intestinal mucosa and creates attaching and effacing (A/E) lesions via the locus for enterocyte effacement (LEE) genes. Studies have shown that HCO_3^- in the medium increases bacterial adherence and the expression of LEE-encoded genes, such as *intimin, Tir, EspA*, and *EspB* [51]. Furthermore, the expression of *ler*, a crucial regulator of LEE-encoded genes, depends on the HCO_3^- concentration in the medium. This suggests that HCO_3^- acts as an intestinal signalling molecule, facilitating EHEC colonization, particularly in the lower intestine, where the HCO_3^- concentration is relatively high [51].

Another study has shown that HCO_3^- is crucial for activating the *rcsDB* and *rcsB* genes. An intact Rcs system and grvA activator are also vital for LEE stimulation, highlighting the role of HCO_3^- in the virulence of intestinal pathogens [52]. Notably, *RcsB* activates and represses LEE transcription and requires HCO_3^- for activation [53]. Both *rcsB* and *GrvA* are essential for this HCO_3^--induced activation [52]. *GrvA* and *RcsB* jointly mediate the role of HCO_3^- in activating the LEE pathway, which is critical for intestinal pathogen colonization (Figure 4).

Effect of HCO_3^- on virulence expression in Vibrio cholerae

V. cholerae, a gram-negative bacterium, causes cholera, which is characterized by severe diarrhoea. The pathogen has two primary biotypes, classical and El Tor, each with unique *in vitro* growth conditions for virulence gene expression [54]. When exposed to specific triggers or upon infection, *V. cholerae* initiates a complex regulatory cascade. This leads to the production of the regulatory protein ToxT, which activates the transcription of key virulence genes, including those encoding cholera toxin (CT), toxin-coregulated pilus (TCP), and other important virulence genes [11].

Studies have shown that HCO_3^- stimulates CT expression in the *V. cholerae* El Tor biotype and enhances ToxT activity, a key regulatory protein for virulence genes [11]. Both the classical and El Tor biotypes showed inactive ToxT in experiments without HCO_3^-. However, adding HCO_3^- significantly upregulated CT and TCP expression in both biotypes without altering ToxT production levels. The presence of ethoxzolamide, a CA inhibitor, disrupted this HCO_3^--mediated virulence induction, suggesting the role of CO_2 to HCO_3^- conversion by CA in enhancing virulence [11]. HCO_3^- is a key chemical trigger for

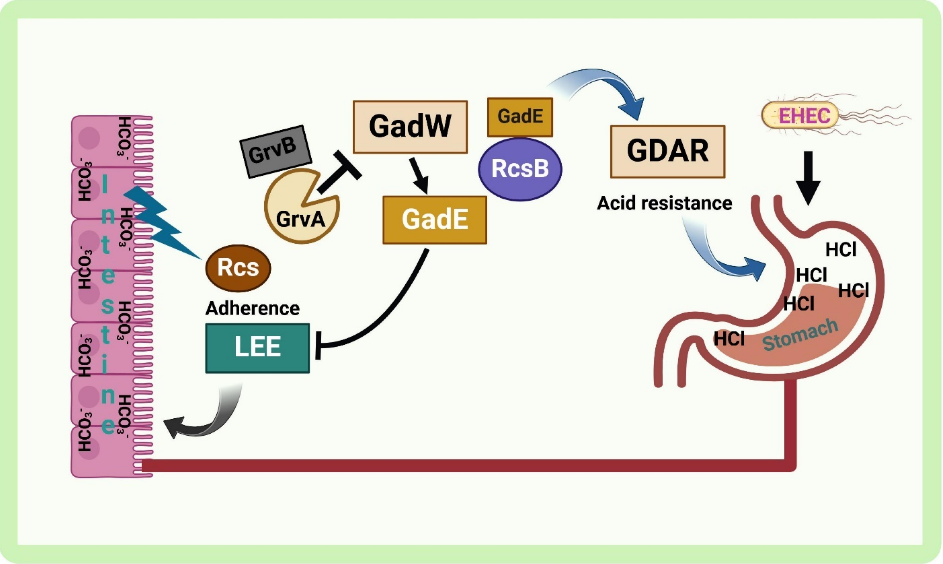

Figure 4. The model explains the GrvA-dependent regulation of acid resistance (GDAR) and lee-dependent adherence by *E. coli*. Credit: created via BioRender.com.

virulence gene activation, as *V. cholerae* colonizes the HCO_3^--rich upper small intestine (Figure 5) [55].

Effect of HCO_3^- on virulence expression in Bacillus cereus *and* Bacillus anthracis

Bacillus cereus (*B. cereus*) is a gram-positive, rod-shaped foodborne pathogen responsible for gastrointestinal symptoms and potentially fatal lower respiratory infections, even with antibiotic treatment [56,57]. Another member of this bacterial group, *Bacillus anthracis* (*B. anthracis*), is known to cause anthrax and is considered a bioterrorism agent because of its lethal nature and the plasmids p×O1and p×O2[58].

HCO_3^- and CO_2, which are essential for pH regulation in the body, increase the expression of genes associated with anthrax toxin components [59]. These components make the environment in mammalian hosts conducive to pathogenic bacteria that thrive in HCO_3^--rich settings. Indeed, both CO_2 and HCO_3^- are vital to host-pathogen dynamics, especially in *B. anthracis* [2,60].

The virulence plasmid p×O1harbors genes for significant anthrax toxins and poly γ-d-glutamic acid capsule (PGA), a key virulence factor [61,62]. PGA transcription is increased by CO_2/HCO_3^- through positive regulation of the capsule biosynthetic operon capBCAD and the plasmid regulators *atxA*, *acpA*, and *acpB* [63]. *In vitro* studies have demonstrated that genes controlled by *atxA*, *acpA*, and *acpB* are induced by HCO_3^- [59,64,65]. A study has shown that in atxA 1 strains, elevated CO_2 /HCO_3^- and temperature increase the expression of toxin genes, namely, *pag*, *lef*, and *cya* [61]. Interestingly, CO_2 /HCO_3^- and temperature combined bolster toxin gene expression, with a sixfold increase in *atxA* mRNA expression at 37°C compared with that at 28°C [59]. These findings indicate that HCO_3^- and temperature jointly regulate the expression of the three *B. anthracis* toxin genes in a coordinated manner [59].

Another study comparing the gene expression profiles of *B. cereus* strain G9241 and an attenuated *B. anthracis* (Sterne 34F 2) strain in high CO_2/HCO_3^- (1666.67 mm) environments versus ambient air identified marked differences in gene expression in the presence of CO_2/HCO_3^- [66,67]]. Intriguingly, gene expression in the G9241 strain differs from that in *B. anthracis*, possibly because it is regulated by PlcR and anthrax toxin activator (AtxA) transcriptional regulators [66,67].

Effect of HCO_3^- on virulence in citrobacter rodentium *and* E. faecalis

C. rodentium is a gram-negative bacterium that predominantly infects mouse intestines and occasionally acts as an opportunistic pathogen in humans [68].

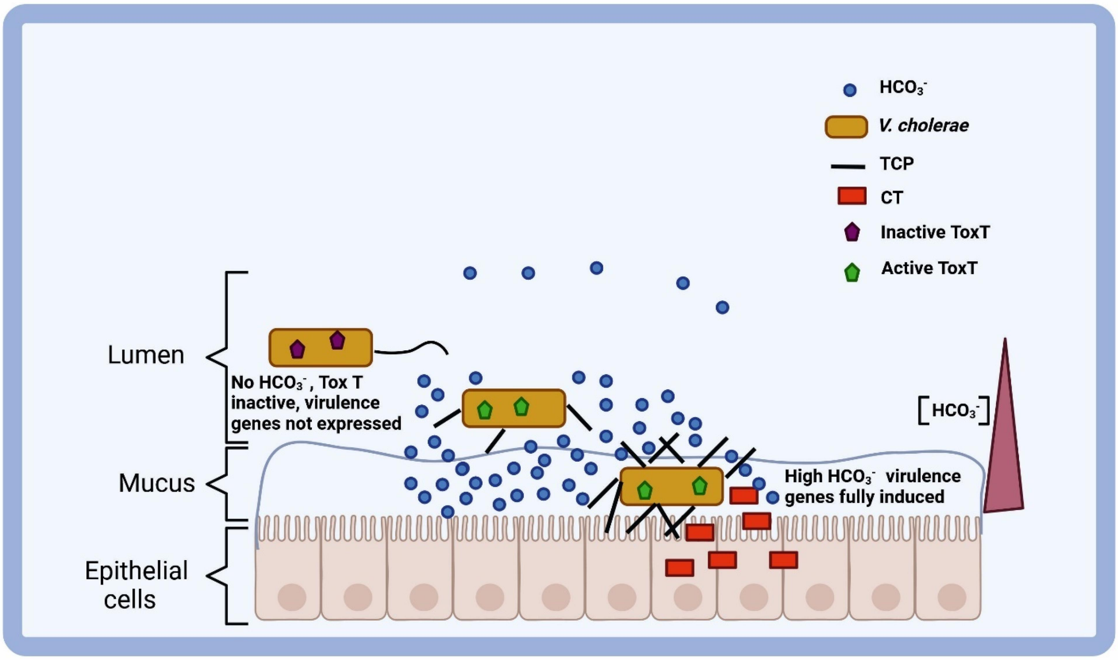

Figure 5. The model illustrates the expression of virulence genes by HCO_3^-. *V. cholerae* strains carrying inactive ToxT protein enter the upper small intestine (left). The HCO_3^- in the intestinal lumen (center), ToxT, activates TCP production. In the mucus layer, higher HCO_3^- levels (right) induce virulence genes and subsequent CT production. Credit: created via BioRender.com.

A study focusing on how a SlyS-like regulator interacts with environmental factors, particularly HCO_3^-, revealed that HCO_3^- activates the transcription of the *adcA* and *kfc* genes. These genes are essential for bacterial adhesion and colonization. This study revealed that HCO_3^- triggers the transcription of these genes and enhances the binding affinity of RegA for its target DNA [2]. These findings highlight the significant role of HCO_3^- in modulating bacterial virulence gene expression, potentially offering new avenues for understanding bacterial colonization and infection processes.

Antibiotic-resistant *E. faecalis* can cause urinary tract infections (UTIs), which present healthcare challenges. In *E. faecalis*, the gene *EbpR*, a member of the *AtxA/Mga* regulator family, influences biofilm development by increasing the expression of the endocarditis and biofilm-associated pilus operon (*ebpABC*) [69,70]. A study showed that treating *E. faecalis* with 100 mm HCO_3^- upregulated *EbpR* and related genes and enhanced pili production and biofilm formation [69]. Research has revealed 73 HCO_3^--responsive genes that are linked mainly to transport systems, indicating that HCO_3^- modulates virulence in *E. faecalis* in a manner similar to its effects on other pathogens, such as *V. cholerae*, *B. anthracis*, *C. neoformans*, and *C. rodentium* [2,55,63,71,72].

Role of HCO_3^- in mycobacterial virulence gene expression

In nontuberculous mycobacteria (NTMs), HCO_3^- has been shown to influence the export of extracellular DNA (eDNA), which is crucial for antibiotic resistance and biofilm formation, and this influence is independent of pH (Table 4) [73]. Interestingly, in *M. avium*, inactivation of CAs diminishes the transport of eDNA, whereas re-establishment of CA activity restores eDNA transport. This highlights the significant role of CAs in the release of eDNA and biofilm formation [73]. Ethoxzolamide (ETZ), a potent CA inhibitor, significantly impairs eDNA export, suggesting that CA plays a role via HCO_3^- [8,75].

In addition, in *M. tuberculosis*, ETZ disrupts the PhoPR signalling pathway, resulting in changes similar to those observed in PhoPR mutants, such as downregulation of the PhoPR regulon, reduction in virulence-associated lipids, and inhibition of Esx-1 protein secretion (Table 4) [74]. This highlights a different aspect of the role of HCO_3^- in bacterial physiology, contrasting its inhibition of gram-positive and gram-negative bacteria, including *M. tuberculosis*.

Bicarbonate and CO_2 as mediators of host immunity and the regulation of pathogenicity

HCO_3^- is the second most abundant anion in the human body; in addition to its role in pH regulation, it controls the activity of many proteins in the body, including immune components [14]. In cystic fibrosis (CF), CF transmembrane regulator (CFTR), an ATP binding cassette, is required for HCO_3^- secretion, and a mutation in CFTR leads to a defect in this anion in the CF lung. The bacteria found in CF are *S. aureus*, *H. influenzae* and *P. aeruginosa*, in which the host immune system responds via leukocytes and other immune components, including AMPs [76]. It has been demonstrated that the use of HCO_3^- enhances this immune response to combat these pathogens in CFs both *ex vivo* and *in vivo* [76]. Furthermore, when combined with vaccines, HCO3- significantly increases the immune response in poultry, suggesting its importance in the immunity of the host [77]. In mice, inoculation of a vaccine with HCO_3^- significantly enhances the protective immune response against *Brucella* compared with that of a formulation without HCO_3^- [78].

An increase in CO_2 or hypercapnia in multiple inflammatory diseases is known to suppress immune cell activity [79]. It has also been shown that bacterial infections and hypercapnic acidosis impair immune cell function, leading to immunosuppression and increased patient mortality after pneumonia [79,80]. A recent study demonstrated that elevated CO_2 reduces monocyte and macrophage migration via inflammatory gene expression and decreases the intracellular pH, which is also dependent on the activity of CA, suggesting that

Table 4. Regulation of genes by HCO_3^- and pathways in pathogenic bacteria.

Pathogen	Genes/Component	Effect	Reference
E. coli	*rcsDB, rcsB, ler, Tir, EspA, and EspB,*	Toxin secretion and virulence	[51–53]
V. cholerae	CT, ToxT, TCP, *pag, lef,* and *cya*	Toxin secretion and virulence	[11]
B. anthracis	PGA capsule, *capBCAD, atxA, acpA, acpB, pag, lef,* and *cya*	Virulence, toxins	[11,61–63,71]
C. neoformans	Cac1adenylyl cyclase	Capsule synthesis	[72]
E. faecalis	*ebpR, ebpABC*	Virulence, biofilm	[69,70]
C. rodentium	*adcA, kfc, RegA*	Virulence	[2]
M. avium	eDNA	Biofilm formation	[73]
M. tuberculosis	Esx-1	Virulence	[74]

CO_2 immunomodulates immune cells through a CA2-coupled change in the intracellular pH [79]. In mice, increased CO_2 altered the immune response to inflammatory agents such as lipopolysaccharide (LPS) and organic dust [81]. Recent studies carried out in mycobacteria revealed that high levels of CO_2 induce PhoPR signalling, which is independent of pH, suggesting that the PhoPR regulon functions as a CO_2 sensor. Interestingly, the CA inhibitor ethoxzolamide (ETZ) inhibited PhoPR signalling, supporting the hypothesis that CO_2 plays a role in regulating PhoPR. Knockdown of CA resulted in a reduction in virulence. Transcriptional profiling studies at 5% CO_2 revealed the induction of PhoPR regulon genes, which include the ESX-1 secretion system [82].

CAs and their inhibition in bacteria as an antimicrobial approach

Pathogenic microbes have become resistant to clinically used drugs, and novel antimicrobial compounds that target novel pathways of these pathogens are needed [83,84]. In the recent past, sequencing the genomes of pathogenic microbes revealed many alternate pathways that are crucial for their life cycle and can be targeted via novel antimicrobials devoid of resistance [84]. Among the alternative pathways associated with these pathogens, carbonic anhydrases play crucial roles and have been used as targets for the development of antimicrobial agents via small-molecule inhibitors. Pathogenic organisms contain both α-CAs and β-CAs that have been cloned, and studies have shown that CAs play crucial roles in the survival and pathogenesis of pathogens [85].

Pathogens sense CO_2 in their environment via HCO_3^-, which is generated by the enzymatic activity of CAs and regulates the expression of genes required for the virulence of these microbes [84]. In addition, studies have demonstrated that CAs are involved in many other functions, such as biofilm formation and survival in the host environment. In *E. faecalis*, disruption of α-CA sensitized bacteria to killing with gentamicin [86]. In *P. aeruginosa*, β-CA is required for calcium deposition and contributes to virulence [4]. The inactivation and inhibition of the activity of this CA chemical inhibitor reduced calcium deposition in this bacterium [73]. In mycobacterial species, CAs are required to transport eDNA, a component of biofilms, and to express virulence factors. Inhibition via EZA or inactivation of CA has been shown to reduce biofilm formation and attenuate virulence [8,9,73,74]. The inhibition of β-CAs with a specific inhibitor significantly reduced the bacterial load *in vivo* in zebrafish larvae [75]. In addition, in *in vivo* studies involving *Neisseria* spp., *H. pylori, B. suis, and S. pneumoniae*, the growth of these pathogens could be impaired via the use of CA inhibitors [87]. *H. pylori* encounter bicarbonate, urea and acid in gastric environment. Analysis of *H. pylori* mutants showed that CAs play a role in maintaining activity of urease and acid resistance through HCO_3^- in an acidic environment, suggesting the requirement of CAs for the bacterium for survival in the gastric niche [88].

The crystal structures of many microbial CAs have been resolved, and inhibition studies using different classes of inhibitors have been performed [85]. The CA inhibitors sulphonamides, dithiocarbamates, and inorganic anions have been shown to inhibit the alpha- and beta-CAs of bacteria and fungi. Among CA inhibitors, sulphonamides/sulfamates represent one of the main classes of CAIs [89]. These compounds are already in clinical use for the treatment of various diseases, including acetazolamide, methazolamide, and ethoxzolamide, which inhibit all CAs, including CAs, from pathogenic microbes. Sulphonamides/sulfamates/sulamides have been shown to inhibit CAs both *in vitro* and *in vivo* in mice infected with antibiotic-resistant strains, confirming their role in contrasting bacterial antibiotic resistance [90]. In addition, phenol inhibitors, either alone or in combination with clinically used antibiotics, inhibit the growth of the bacterium and biofilm formation [87]. Both *in vitro* and *in vivo* inhibition studies have shown that the CAs of these pathogens can be novel targets for combating microbial infections that are devoid of resistance [8,9,75,91,92].

Concluding remarks

HCO_3^- plays a pivotal role in human health and disease, and its role in cellular physiology involves pH regulation, suggesting its impact on various metabolic and signalling pathways. In the realm of microbial studies, HCO_3^- has emerged as an agent of interest owing to its importance not only in microbial physiology but also in human physiology. The antimicrobial effects of HCO_3^- are manifested in diverse ways, first by inhibition of the growth of pathogenic bacteria directly and second by increasing the effects of different antibiotics on pathogenic bacteria.

However, HCO_3^- is a double-edged sword, as HCO_3^- also regulates genes and increases virulence in certain pathogenic bacteria. This underlines the need for caution before integrating HCO_3^- into clinical applications that target bacterial infections. As researchers dive deeper, the dichotomous nature of the effects of HCO_3^-, both inhibitory and promotional, on bacterial virulence becomes evident. The path ahead necessitates a thorough and nuanced understanding of HCO_3^- before its widespread adoption in microbial therapies.

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Author contributions

Ashok Aspatwar: Conceptualization, preparing the original draft, preparing figures, and visualization; **Jenny Parkkinen**: Writing-reviewing and editing; Seppo **Parkkila**: Writing of the manuscript, reviewing and editing, supervision and financial support.

Author contributions

Original idea and original manuscript draft preparation: A.A. Data collection: A.A., review, writing, and editing: A.A., J.P., and S.P., Funding acquisition: A.A. (grants from the Finnish Cultural Foundation, Tampere Tuberculosis Foundation), S. P. (grants from the Academy of Finland, Jane and Aatos Erkko Foundation). Final approval: All the authors (A.A., J. P., and S.P.) read, agreed upon, and approved the final version of the manuscript for publication.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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