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ANTIMICROBIAL RESISTANCE IN *KLEBSIELLA PNEUMONIAE*: MECHANISMS, EPIDEMIOLOGY, AND EMERGING THREATS

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Abstract: Currently, one of the most serious threats to public health is the rapid spread of multidrug–resistant (MDR) and highly virulent *Klebsiella pneumoniae* (hvKp). In these strains, various antibiotic resistance genes (ARGs) are frequently detected, including several novel ones first identified in this bacterial species, such as carbapenemases of the KPC, NDM–1, and OXA–48 types. The widespread prevalence of environmental ARGs, which can be transferred between microbiota, particularly among clinical pathogens and human commensals, has significantly contributed to the emergence of antimicrobial resistance (AMR) in *K. pneumoniae*. Resistance is caused by mechanisms such as drug inactivation, increased efflux, or altered binding to target sites. Moreover, this resistance is further enhanced by the production of various beta-lactamases, mainly extended–spectrum beta-lactamases (ESBLs), or by biofilm formation. Particularly concerning in *K. pneumoniae*, the main species representing the carbapenemase–producing *Enterobacterales* (CPE) group, is the emergence of plasmid–mediated resistance to carbapenems and polymyxins, which are considered "last–resort drugs" for treating infections caused by these pathogenic microorganisms. The growing resistance of *K. pneumoniae* to antibiotics and other antimicrobial agents remains one of the most important problems in epidemiological surveillance, microbiological diagnostics, and the global treatment of infections. The high mortality rate among infected patients and the difficulty in treating infections caused by MDR *K. pneumoniae* have led to this bacterium being classified as one of the most common and dangerous human pathogens in both Polish and global healthcare. This article characterizes the factors determining antibiotic resistance in *K. pneumoniae*, both in terms of biochemical mechanisms and molecular features. In addition, the current epidemiological situation regarding the spread of invasive, resistant *K. pneumoniae* isolates in Europe and Poland is described, and treatment regimens for infections caused by these high–alert pathogens are presented.

Keywords: *Klebsiella pneumoniae*, antibiotic resistance determinants, carbapenems, epidemiology, extended spectrum beta-lactamases (ESBL), treatment of infections

1. Introduction

Klebsiella pneumoniae bacilli are part of the natural gut microbiota in humans, they also colonize the nasopharynx and skin in many healthy people, usually without causing symptoms. These bacteria can also act as opportunistic pathogens. They cause serious infections: pneumonia, urinary tract infections, septic infections, intra–organ abscesses, and other diseases (Ochońska *et al.* 2024).

Resistance of *K. pneumoniae* to antibiotics and other antimicrobials is one of the greatest challenges for

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modern medicine, spanning epidemiological surveillance, microbiological diagnostics, and infection therapy. Multidrug–resistant (MDR) and highly virulent strains of *K. pneumoniae* (hvKp) are among the most important bacterial pathogens responsible for healthcare–associated infections (HAIs). These microorganisms, in particular, cause dangerous nosocomial infections and are increasingly etiological factors in severe community–acquired infections (van Duin *et al.* 2015; van Duin and Paterson 2020).

2. Drug resistance in *K. pneumoniae*

For *K. pneumoniae*, natural resistance is characteristic, as is its high capacity to acquire resistance through various genetic processes (Lee *et al.* 2016; Navon–Venezia *et al.* 2017). Acquired drug resistance in *K. pneumoniae* to antibiotics and/or other antimicrobial agents arises from spontaneous mutations in the bacterial genome or from the acquisition of genetic information through horizontal gene transfer (HGT). HGT mechanisms significantly affect the spread of resistance among *K. pneumoniae* strains and across other bacterial genera and species, including phylogenetically unrelated ones (Fig. 1) (Lee *et al.* 2016; Navon–Venezia *et al.* 2017). HGT occurs *via* mobile genetic elements (MGEs), which can constitute up to 40% of the total genetic information in a given strain (Dziewit and Bartosik 2011). In addition to plasmids and bacteriophage genomes, MGEs include transposable elements (insertion sequences and transposons), DNA–integrating elements such as integrative and

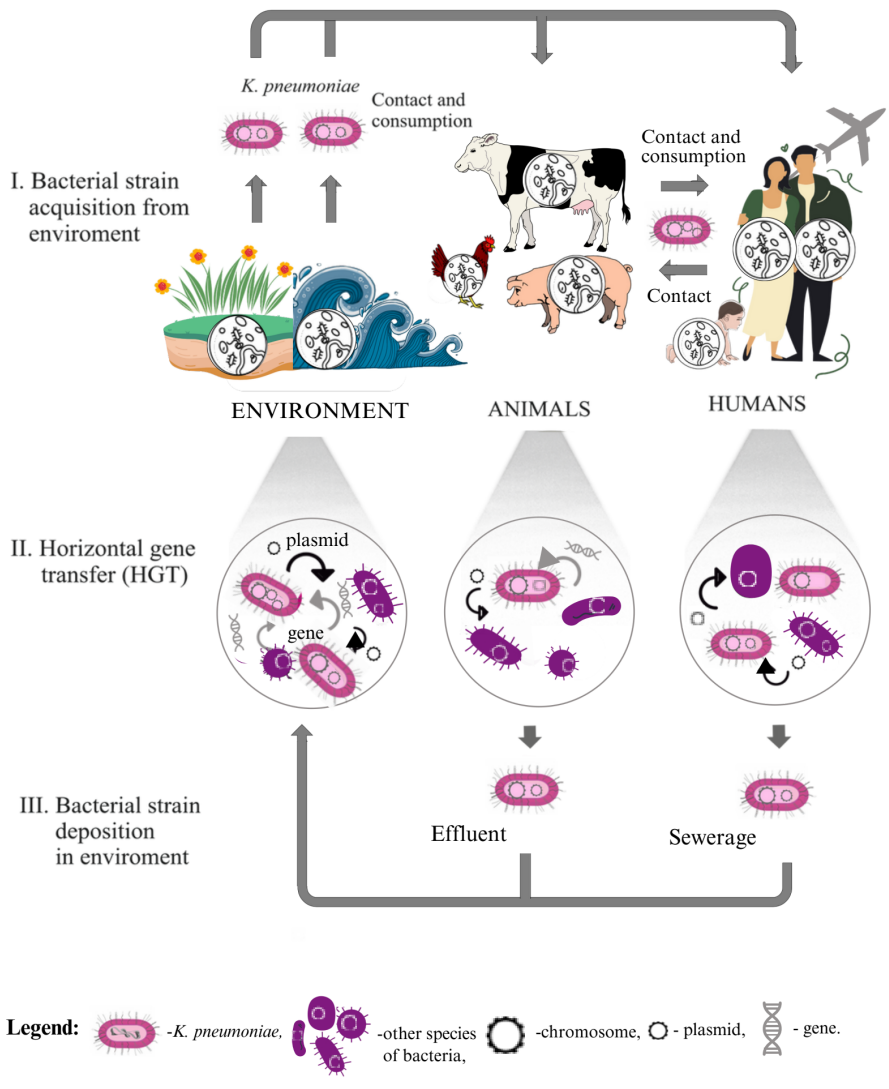

Fig. 1. Diagram illustrating horizontal gene transfer (HGT) of resistance to antibiotics and/or other antimicrobial agents (ARGs) among *K. pneumoniae* strains. Own graphic design based on (Meek *et al.*, 2015; Wyres *et al.*, 2018 and Catalano *et al.*, 2022).

conjugative elements (ICEs) and integrative and mobilizable elements (IMEs), and introns (Dziewit and Bartosik 2011). MGEs are among the most recombinogenic agents, as they encode various recombinases whose activity can alter the structure of the microorganism's genetic material (Dziewit and Bartosik 2011). Exposure to antimicrobial substances from different classes can lead to the emergence of cross–resistance and the selection of genes that carry them (Catalano *et al.* 2022).

The most common causes of the emergence and development of antibiotic resistance in *K. pneumoniae* strains are the overuse or inappropriate use of antimicrobials, not only in medicine but also in other areas of life (e.g., veterinary medicine, agriculture, fisheries, and horticulture) (Meek *et al.* 2015). The spread of resistance to antimicrobials, including antibiotics, is associated with the transfer of antibiotic resistance genes (ARGs) to other bacteria, through direct contact – i.e., from colonized animals to humans (especially from farm animals) – and through indirect contact (an environment contaminated with resistant strains) (Meek *et al.* 2015; Wyres *et al.* 2018). Indirect carriers of antibiotic–resistant bacteria or their ARGs can be: aerosols, raw materials and food of animal origin, feces, animal manure, soil, farmlands and crops, water resources, sewage, and other environmental pollutants. Hospitals, livestock farms, veterinary clinics, food processing plants, and food warehouses should also be considered concentration sites for antibiotic–resistant bacteria and their ARGs. The spread of drug–resistant strains is also facilitated by the growing phenomenon of travel and long–distance movement of people (Wyres *et al.* 2018).

2.1 Mechanisms of resistance in *K. pneumoniae*

K. pneumoniae has developed three major mechanisms of resistance to antibiotics and/or chemotherapeutics, i.e., enzyme resistance, receptor resistance, and transport resistance (Galani *et al.* 2022). This resistance can arise from inactivation, altered binding to the target site, or increased efflux of the drug. In addition, this resistance can be potentiated by the production of various beta-lactamases, mainly extended–spectrum beta-lactamases (ESBLs), or by biofilm formation (Fig. 2) (Galani *et al.* 2022).

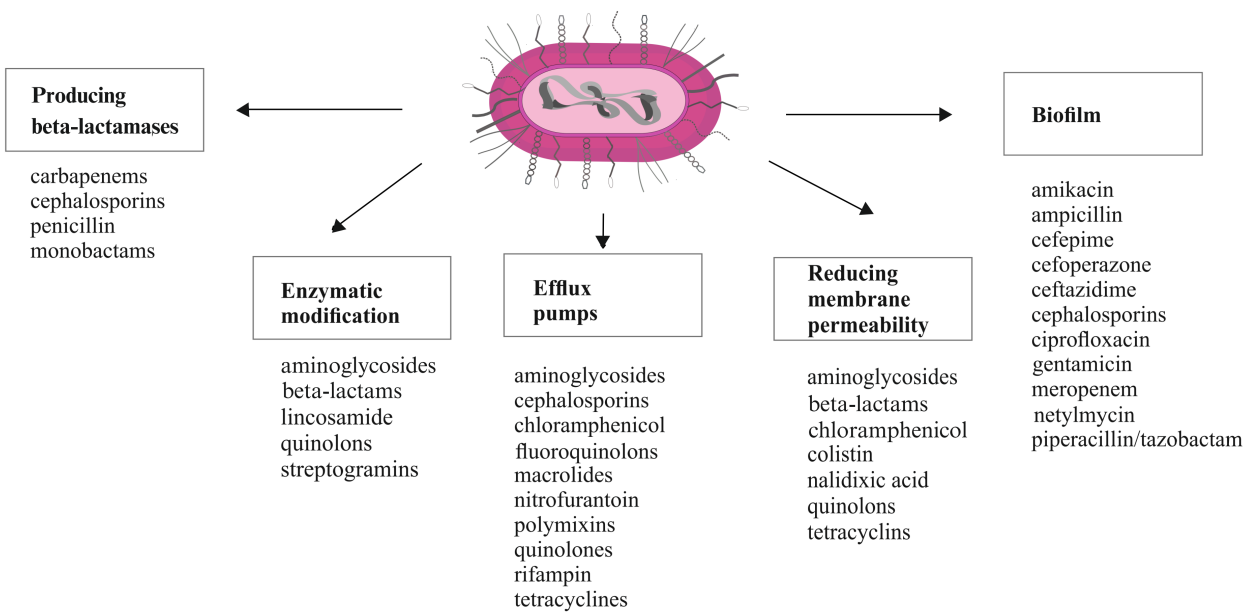

Fig. 2. Diagram showing the resistance mechanisms of *K. pneumoniae,* along with the classes of antibiotics and/or chemotherapeutics to which each mechanism applies. Own graphic design based on (Hughes *et al.,* 2020; Karami-Zarandi *et al.,* 2022; Dzierżanowska-Fangrat, 2023).

2.1.1 Enzymatic resistance

Enzymatic resistance is the best understood, most common, and most extensively studied mechanism of resistance to beta-lactam antibiotics in *K. pneumoniae* strains. This type of resistance is associated with the production of extracellular beta-lactamases that degrade antibiotics in this class (Nordmann and Poirel 2014; Calbo and Garau 2015; Caltagirone *et al.,* 2017; Chong *et al.,* 2018).

A fundamental framework for beta-lactamase subdivisions, including all known classes, groups, and subgroups of enzymes, was established long before beta-lactam antibiotics were introduced into therapeutics (Paterson *et al.* 2003; Chong *et al.* 2018; Galani *et al.* 2022). The current vast diversity of beta-lactamases results from the spread of resistance genes among bacterial strains through conjugation, transduction, and transformation (Galani *et al.,* 2022). As with other ARGs, a variety of MGEs play a key role in the mobilization and HGT of beta-lactamase genes. Moreover, over evolutionary time, additional mutations arise, and homologous recombination drives differentiation of these enzymes, driven by the massive use of antibiotics and/or chemotherapeutics (Meek *et al.* 2015; Wyres *et al.* 2018).

Currently, there are two main classification systems for beta-lactamases, i.e., the structural and functional systems (Table I) (Galani *et al.* 2022). The structural system proposed by Ambler is based on the analysis of the amino acid sequences of beta-lactamases and classifies the enzymes into four classes designated A through D, based on evolutionary affinity (Ambler 1991; Galani *et al.* 2022). Classes A, C, and D include serine beta-lactamases, while class B enzymes are metallo-beta-lactamases (Galani *et al.,* 2022). All of these beta-lactamases from classes A, C, and D are described in more detail later in this article.

Table I.

Classification of preferred substrates for beta-lactam antibiotics, beta-lactamase inhibitors (BLIs), and bacterial beta-lactamases. Own graphic design based on (Livermore 1995; Bush and Jacoby 2010; Mączyńska 2015; Bush 2018).

Molecular class according to Ambler	Bush-Jacoby-Mederios functional group	Preferred substrates in the group of beta-lactam antibiotics	Inhibited by beta-lactamase inhibitors (BLIs):			Bacterial beta-lactamases - representative enzymes
			AV	CA or TZB	EDTA	
A	2a	P	+	+	-	PC1
	2b	P, Cp	+	+	-	TEM-1, TEM-2, SHV-1
	2be	P, Cp, E, Mb	+	+	-	ESBL (e.g. CTX-M), TEM, SHV, K1 (OXY) in *K. oxytoca*
	2br	P	+	-	-	TEM-30, SHV-10
	2c	P, Cbp	+	±	-	CARB-1, PSE-1, PSE-3, PSE-4
	2e	Cp	+	+	-	Inducible cephalosporinases *Proteus vulgaris*
	2f	P, Cp, Cb, E, Mb	+	±	-	KPC
B	3a	P, Cp, E, Cb	+	-	+	IMP-1, VIM-1, NDM-1, L1
	3b	Cb	-	-	+	CphA
C	1	Cp	+	-	-	AmpC (e.g. MIR-1), CMY
	1e	Cp, E	+	-	-	GC1
D	2d	P, Clox	+	±	-	OXA-1, OXA-10 (PSE-2)
	2de	P, Cb	+	-	-	OXA-23, OXA-48
	2df	P, E, Mb	+	±	-	OXA-11, OXA-15

Abbreviations: AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cbp, carboxypenicillins; Cp, cephalosporin; Clox, cloxacillin; E, extended-spectrum cephalosporin; EDTA, ethylenediaminetetraacetic acid; Mb, monobactam; P, penicillin; TZB, tazobactam; (+), hydrolyzed; (-), unhydrolyzed; (±), barely hydrolyzed.

The second classification system, developed by Bush and Jacob, groups beta-lactamases based on the rates of hydrolysis of beta-lactam antibiotics and on an assessment of these enzymes' susceptibility to classic beta-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) and other compounds, including aztreonam, cloxacillin, EDTA, and NaCl (Table I) (Bush and Jacoby 2010; Galani *et al.* 2022). However, aztreonam and cloxacillin are not classic beta-lactamase inhibitors; rather, they are compounds that can be inactivated by beta-lactamases or that inhibit these enzymes through mechanisms distinct from those of classic inhibitors (clavulanic acid, sulbactam, tazobactam). Aztreonam is a synthetic antibiotic in the monobactam class, with a structure that differs significantly from other beta-lactam antibiotics. It is highly resistant to hydrolysis by most beta-lactamases produced by Gram-negative bacteria. However, it is hydrolyzed by many enzymes, including: extended-spectrum plasmid-mediated beta-lactamases (ESBLs) of class A (mutated forms of classical enzymes such as TEM and SHV-1) and class D enzymes (e.g., OXA-1); chromosomal AmpC cephalosporinase produced by mutants of certain *Enterobacterales* species in the genera *Enterobacter*, *Serratia*, *Citrobacter*, and indole-positive *Proteus*, as well as non-fermenting rods of the genus *Pseudomonas*; and plasmid-mediated enzymes derived from AmpC-type beta-lactamases produced by *Klebsiella* spp. and *Escherichia* spp. (Dzierżanowska 2024). The presence of a characteristic monocyclic beta-lactam ring makes this antibiotic naturally resistant to class B beta-lactamases (MBLs). This resistance is due to spatial separation from the nucleophilic zinc ion present in the active site of these enzymes (Musawa *et al.* 2024). Because ESBL and AmpC beta-lactamases are widely co-produced in MBL-positive pathogens, aztreonam monotherapy has proven clinically ineffective. For strains that co-produce MBLs, ESBLs, and AmpCs, combination therapy or the use of the latest molecules has become the standard of care (Musawa *et al.* 2024; Khattab *et al.* 2025). Cloxacillin is an isoxazolyl penicillin resistant to staphylococcal beta-lactamases (penicillinases). Cloxacillin acts as a "suicide substrate," binding the beta-lactamase active site and forming a stable acyl-cloxacillin-enzyme complex that prevents the enzyme from degrading other beta-lactam antibiotics in the vicinity (Kiener *et al.* 1980). EDTA (ethylenediaminetetraacetic acid) inhibits metallo-beta-lactamases (MBLs), enzymes classified as class B by Ambler, by chelating (binding) zinc ions (Zn^{2+}), which are essential for their catalytic activity. MBLs (e.g., NDM, VIM, IMP) require one or two zinc ions in their active site to cleave the beta-lactam ring of an antibiotic. MBLs deprived of zinc become inactive, restoring bacterial susceptibility to carbapenems and other beta-lactam antibiotics (Bahr *et al.* 2021). There is no scientific evidence that sodium chloride (NaCl) directly binds to or inactivates beta-lactamase enzymes, the target of classic beta-lactamase inhibitors. However, high salt concentrations (high ionic strength) may indirectly affect enzyme function through several mechanisms: a) changes in protein structure – high salt concentrations can affect the conformational stability of proteins, including enzymes, which in some cases can lead to changes in their active site and a reduction in catalytic activity (Sinha and Khare 2014); b) effects on bacteria–resistance mechanisms, such as the production of beta-lactamases, are often linked to the life processes of bacteria. Changes in the osmotic environment (e.g., high salinity) can affect bacterial metabolism and, consequently, the production or secretion of these enzymes (Chambers and Hackbarth 1987); c) inhibition of autolysis (self-destruction) – beta-lactam antibiotics inhibit bacterial cell wall synthesis, which activates the bacteria's own "suicide" enzymes (autolysins). NaCl blocks the action of autolysins, preventing the bacterium from "falling apart" even though its cell wall is damaged (Chambers and Hackbarth 1987; Koch *et al.* 2001); d) effect on overall survival – rather than neutralizing the drug, NaCl alters the physiology of the bacteria (e.g., by increasing osmotic pressure or strengthening the cell wall) (Chambers and Hackbarth 1987; Esumeh *et al.* 2017). Accordingly, some of the listed substances can be used in phenotypic screening tests to detect enzymes such as ESBL–type beta-lactamases or carbapenemases (KPC, MBL, OXA-48), even though they are not inhibitors *sensu stricto* (Chambers and Hackbarth 1987; Koch *et al.* 2001; Sinha and Khare 2014; Esumeh *et al.* 2017). Bush and Jacoby's system classifies beta-lactamases into four major functional groups, numbered 1 to 4 (Paterson *et al.* 2003; Bush and Jacoby 2010).

Group 1 includes chromosomal AmpC cephalosporinases, which are completely inhibited by cloxacillin but respond poorly to clavulanic acid. Cephalosporins, penicillins, and monobactams (aztreonam) are preferred substrates of AmpC cephalosporinases (Livermore 1995; Nordmann and Poirel 2014). Group 2, the most structurally and functionally diverse, includes 12 subgroups (2a, 2b, 2be, 2br, 2ber, 2c, 2ce, 2e, 2f, 2d, 2de,

2df) of enzymes that include both penicillinases and narrow–spectrum cephalosporinases, such as: LEN-1 (from *K. pneumoniae* strain), OKP (other *K. pneumoniae* beta-lactamases), and SHV (sulfhydryl variable) (Livermore 1995; Haeggman *et al.* 2004; Nordmann and Poirel 2014; Arakawa 2020). Group 2 also includes broad-spectrum beta-lactamases (BSBL), also known as secondary beta-lactamases, subsequently extended–spectrum beta-lactamases (EBSL), and beta-lactamases with an extremely broad substrate spectrum covering virtually all beta-lactam antibiotics (Nordmann and Poirel 2014). The latter include carbapenemases of the KPC type (*Klebsiella pneumoniae* carbapenemases), acquired beta-lactamases hydrolyzing class D carbapenems (subgroup 2df), or CHDL enzymes (carbapenem-hydrolysing class D beta-lactamases), including OXA-48 (oxacilinase-48). The activity of enzymes of the OXA group, including OXA-48, is not inhibited by clavulanic acid, sulbactam, and tazobactam, with the exception of certain narrow–spectrum OXA enzymes (e.g., OXA-1), which may exhibit partial susceptibility to classical beta-lactamase inhibitors (Pitout *et al.* 2019). Group 3 includes metallo-beta-lactamases (MBLs) represented by imipenemase (IMP)-type beta-lactamases, Verona integron-encoded metallo-beta-lactamases (VIMs), and New Delhi metallo-beta-lactamase (NDM-1) (Livermore 1995; Nordmann and Poirel 2014). MBLs hydrolyze penicillins, cephalosporins, and carbapenems. These enzymes are encoded on class 1 and 3 integrons, are inhibited by EDTA, and are not inhibited by beta-lactamase inhibitors, distinguishing them from the activity of group 2 enzymes. Group 4 includes several long–identified penicillinases, while these poorly understood enzymes were omitted from the latest version of the functional classification system (Livermore 1995).

In *K. pneumoniae* strains, alongside the evolution of enzymatic resistance to beta-lactam antibiotics, the acquisition of enzymatic resistance to aminoglycoside antibiotics also occurred (Butler *et al.* 2021). Compared with beta-lactams, aminoglycosides have a much narrower spectrum of activity and are less likely to induce resistance in bacteria. The primary mechanism of bacterial resistance to this class of antibiotics is the production of plasmid-encoded enzymes. These enzymes modify aminoglycoside antibiotics by blocking the functionally active amine and hydroxyl groups (Butler *et al.* 2021). Among the most common aminoglycoside-modifying enzymes (AMEs) are: aminoglycoside N-acetyltransferases (AAC), which modify amino groups using acetyl–coenzyme A as the acetyl donor; O-phosphotransferases (AMEs), in turn, aminoglycoside phosphotransferases (APH), which modify hydroxyl groups using adenosine-5′-triphosphate (ATP); and O-nucleotidyltransferases (ANT), which modify hydroxyl groups using ATP (Butler *et al.* 2021). Natural aminoglycosides such as gentamicin, kanamycin, streptomycin, and tobramycin are readily modified (Butler *et al.* 2021). Aminoglycoside resistance may also result from reduced affinity of the 30S ribosomal subunit for the antibiotic, caused by mutations in bacterial DNA, 16S rRNA, and ribosomal proteins, as well as from reduced antibiotic uptake into the bacterial cell due to activation of efflux pumps that actively remove the drug from the cell (Butler *et al.* 2021).

2.1.1.1 Species-specific beta-lactamases

In *K. pneumoniae*, species–specific class A beta-lactamases were present even before the era of widespread antibiotic use. These enzymes include broad-spectrum beta-lactamases (LEN-1, OKP or SHV) that primarily hydrolyze penicillins (ampicillin, amoxicillin, carbenicillin, and ticarcillin) (Haeggman *et al.* 2004; Fevre *et al.* 2005; Arakawa 2020). These enzymes are chromosomally encoded and are constitutively expressed at low levels. Species-specific beta-lactamases do not hydrolyze cephalosporins and are sensitive to beta-lactamase inhibitors (Livermore 1995; Haeggman *et al.* 2004; Bush 2018).

2.1.1.2 Broad-spectrum beta-lactamases (BSBLs)

The emergence of broad-spectrum beta-lactamases (BSBLs), also known as secondary beta-lactamases, results from mutations and the transfer of genes for new beta-lactamases with a broader substrate hydrolysis spectrum onto a plasmid. BSBLs are usually plasmid-encoded, and the genes encoding them are often located within transposons (Haeggman *et al.* 2004; Dzierżanowska *et al.* 2010). In *Enterobacteriaceae*, the most common BSBLs are TEM-1, TEM-2, and SHV-1 (Dzierżanowska *et al.* 2010). TEM-1 was the first enzyme, discovered in 1965 in a strain of *Escherichia coli* isolated from a patient–resident of Athens, Greece, named Temoneira, after whom it was given its shortened name (Fig. 3). The TEM-1 enzyme hydrolyzes aminopenicillins most strongly, carboxypenicillins and first-generation cephalosporins to a lesser extent, does not degrade broad-spectrum cephalospo-

rins, carbapenems, and monobactams, and is inhibited by clavulanic acid. TEM-2, like TEM-1, is a beta-lactamase with a broad substrate spectrum and exhibits high hemolytic activity. SHV-1 is another secondary beta–lactamase first described in 1972 in *K. pneumoniae*, also referred to as PIT-2 after its discoverer (Pitton 1972). Currently, SHV-1 is commonly found in this bacterial species (Haeggman *et al.* 2004; Dzierżanowska *et al.* 2010).

2.1.1.3 Extended–spectrum beta-lactamases (ESBLs)

Extended–spectrum beta-lactamases (ESBLs) were first detected in *K. pneumoniae* in 1983 in Europe (France, Germany) and then in the United States in 1989 (Bush 2018). These enzymes arose from point mutations in the genes responsible for the synthesis of BSBLs. ESBLs hydrolyze nearly all penicillins (except temocillin), cephalosporins (except cephamycins), and monobactams (aztreonam). ESBLs do not hydrolyze carbapenems. The activity of most of these beta-lactamases is inhibited by clavulanic acid, sulbactam, and tazobactam. Currently, among the numerous known ESBL variants, the most common are beta-lactamases from the TEM, SHV, CTX-M, and OXA families, described in more detail below. Lesser–known endemic enzymes include those from the BEL, BES, GES, PER, SFO, TLA, and VEB families, the description of which is beyond the scope of this publication (Bush 2018; Castanheira *et al.* 2021).

Beta-lactamases of the TEM family are common ESBL enzymes in *K. pneumoniae*. The first described enzyme from this family, CTX-1 (TEM-3), was detected in this bacterial species in 1984 (Philippon *et al.* 1989). Evolution has produced a large diversity of TEM-type beta-lactamase variants worldwide, with about 243 known (Bradford 2001; Bush 2018; Castanheira *et al.* 2021). In Poland, the scientific team of Gniadkowski *et al.* (1998) traced two evolutionary paths of the TEM family enzymes, i.e., the first path leading from the secondary beta-lactamase TEM-1 to the ESBL type TEM-93 and the second evolutionary path leading through different variants of ESBL types: TEM-25, TEM-29 and TEM-85, and, successively, through TEM–48, TEM-49 and TEM-47 (Gniadkowski *et al.* 1998). Moreover, in the studies of the group of Gniadkowski *et al.* (1998), a new variant TEM-68 resulting from a point mutation of TEM-47 was described (Gniadkowski *et al.* 1998). After 2000, few strains of *K. pneumoniae* producing TEM-30, TEM-32 and TEM-37 were detected (Gniadkowski *et al.* 1998).

SHV family beta-lactamases that contain a sulfhydryl group in the active site are also among the ESBL enzymes frequently produced by *K. pneumoniae* (Tsang and KlebNETGSP AMR Genotype-Phenotype Group *et al.* 2024). After the first SHV-2 enzyme was detected in *Klebsiella ozenae*, subsequent *Klebsiella* strains producing diverse, heterogeneous SHV variants have spread worldwide (Gniadkowski *et al.* 1998; Paterson *et al.* 2003; Dzierżanowska *et al.* 2010; Bush 2018; Castanheira *et al.* 2021). Currently, 228 variants of these widespread (cosmopolitan) enzymes are known. In Poland, the most frequently detected SHV variants are SHV-2, SHV-5, and SHV-12 (Baraniak *et al.* 2005; Liakopoulos *et al.* 2016; Castanheira *et al.* 2021).

Enzymes from the CTX-M family (cefotaximase from Munich) are considered the dominant group of ESBLs, replacing TEM and SHV. The first CTX-M-type beta-lactamases were detected in the 20th century, appearing simultaneously in several locations around the world, including France, Japan, Germany, and Italy (Castanheira *et al.* 2021). These initial reports were followed by the widespread dissemination of CTX-M in several countries. The worldwide expansion of isolates carrying these ESBLs was later termed the "CTX-M pandemic" (Castanheira *et al.* 2021). CTX-M enzymes originate from plasmid–transferred and mutated chromosomal genes of *Klebsiella oxytoca* OXY (K1) beta-lactamases. CTX-M enzymes exhibit strong hydrolytic activity toward cefotaxime, while they hydrolyze ceftazidime less effectively. Currently, CTX-M enzymes are the most common type of ESBL, with the dominant variants worldwide being CTX-M-15, CTX-M-14, and CTX-M-27 (Castanheira *et al.* 2021). In Poland, the most common variants of CTX-M enzymes are CTX-M-3 and CTX-M-15 (Baraniak *et al.* 2005).

OXA-type beta-lactamases constitute the majority of class D beta-lactamases in the Ambler classification and Bush–Jacoby–Medeiros functional group 2d (Yoon and Jeong 2021). This broad group of enzymes shows substantial variability in substrate profiles and amino acid sequences. These enzymes hydrolyze oxacillin. Several variants of OXA–type beta-lactamases have been reported that hydrolyze cephalosporins, cephems, and/or monobactams. According to Yoon and Jeong 2021, a total of 965 OXA-type beta-lactamases were detected in the beta-lactamase database (BLDB) used in this work (Yoon and Jeong 2021). Most extended–spectrum oxacillinases are derived from OXA-2 and OXA-10. OXA-2 derivatives include:

OXA-15, OXA-32, OXA-34, OXA-36, OXA-53, OXA-141, OXA-161, OXA-210, and OXA-226. OXA-10 derivatives include OXA-11, OXA-13, OXA-14, OXA-16, OXA-17, OXA-19, and OXA-28 (Castanheira *et al.* 2021). One of the most common is OXA-1. Importantly, this group of enzymes gives rise to CHDL-class D beta-lactamases (e.g., OXA-48) that hydrolyze carbapenems, as described below (Pitout *et al.* 2019).

2.1.1.4 AmpC cephalosporinases

AmpC cephalosporinases are a distinct group of beta–lactamases that have evolved along a distinct evolutionary path (Tamma *et al.* 2019; Rodríguez–Guerrero *et al.* 2022). Chromosomal enzymes (chromosome–mediated AmpC beta-lactamase, cAmpC) are encoded by evolutionarily "old" *amp*C genes located in the chromosomes of many species of Gram-negative bacilli, including *Acinetobacter* spp., *Aeromonas* spp., *Citrobacter freundii*, *Enterobacter* spp., *Hafnia alvei*, *Morganella morganii*, *Proteus vulgaris*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Yersinia enterocolitica* (Jacoby *et al.* 2009; Meini *et al.* 2019; Tamma *et al.* 2019). These bacteria gave rise to several families of acquired AmpCs, such as ACT, MIR, CMY-2, DHA, ACC, MOX, FOX, and LAT. The most common beta-lactamases from the CMY-2 family are derived from the *C. freundii* AmpC (Papanicolaou *et al.* 1990; Bauernfeind *et al.* 1999; Meini *et al.* 2019; Tamma *et al.* 2019; Rodríguez-Guerrero *et al.* 2022). Variants of these enzymes have been identified, among others, in Greece (LAT-1, LAT-2, MOX-2, CMY-2), France (DHA-2, MOX-2, ACC-1), the United States (ACT-1, FOX-5, MIR-1), South Korea (CMY-1), India and Sweden (CMY-4), Taiwan (CMY-8), Germany and Tunisia (ACC-1), Japan (MOX-1), Argentina (FOX-1), Guatemala (FOX-2), and Italy (FOX-3) (Fig. 3) (Jacoby *et al.* 2009; Dzierżanowska *et al.* 2010; Mączyńska *et al.* 2015; Castanheira *et al.* 2021). In *K. pneumoniae* and other bacterial species (e.g., *K. oxytoca*, *Proteus mirabilis*, *Salmonella enterica*) that normally do not produce cAmpC or produce it in negligible amounts (e.g., *E. coli*), plasmid-mediated AmpC beta-lactamase (pAmpC) can be found (Jacoby *et al.* 2009; Meini *et al.* 2019; Tamma *et al.* 2019).

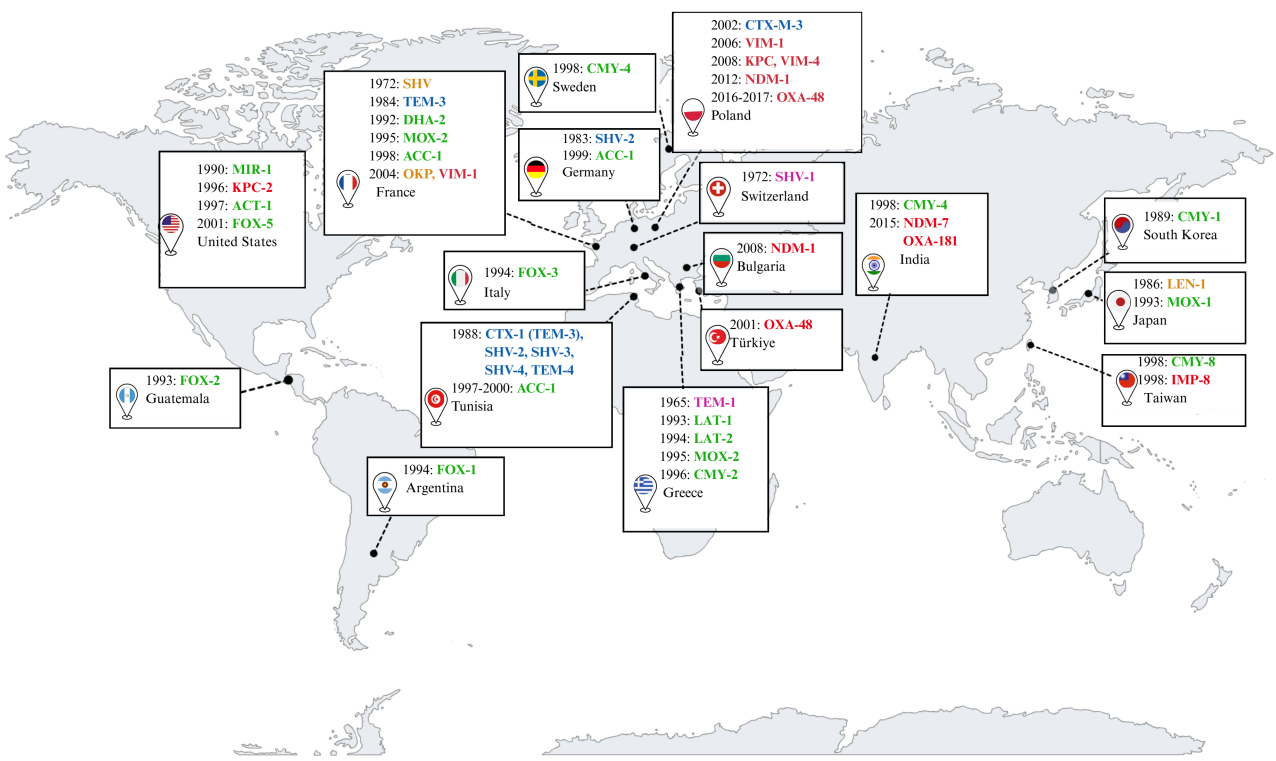

Fig. 3. World map showing the spread and origin of *K. pneumoniae* strains producing: species-specific beta-lactamases (marked in orange); selected BSBL-type beta-lactamases (marked in purple); selected ESBL-type beta-lactamases (marked in blue); selected plasmid-encoded AmpC beta-lactamases (marked in green); and selected carbapenemases KPC, NDM, and OXA-48 (marked in red). Own graphic design based on (Jacoby 2009; Dzierżanowska *et al.* 2010; Mączyńska *et al.* 2015; Castanheira *et al.* 2021).

The production of the AmpC enzyme depends on the *amp*D, *amp*G, and *amp*R gene system. AmpC production can be either induced or constitutive, resulting in different resistance phenotypes. Constitutive expression of the *amp*C gene means that AmpC enzymes are produced constantly at a steady level, whereas inductive expression involves production of the AmpC enzyme only after an inducer (e.g., an antibiotic) appears in the environment (Jacoby 2009; Meini *et al.* 2019; Tamma *et al.* 2019). The level of AmpC enzyme production may differ between the two expression types, reflecting variations in the rate of gene transcription that depend on the nucleotide sequence of the promoter, i.e., the site where expression begins. The speed of the process depends primarily on whether the antibiotic is a so-called weak or strong inducer. Antibiotics identified as strong inducers of the AmpC production pathway include aminopenicillins, amoxicillin/clavulanic acid, amoxicillin, narrow-spectrum (i.e., first-generation) cephalosporins, and cephamycins (Jacoby 2009; Meini *et al.* 2019; Tamma *et al.* 2019). Mutation of the regulatory gene (e.g., *amp*D) or deletion of the *amp*R gene causes permanent unblocking of expression, resulting in constant, very high-level beta-lactamase production regardless of the presence of the inducer (Jacoby 2009; Meini *et al.* 2019; Tamma *et al.* 2019). This phenomenon, referred to as AmpC derepression, enables bacteria to hydrolyze a wide range of substrates, i.e., oxyimino-beta-lactams (penicillins, cephalosporins, and monobactams) and 7-alpha-methoxycephalosporins (cefoxitin and cefotetan). At the same time, AmpC enzymes are not inhibited by commonly known inhibitors (clavulanate, sulbactam, and tazobactam). For infections caused by AmpC cephalosporinase-producing *Enterobacterales*, cefepime or carbapenems are the primary treatment options. The choice of regimen depends on the severity and location of the infection, with carbapenems the drugs of choice for the most serious infections (e.g., bacteremia, sepsis, pneumonia, intra-abdominal infections). Cefepime is a recommended, safe alternative to carbapenems, especially when the antibiogram indicates susceptibility to cefepime. Treating selected infections with cefepime is an important component of antimicrobial stewardship strategies aimed at reducing the overuse of carbapenems, thereby minimizing the risk of carbapenem-resistant organisms (the so-called "carbapenem-sparing" approach) (Tebano *et al.* 2024; Slain *et al.* 2025). For urinary tract infections caused by AmpC cephalosporinase-producing *Enterobacterales*, current treatment options include nitrofurantoin, fosfomycin, pivmecillinam, fluoroquinolones, cefepime, piperacillin with tazobactam, and carbapenems (Bader *et al.* 2020; Tebano *et al.* . 2024; Slain *et al.* 2025).

Strains producing cAmpC in an inducible manner usually appear susceptible to third-generation cephalosporins, which are weak inducers, but can readily generate constitutively expressing mutants resistant to these drugs (which are good substrates), resulting in treatment failure. pAmpC is usually constitutively expressed. pAmpC production is common in community–acquired infections, while cAmpC producers are mainly involved in healthcare–associated infections (HAIs) (Meini *et al.* 2019).

K. pneumoniae belongs to the bacilli that lack the natural chromosomal AmpC mechanism (with the exception of *Klebsiella mobilis*). Currently, chromosomal *amp*C genes, both inducible and derepressed, have been transferred to plasmids and spread in the environment, especially in hospitals (Papanicolaou *et al.* 1990; Jacoby 2009; Meini *et al.* 2019; Tamma *et al.* 2019; Rodríguez–Guerrero *et al.* 2022). The elements IS*Ecp1*, IS*CR1,* and IS*26* are responsible for the transfer of natural *amp*C genes. This process has been accelerated by the widespread use of antibiotics from the 7-alpha-methoxycephalosporin group (cefoxitin and cefotetan) and combinations containing beta-lactamase inhibitors (amoxicillin with clavulanic acid, ampicillin with sulbactam, or piperacillin with tazobactam) (Rodríguez–Guerrero *et al.* 2022). Currently, outbreaks caused by *K. pneumoniae* strains with an acquired, derepressed AmpC mechanism, carrying plasmids encoding AmpC beta-lactamase genes of molecular class C, have been reported (Rodríguez–Guerrero *et al.* 2022). Moreover, in *K. pneumoniae* strains with reduced envelope permeability, AmpC enzymes may cause resistance to carbapenems, as observed in epidemic outbreaks registered, among others, in the United States and in numerous European countries: Spain, the Netherlands, Sweden, and the United Kingdom (Papanicolaou *et al.* 1990; Rodríguez–Guerrero *et al.* 2022).

K. pneumoniae remains a major producer of acquired AmpC cephalosporinase variants, first observed in 1988 (Jacoby 2009). Unlike their induced precursors, most acquired cephalosporinases are produced constitutively because the *amp*C gene is often mobilized without the accompanying regulatory gene *amp*R on the chromosome. Acquired AmpC confers resistance to penicillins, cephalosporins (except

fourth–generation cephalosporins), monobactams, and beta-lactam-inhibitor combinations. As with other beta-lactamases, the level of resistance in strains depends on the level of enzyme expression. In clinical terms, acquired AmpC-producing *K. pneumoniae* strains are as dangerous as ESBL (+) strains, but epidemiologically they pose little threat due to their much lower incidence (Papanicolaou *et al.* 1990; Jacoby 2019; Meini *et al.* 2019; Tamma *et al.* 2019; Rodríguez–Guerrero *et al.* 2022).

2.1.1.5 Carbapenemases of classes A, B and D in the Ambler classification

2.1.1.5.1. Class A: KPC type carbapenemases

Among the known families of carbapenemases classified as molecular class A are: NMC-A/IMI, SME, KPC, SFC, BKC, FRI, and GES (Sawa *et al.* 2020; Ding *et al.* 2023). The chromosome is the primary location of genes encoding enzymes such as NMC-A/IMI, SME, and SFC, whereas genes encoding enzymes such as KPC, BKC, FRI, and GES are located on plasmids (Sawa *et al.* 2020; Ding *et al.* 2023). These genes can be located on various genetic elements, including simple or complex transposons, integrons, and phage-like genomic islands. Among class A carbapenemases, most enzymes are detected predominantly or partially in *Enterobacterales* as acquired beta-lactamases (Sawa *et al.* 2020; Ding *et al.* 2023).

KPCs (*Klebsiella pneumoniae* carbapenemases), one of the three most common types of carbapenemases in *Enterobacterales*, are important enzymes from the clinical and epidemiological point of view (Fig. 3) (Baraniak *et al.* 2011; Bush and Bradford 2019; Sawa *et al.* 2020). The first description of *K. pneumoniae* producing a carbapenemase KPC appeared in 1996 in North Carolina, USA. Since then, *K. pneumoniae* strains with this resistance phenotype have spread to many places around the world (Szewczyk 2019; Ochońska *et al.* 2021; Parra–Sellera *et al.* 2024). *K. pneumoniae* remains the dominant producer of KPCs, which constitute the largest category of class A carbapenemases in terms of the number of types and variants, while serine carbapenemases within this class constitute a significant minority (Bush and Bradford 2019; Sawa *et al.* 2020).

The production of KPC carbapenemases is determined by the gene $bla_{KPC,}$ which has a highly conserved nucleotide sequence. To date, several blaKPC haplotypes have been described, encoding KPC variants that differ by single amino acids (Ding *et al.* 2023). Currently, there are over 150 variants of the bla_{KPC} gene, including most of the new variants detected over the last 3 years (Ding *et al.* 2023). Globally, the most commonly observed gene variants *are* $bla_{KPC,}$ and the two oldest variants remain, i.e., KPC-2 and KPC-3 (Ojdana *et al.* 2015; Machulska *et al.* 2017; Ochońska *et al.* 2021). In most *K. pneumoniae* strains producing KPC-type carbapenemases, bla_{KPC} genes are located within specific elements of Tn*4401, which* belongs to the family of simple transposons Tn3 (Ding *et al.* 2023). Tn*4401* fragments carrying bla_{KPC} genes are located mainly on plasmids, among which the key role is played by specific molecules with two replicons, $IncFII_K$ and $IncFIB_K$ (pKpQIL type), but also other plasmids: IncA/C, IncI2, IncR, IncX, and ColE1 (Ding *et al.* 2023).

KPC-type carbapenemases have the broadest substrate spectrum (Baraniak *et al.* 2011; Bush and Bradford 2019; Sawa *et al.* 2020). These enzymes effectively hydrolyze virtually all classical beta-lactam antibiotics, i.e., penicillins, 1^{st}-4^{th} generation cephalosporins, monobactams, and carbapenems (Codjoe *et al.* 2018). Among these groups, cefoxitin and ceftazidime remain poor substrates for KPC. This does not translate into susceptibility of KPC (+) strains to these antibiotics; moreover, in the common KPC-3 variant, mutations are known that increase KPC activity toward ceftazidime (Mehta *et al.* 2015). Cefiderocol, a new fifth-generation cephalosporin, has very good *in vitro* activity against KPC (+) strains, although, according to current reports, clinical experience with this drug is limited (McCreay *et al.* 2021; Syed *et al.* 2021). In the treatment of infections caused by KPC (+) strains, non-beta-lactam beta-lactamase inhibitors (avibactam, relebactam, and vaborbactam) in the following combinations are effective: ceftazidime with avibactam, imipenem with relebactam, and meropenem with vaborbactam (Bush *et al.* 2019).

On a global scale, the spread of KPC-type carbapenemases occurs through clonal expansion, i.e., the transmission of microorganisms containing bla_{KPC} genes. Baraniak *et al.* (2017) presented an example of clonal spread of *K. pneumoniae* ST258 strains belonging to the CG258 clonal group, which includes closely related genotypes ST437 and ST512 (Baraniak *et al.* 2017). Hyperepidemic CG258 clones of *K. pneumoniae* are responsible for nationwide epidemics in Brazil, China, the Netherlands, Israel, Canada, Korea, Norway, Sweden, Italy, and many other countries (Pitout *et al.* 2015; Dautzenberg *et al.* 2016; Baraniak *et al.*

2017; Liu *et al.* 2022). Over the last 10 years, in Poland and in other countries, including France and Italy, significant changes have been noted in *K. pneumoniae* KPC (+) populations, consisting of the replacement of CG258 clones by other clones (emerging clones) with outstanding expansiveness, such as ST101, ST147, and ST307 (Loconsole *et al.* 2020; Peirano *et al.* 2020; Ochońska *et al.* 2021). Such clones, unlike CG258, are not closely related to KPC, but their variants producing ESBL-type beta-lactamases and/or other carbapenemases are often observed (Peirano *et al.* 2020). International CG258 *K. pneumoniae* clones are very often characterized by a heteroresistance phenotype, meaning that strains are simultaneously resistant to carbapenems and to antibiotics and/or chemotherapeutics from other therapeutic groups, mainly aminoglycosides, fluoroquinolones, tetracyclines, sulfonamides, and chloramphenicol (Liu *et al.* 2022; Krul and Dalla Costa 2024).

2.1.1.5.2. Class B: carbapenemases type MBL/NDM

Molecular class B includes metallo-beta-lactamases (MBLs) (Bush *et al.* 2019). Natural MBLs occur in many environmental bacteria of limited clinical importance, such as *Aeromonas hydrophila*, *Bacillus cereus*, *Bacteroides fragilis*, *Chryseobacterium indologenes*, *Elisabethkingia meningoseptica*, *Myroides odoratimimus*, and *Stenotrophomonas maltophilia* (Boyd *et al.* 2020). MBLs constitute the most distinct evolutionary and structural lineage. Functionally, these enzymes are distinguished from other carbapenemase classes by their dependence on zinc ions and their natural ability to hydrolyze carbapenems (Codjoe *et al.* 2018). The substrate spectrum of most MBL enzymes is broad and includes penicillins and cephalosporins from the first to the fourth generation. MBLs do not hydrolyze monobactams (aztreonam) and are not inhibited by beta-lactam inhibitors (clavulanate, sulbactam, and tazobactam). Similarly to KPC (+) strains, cefiderocol shows *in vitro* activity against MBL (+) strains. Ceftazidime with avibactam, ceftolozane with tazobactam, imipenem with relebactam, and meropenem with vaborbactam remain inactive against strains producing MBLs (Bush *et al.* 2019; Syed *et al.* 2021; Shortridge *et al.* 2022). In the treatment of infections caused by strains with this resistance phenotype, attempts are being made to use substances that demonstrate the ability to inhibit MBL enzymes, such as diazabicyclooctanes (zidebactam and nacubactam), cyclic boronic acids (taniborbactam), or indole-2-carboxylates (InCs) (Bush *et al.* 2019; Yahav *et al.* 2020; Brem and Schofield 2022).

Among the 20 families of acquired MBL-type carbapenemases commonly found in *K. pneumoniae* enterobacteria, the most clinically and epidemiologically significant are the NDM (New Delhi metallo-beta-lactamases) enzymes (Boyd *et al.* 2020; Ochońska *et al.* 2021; Biedrzycka *et al.* 2022). About 65 variants of NDM-type enzymes are known, with NDM-1 and NDM-5 predominating in pathogenic microorganism populations (Reference Gene Catalog - Pathogen Detection, https://www.ncbi.nlm.nih.gov/pathogens/refgene/). The production of NDM-type carbapenemases is encoded by the bla_{NDM} gene, which, together with the bleomycin resistance gene (ble_{MBL}), forms an operon that is part of the complex transposon Tn125 (Fiett *et al.* 2014). In *Enterobacterales* bacilli, larger or smaller fragments of Tn125 elements are observed, and the bla_{NDM} genes are located on plasmids of many groups: IncC, IncF, IncH, IncM, IncN, IncR and IncX, and their various subgroups. This localization indicates high transposition and recombination activity of Tn125 and/or other "secondary" elements containing Tn125 fragments (Fiett *et al.* 2014).

The diversity of plasmids carrying bla_{NDM} genes means that, both globally and in individual countries, the distributions of *K. pneumoniae* MBL (+) clones (genotypes) are more diverse than those of *K. pneumoniae* KPC (+) strains (Parra Selerra *et al.* 2024). Uncontrolled spread of a given *K. pneumoniae* clone (genotype) can lead to its dominance in a given region (Fiett *et al.* 2014; Loconsole *et al.* 2020; Peirano *et al.* 2020; Krul and Dalla Costa 2024).

The Indian subcontinent is the primary reservoir of microorganisms producing NDM-type enzymes, which are widely distributed in the environment and have been spreading worldwide since 2006 among hospital pathogens (Bose *et al.* 2022). The endemic state of NDM has been reported in the Persian Gulf countries, North African countries, and the southwestern Balkans (Subramanian *et al.* 2016). Since 2008, *K. pneumoniae* NDM (+) strains have been systematically detected across Europe (Baraniak *et al.* 2016; Baraniak *et al.* 2019; Parra Sellera *et al.* 2024).

2.1.1.5.3 Class D: carbapenemases type OXA–48

Class D is the most diverse category of beta–lactamases in terms of evolution and structure. Enzymes in this group most effectively hydrolyze isoxazolyl

penicillins (oxacillin and cloxacillin), which is why they are called oxacillinases. CHDL carbapenemases (CHDL) are a minority among oxacillinases and mainly comprise serine beta-lactamases from the OXA family, produced naturally by bacilli of the genera A*cinetobacter*, *Aeromonas*, and *Shewanella* (Bush *et al.* 2019; Yoon and Jeong 2021). Most OXA enzymes are unable to hydrolyze carbapenems and are classified as ESBL enzymes. New variants that have emerged during evolution and exhibit an extended substrate-hydrolysis spectrum show weak hydrolytic activity toward carbapenems (Bush *et al.* 2019; Yoon and Jeong 2021).

In *K. pneumoniae*, the plasmid variant of OXA-47 located in class 1 integrons does not show hydrolytic activity against ceftazidime and imipenem, whereas the acquired CHDL (OXA-48 variant) already shows strong carbapenemase properties (Nordmann–Poirel 2014; Mączyńska 2015). The OXA-48 mechanism was first detected in a strain of *K. pneumoniae* in Turkey in 2001. After this discovery, observational studies showed a high prevalence of OXA-48-type carbapenemase in the Arabian Peninsula, the Middle East, North Africa, and Europe (Pitout *et al.* 2019). The activity of OXA-48-type oxacillins, similar to other CHDLs, is highly specific among carbapenemases, as it covers only penicillins, first-generation cephalosporins, and carbapenems, while the efficiency of carbapenem hydrolysis is relatively low. Resistance to temocillin is characteristic of OXA-48-producing strains. These enzymes are not sensitive to EDTA and clavulanic acid (Nordmann–Poirel 2014; Mączyńska 2015; Bush *et al.* 2019; Yoon and Jeong 2021). CHDLs do not inactivate third- and fourth-generation cephalosporins, monobactams, and cefiderocol. OXA-48 and other CHDLs are not inhibited by clavulanate, sulbactam, and tazobactam, which is why antibiotic combinations containing them are not used. The preferred first-line treatment for severe infections caused by OXA-48-producing bacterial strains is typically a combination of beta-lactam antibiotics and beta-lactamase inhibitors (e.g., ceftazidime with avibactam) (Pitout *et al.* 2019). In cases of non-response, resistance, or mixed infections (e.g., those additionally producing MBLs), cefiderocol demonstrates high efficacy (Tarski *et al.* 2024). In the absence of newer drugs or due to specific clinical circumstances, the use of older antibiotics in combination (e.g., colistin, tigecycline, aminoglycosides) is considered, always based on an individual antibiotic susceptibility test (Stewart *et al.* 2018). It is worth noting that OXA-48 microorganisms (as in other CPE) commonly harbor ESBLs, which means that these two beta-lactamases together hydrolyze all classical beta-lactams (Nordmann–Poirel 2014; Mączyńska 2015; Bush *et al.* 2019; Yoon and Jeong 2021).

The formation of the group of OXA-48 derivatives, i.e., OXA-181, OXA-204, OXA-232, OXA-163, OXA-244, and OXA-245, was associated with substitution or deletion of single amino acids (Pitout *et al.* 2019). $bla_{\text{OXA-48}}$-type genes occur in various mobile genetic elements, most often in complex transposons of the Tn1999 type. These genes can also be located within the IS*Ecp1* transposition modules defined as Tn2016-type elements (Izdebski *et al.* 2018). These transposons are most often located on plasmids, but their diversity is lower than that of the bla_{KPC}, bla_{NDM}, and bla_{VIM} genes. Tn1999-type elements usually lie on plasmids from the IncL group. The evolutionary line pOXA-48a is characterized by an exceptionally high potential for conjugative transfer of $bla_{\text{OXA-48}}$ genes and their derivatives in *Enterobacterales* populations at the scale of single hospitals, countries, and the world. Tn2013 modules with the $bla_{\text{OXA-181}}$ gene or its variants often correlate with plasmids from the IncX3 group, but also with IncM, IncT, and ColE (Izdebski *et al.* 2018).

K. pneumoniae is one of the most commonly reported species of redundant carbapenemase–producing (RCP) bacteria. These bacteria possess dual or multiple carbapenemases in various combinations, are widely distributed worldwide, and their incidence is increasing over time (Yuan *et al.* 2024). In the study by Yuan *et al.* (2024), it was shown that *K. pneumoniae* carrying certain carbapenemase combinations, i.e., NDM + OXA (56.76%) and KPC + VIM (50.00%), are associated with high mortality. In patients with RCP strains isolated from the bloodstream and respiratory tract, the mortality rates are 58.70% and 69.23%, respectively. Plasmid analysis of RCP strains suggests that they may acquire additional antibiotic resistance phenotypes and virulence factors (Yuan *et al.* 2024).

2.1.2 Receptor resistance

Receptor resistance in *K. pneumoniae* concerns beta–lactam antibiotics, aminoglycosides and fluoroquinolones. This type of resistance involves modifying the antibiotic's target binding site, called the receptor, where it binds to the bacterial cell. The effect of this process is various conformational changes, depending on the type of antibiotic. In the case of beta-lactam antibiotics, the mechanism of receptor resistance

is associated with permeability barriers or with a decrease in affinity for penicillin-binding proteins (PBPs) belonging to the genera PBP-3, PBP-4 and PBP-5. This mechanism is recorded in 60% of *Enterobacteriaceae* (Dzierżanowska–Fangrat 2023). Receptor mechanisms of resistance to aminoglycosides may result from changes in ribosomal proteins (mainly streptomycin) or, less frequently, from mutations occurring in ribosomal RNA (rRNA). In the epidemiological aspect, the most important mechanism determining the resistance of *K. pneumoniae* to most aminoglycosides used in therapy (amikacin, gentamicin, kanamycin, netilmicin and tobramycin) is the production and expression of plasmid-encoded methyltransferases that methylate the 16S rRNA subunit: ArmA (aminoglycoside resistance methyltransferase), RmtA (resistance-associated methyltransferase A), RmtB (resistance-associated methyltransferase B), RmtC (resistance-associated methyltransferase C), RmtD (resistance-associated methyltransferase D), RmtG (resistance-associated methyltransferase G) (Ishizaki *et al.* 2018, Yang *et al.* 2022).

For fluoroquinolone antibiotics, the primary mechanism of resistance is mutations in the quinolone resistance-determining region (QRDR). The most common mutations occur in the genes responsible for the synthesis of gyrase (*gyr*A) or topoisomerase IV (*par*C). The first enzyme introduces negative supercoils into the DNA helix, and the second enzyme separates DNA strands necessary for the replication process (Dzierżanowska–Fangrat 2023). Mutations in the gyr gene lead to a change in a single amino acid in the enzyme. Resistance associated with topoisomerase IV results from stepwise mutations, through which bacterial strains gradually acquire higher levels of resistance (Dzierżanowska–Fangrat 2023).

2.1.3 Transport resistance

Transport resistance may result from mutations in genes encoding outer membrane proteins (OMPs). OMPs are anchored in the bacterial cell membrane and constitute the first barrier encountered by antibacterial agents. The ability to penetrate the cell membrane is necessary for inhibiting bacterial division or killing bacteria (Rocker 2020). In *K. pneumoniae*, two major porin proteins (OmpK35 and OmpK36), present in large numbers of copies in the membrane, play a key role in antibiotic transport. Resistant strains may be characterized by a deficiency of porins in the cell membrane, in which case their functions are taken over by minor proteins (LamB, OmpK37/26 and PhoE), the expression of which in the membrane depends on the needs of the cell. These proteins compensate for the deficiency of non–specific porins by allowing the free flow of nutrients while blocking the flow of antibiotics (Rocker 2020).

The second possible variant of transport resistance used by *K. pneumoniae* bacilli is the pumping of antibiotics out of the cell *via* the efflux mechanism. The transport of antibiotics is determined by the presence of transport proteins in the cell membrane. Depending on structure, the number of transmembrane sequences, substrate specificity, and mechanism of action, five families of pumps are distinguished: MFS (major facilitator superfamily), SMR (small multidrug resistance family), MATE (multidrug and toxic compound extrusion family), ABC (ATP–binding cassette superfamily), and RND (resistance–nodulation–cell–division family) (Huang *et al.,* 2022). Due to the complex structure of the cell envelopes in Gram-negative bacteria, transport proteins form a tripartite structure composed of a protein of the inner cytoplasmic membrane, a protein of the outer membrane, and a protein of the periplasmic space.

In *K. pneumoniae*, there is an AcrAB-TolC pump previously described in *E. coli* (Huang *et al.* 2022). This pump belongs to the RND family of pumps that use a proton gradient across the cell membrane to trigger the movement of substrate particles (Huang *et al.* 2022). The AcrAB-TolC pump is responsible not only for the removal of fatty acid salts from the bacterial cell, but also for the ejection of antibiotics, including aminoglycosides, beta-lactams, fluoroquinolones, glycocyclines, and tetracyclines. As a result of derepression of the *acr*AB gene, the cell membranes of resistant *K. pneumoniae* strains are characterized by an increased number of pumps, which results in increased active removal of the antibiotic from the bacterial cell (Huang *et al.* 2022).

3. Treatment of infections caused by *K. pneumoniae*

Antibiotics and other antimicrobial agents active against *K. pneumoniae* include: aminoglycosides (amikacin, gentamicin, netilmicin, tobramycin), second-generation cephalosporins (cefuroxime), third-generation cephalosporins (ceftazidime, cefotaxime) and fourth-generation cephalosporins (cefepime), fluoroquinolones (ciprofloxacin, levofloxacin, moxi-

floxacin, ofloxacin), carbapenems (doripenem, ertapenem, imipenem, meropenem), monobactams (aztreonam), piperacillin with tazobactam, ticarcillin with clavulanic acid, trimethoprim with sulfamethoxazole (co-trimoxazole) and ureidopenicillins (azlocillin, mezlocillin). In empirical therapy, the most commonly used groups of antibiotics are aminoglycosides and cephalosporins (Dzierżanowska-Fangrat 2023).

In the case of isolation of multi-drug resistant (MDR) *K. pneumoniae* strains and strains belonging to CPE, it is necessary to determine the sensitivity to available antibacterial drugs in order to select the most effective therapeutic option (Chung *et al.* 2016; Wang *et al.* 2019; Wyres *et al.* 2020). Older generation antibiotics used to treat infections caused by CPE strains include: aminoglycosides (amikacin, gentamicin, tobramycin), meropenem, colistin methanesulfonate sodium (colistimethate), fosfomycin sodium, temocillin, and sulfamethoxazole with trimethoprim (Hughes *et al.* 2020; Kuch *et al.* 2022). Moreover, several new antibiotics have recently been registered for the treatment of infections caused by CPE strains, including: cefiderocol, plazomicin, tigecycline, eravacycline, omadacycline, ceftazidime with avibactam, meropenem with vaborbactam, and imipenem with cilastatin and relebactam (Table II) (Bush *et al.* 2019; Hughes *et al.* 2020; McCreary *et al.* 2021; Kuch *et al.* 2022). It should be emphasized that these drugs often do not cover all classes of carbapenemases in their spectrum of action, and are often registered for very narrow indications (Hughes *et al.* 2020; Krajewska and Laudy 2021; Kuch *et al.* 2022).

In Poland, examples of the use of newly registered drugs were given in the work of Sękowska *et al.* (2024) covering a group of 41 strains of *K. pneumoniae* (pandrug resistant, PDR) and in which 43.9% of isolates were sensitive to colistin, 24.4% of isolates were sensitive to fosfomycin and ceftazidime with avibactam, 14.6% of isolates were sensitive to meropenem with vaborbactam (Sękowska *et al.* 2024). In another study by the same scientific team, Sękowska *et al.* (2024) demonstrated the efficacy of plazomicin, a new aminoglycoside with a broad spectrum of activity against the analyzed strains of *K. pneumoniae* MDR (Sękowska *et al.* 2024). In a study of ESBL-positive *K. pneumoniae* strains, plazomicin showed high activity with MIC values ranging from 0.19 to 4 μg ml-1, whereas for carbapenemase-positive strains, MIC values ranged from 0.25 to 256 μg ml-1 (Sękowska *et al.* 2024). In turn, in the case report presented by Tarski *et al.* (2024), the efficacy of cefiderocol in the treatment of a 72-year-old patient with sepsis caused by *K. pneumoniae* NDM and OXA-48 was demonstrated (Tarski *et al.* 2024). Polymyxins are useful in the treatment of patients infected with *K. pneumoniae* NDM, and the efficacy of therapy is greater when colistin is used in combination with carbapenem or rifampicin or tigecycline. Revealing the *in vitro* interactions of such antibiotic combinations was the aim of the study conducted by Müderris *et al.* (2024) covering a group of 30 *K. pneumoniae* isolates forming biofilm (Müderris *et al.* 2024). Based on the obtained results, the study showed that the biofilm inhibitory concentration (BIC) of colistin, meropenem, tigecycline and rifampicin significantly increased after biofilm formation. The synergistic effect observed in the sessile form was independent of the planktonic form. Although a high synergistic effect of the combination of meropenem and colistin on sessile bacteria was observed, colistin had a very high BIC in all combinations (Müderris *et al.* 2024).

The introduced avibactam in combination with ceftazidime and aztreonam is another promising therapeutic combination in severe infections caused by *K. pneumoniae*, in particular by (MDR) strains. The synergistic effect of the combination of ceftazidime, avibactam, and aztreonam results in the protection of both beta-lactam antibiotics, ceftazidime and aztreonam, by avibactam, which shields them from degradation by bacterial enzymes (serine beta-lactamases). Avibactam belongs to a new class of non-beta-lactam beta-lactamase inhibitors. This multifunctional organic compound contains a 5-membered urea ring at its core, which significantly enhances ceftazidime's activity against multidrug-resistant Gram-negative bacilli. Like beta-lactam inhibitors, avibactam covalently binds to serine at the active site of beta-lactamases. Unlike other inhibitors, however, this process is reversible; the inhibitor does not undergo hydrolysis but is reconstituted (the 5-membered molecule is less susceptible to irreversible deformation). Due to avibactam's affinity for serine, this inhibitor is active against serine beta-lactamases, specifically those belonging to classes A (ESBL and KPC enzymes), C (chromosomal and plasmid-mediated AmpC), and certain class D enzymes (e.g., OXA-48) according to the Ambler classification (Kowalska-Krochmal *et al.* 2019). Avibactam does not inhibit class B (MBL) enzymes and is unable to inhibit the activity of many class D enzymes. Aztreonam is naturally resistant to hydrolysis by metallo-beta-lactamases (e.g., NDM). Avibactam, which

Table II.

Table listing currently used antibiotics for treating infections caused by carbapenem-resistant Gram-negative bacteria (CR-GNB) and the recommended and approved indications for their use. Own graphic design based on Paul *et al.,* (2022).

	ESBLs	**CRE non--CP**	**CRE--KPC**	**CRE--O-XA-48**	**CRE--MBL**	**Current clinical indications**	**Approval**
New antibiotics							
Cefiderocol	Yes	Yes	Yes	Yes	Yes	cUTIs, HAP, VAP	FDA
						for the treatment of infectious due to aerobic Gram-negative organisms in adults with limited treatment options	EMA
Ceftolozane-tazobactam	Yes	No	No	No	No	cIAI, cUTIs, HAP, VAP	FDA and EMA
Ceftazidime-avibactam	Yes	+/-	Yes	Yes	No	cIAI, cUTIs, HAP, VAP	FDA and EMA
						for the treatment Gram-negative infections in patients with limited treatment options	EMA
Eravacycline	Yes	Yes	Yes	Yes	Yes	cIAI	FDA and EMA
Imipenem-cilastatin-relebactam	Yes	+/-	Yes	No	No	cIAI, cUTIs	FDA
						HAP, VAP, BSI with a suspected respiratory source, and for the treatment Gram-negative infections in patients with limited treatment options	EMA
Meropenem-vaborbactam	Yes	+/-	Yes	No	No	cUTIs	FDA
						cUTIs, HAP, VAP, and for the treatment Gram-negative infections in patients with limited treatment options	EMA
Plazomicin	Yes	Yes	Yes	Yes	+/-	cUTIs	FDA
							EMA application withdrawn
Old antibiotics							
Aminoglycosides	+/-	+/-	+/-	+/-	+/-	for the treatment of a variety of bacterial infections	FDA and EMA
Aztreonam	No	No	No	No	+/-	for the treatment of infections caused by susceptible Gram-negative microorganisms	FDA and EMA
Fosfomycin iv	Yes	+/-	+/-	+/-	+/-	to treat serious infections when other antibiotics treatment are not suitable	EMA
							FDA under review
Polymyxins	Yes	Yes	Yes	Yes	Yes	to treat serious infections caused by susceptible strains, when less potentially toxic drugs are ineffective or contraindicated	FDA
						to treatment of serious infections due to aerobic Gram-negative pathogens in patients with limited treatment options	EMA
Tygecycline	Yes	Yes	Yes	Yes	Yes	complicated SSTI and IAI	FDA and EMA
						CAP	FDA

Abbreviations: BSI, bloodstream infection; CAP, community-acquired pneumonia; cIAI, complicated intra-abdominal infections; CRE non-CP, non-carbapenemase-producing carbapenem-resistant *Enterobacterales*; cUTIs, complicated urinary tract infections; EMA, European Medicines Agency; ESBLs, extended-spectrum beta-lactamases; FDA, US Food and Drug Administration; HAP, hospital-acquired pneumonia; iv, intravenous administration; MBL, metallo-beta-lactamases; SSTI, skin and soft-tissue infections; VAP, ventilator-associated pneumonia; +/-, variable antibiotic activity.

inactivates beta-lactamases that degrade aztreonam, allows the antibiotic's efficacy to be restored. The combination of both drugs, ceftazidime with avibactam and aztreonam, allows for the inhibition of both ESBL and MBL resistance mechanisms, which is crucial in the fight against MDR strains of *K. pneumoniae* (Kowalska–Krochmal *et al.* 2019).

The use of this drug combination is supported by numerous studies conducted by Polish and international research teams (Guzek *et al.* 2024; Słabisz *et al.* 2024; Szymański *et al.* 2024; Khattab *et al.* 2025; Rajan and Sasikala 2025). For example, the scientific team of Słabisz *et. al.* (2024) examined 60 strains of *K. pneumoniae* producing NDM-type carbapenemase (Słabisz *et. al.* 2024). This study showed that all tested *K. pneumoniae* strains (100%) were resistant to ceftazidime with avibactam and 92% of strains were resistant to aztreonam when these drugs were used individually (Słabisz *et al.* 2024). The strip stacking method confirmed the synergistic effect of ceftazidime/avibactam (CZA) and aztreonam (AT) and demonstrated 100% *in vitro* sensitivity to this combination of antibiotics among the tested strains (Słabisz *et al.* 2024). Similarly, in a study by another Polish scientific team, (Guzek *et al.*, 2024), the efficacy of the combination of ceftazidime/avibactam with aztreonam in the treatment of 23 patients with hospital-acquired KP-NDM infection was confirmed (Guzek *et al.* 2024). In the study, the synergistic effect of all compounds resulted in good agreement between the clinical efficacy of CZA + AT and the results of *in vitro* susceptibility tests (Guzek *et al.* 2024). According to the research conducted by the scientific team of Szymański *et al.* (2024), in the group of patients treated with CZA + AT, statistically lower mortality and faster clinical response to treatment were demonstrated due to the therapy used compared to the group of patients treated with alternative combinations of antibiotics including: colistin sodium, fosfomycin, an aminoglycoside, and tigecycline (Szymański *et al.* 2024). Similar positive clinical assessments were obtained for sulbactam, especially in combination with meropenem and colistin, which was confirmed in the study conducted by (Laishram *et al.* 2016). Combinations of sulbactam, meropenem, and colistin were studied for their synergistic activity against 100 invasive carbapenem-resistant *K. pneumoniae* isolates from cancer patients hospitalized in the ICU (Laishram *et al.* 2016). In recent years, researchers have explored several combination therapies, such as heat shock in combination with aminoglycosides, polymyxin B combined with fosfomycin, zidovudine combined with rifampicin, FAS (fatty acid synthesis) inhibitors in conjunction with colistin, as

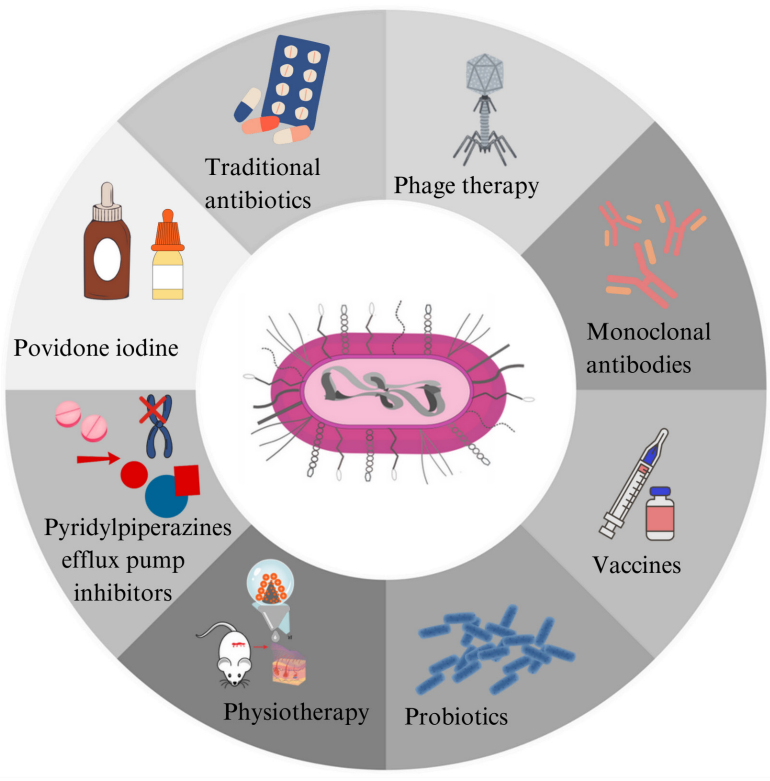

Fig. 4. Diagram showing therapeutic strategies for treating infections caused by multidrug-resistant (MDR) *K. pneumoniae*. Own graphic design based on (Wantuch and Rosen, 2023; Lei *et al.*, 2024 and Li *et al.*, 2024).

well as new therapeutic approaches such as monoclonal antibodies (MAb), vaccines, phage therapy, pyridylpiperazine efflux pump inhibitor (PyrPips), probiotics, and physiotherapy (Fig. 4) (Wantuch and Rosen 2023; Lei *et al.* 2024).

4. Epidemiology of *K. pneumoniae* resistant to specific classes of antibiotics in Europe and Poland

Antibiotic resistance in *K. pneumoniae* bacilli is a major medical and epidemiological problem both across Europe and in Poland (Asokan *et al.* 2025, Shah *et al.* 2025). Data from national and international antibiotic resistance surveillance networks provide insights into the scale of this problem. In Europe, for the countries of the European Union (EU) and the European Economic Area (EEA) comprising Iceland and Norway, the monitoring of antibiotic resistance in bacterial strains cultured from blood and cerebrospinal fluid samples is conducted by the European Centre for Disease Prevention and Control (ECDC) in Stockholm as part of the European Antimicrobial Resistance Surveillance Network (EARS-Net, https://www.ecdc.europa.eu) (EARS-Net 2024; ECDC 2026). EARS-Net uses standardized methodologies for data collection and analysis. However, given that each national surveillance system is shaped by country-specific protocols and practices, particular caution should be exercised when comparing antimicrobial resistance patterns across countries. In 2024, the EARS-Net network published data submitted by 30 European countries, from which 62 774 *K. pneumoniae* isolates derived from invasive infections (including blood) were reported. Data collected by EARS-Net laboratories, after being verified and prepared for transmission, are submitted to the EpiPulse database at the ECDC. The EARS-Net network in Europe is complemented by the CAESAR network (The Central Asian and European Surveillance of Antimicrobial Resistance Network) coordinated by the World Health Organization (WHO). Both networks: EARS-Net and CAESAR submit data to the WHO GLASS (Global Antimicrobial Resistance and Use Surveillance System) database to the extent that their systems overlap (https://www.who.int/initiatives/glass, https://www.who.int/) (EARS-Net 2024; WHO GLASS 2025; ECDC 2026).

In Poland, monitoring within the EARS-Net network includes laboratories from across the country that perform microbiological testing for selected university, provincial, and county hospitals. In 2024, data provided by 54 EARS-Net laboratories from across Poland, performing microbiological testing for 61 hospitals, were analyzed (EARS-Net 2024). In Poland, the dataset is funded from resources at the disposal of the Minister of Health as part of the implementation of the task titled "Preventing the Development of Antibiot-

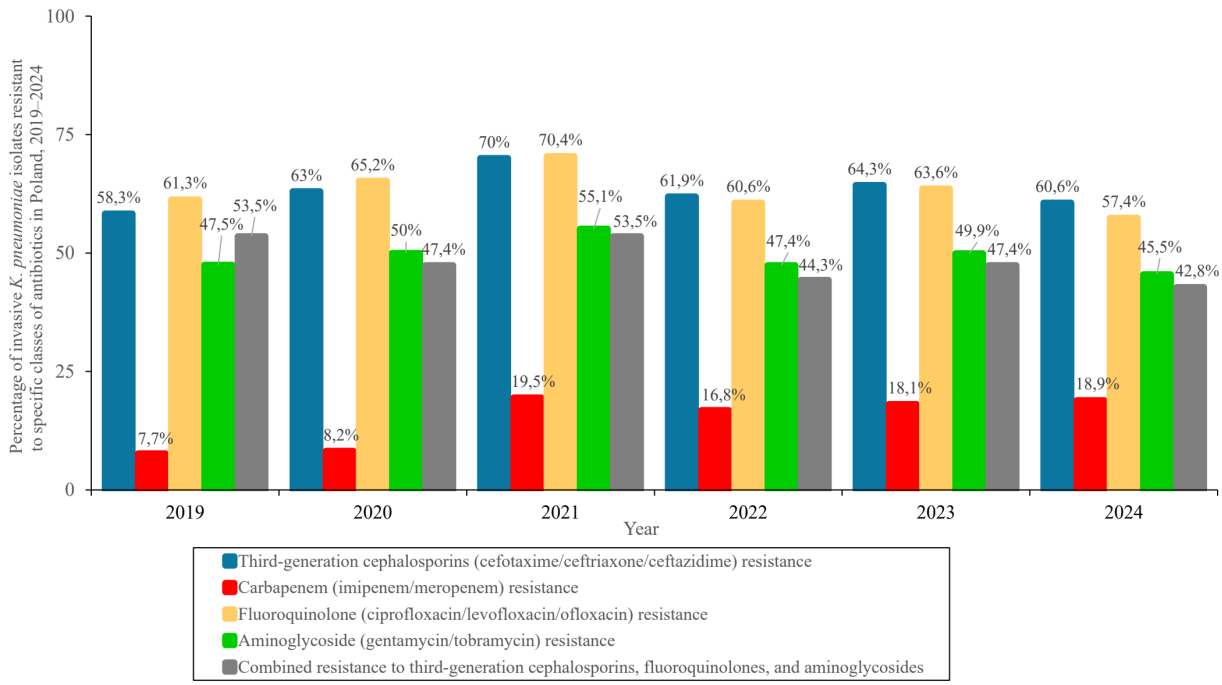

Fig. 5. Graph showing the percentage of invasive *K. pneumoniae* isolates resistant to specific antibiotic classes in Poland, 2019–2024. Own graphic design based on data from EARS-Net (2024) and ECDC (2026).

ic Resistance in Microorganisms" under the National Health Program for 2021–2025, and is coordinated by the Polish National Reference Centre for Antimicrobial Susceptibility Testing (KORLD) operating at the National Medicine Institute in Warsaw (Journal of Laws of the Republic of Poland, Warsaw, April 8, 2021, Item 642: Regulation of the Council of Ministers of March 30, 2021, on the National Health Program for 2021–2025, KORLD).

In EU/EEA countries, the estimated incidence of invasive *K. pneumoniae* isolates (per 100 000 population) was 19.2 cases in 2020, 19.3 in 2021, in 2022 – 20.5, in 2023 – 22.6, and in 2024 – 25.3, indicating an increase of +31.8% (EARS-Net 2024; ECDC 2026). According to the latest EARS-Net report, between 2019 and 2024, a statistically significant increase in the incidence rate of bloodstream infections caused by isolates belonging to this species was observed across the EU among invasive infections caused by *K. pneumoniae*. In 2024, when broken down by resistance phenotypes, the highest estimated prevalence of bloodstream infections caused by third-generation cephalosporin-resistant *K. pneumoniae* was reported in EU/EEA countries (9.03 per 100 000 population), followed by isolates resistant to fluoroquinolones (8.53 per 100 000 population) and, subsequently, isolates resistant to aminoglycosides (5.58 per 100 000 population). Worryingly, a statistically significant upward trend in the proportion of carbapenem-resistant isolates was observed among invasive *K. pneumoniae* isolates, with the estimated total incidence of carbapenem-resistant *K. pneumoniae* bloodstream infections in the EU in 2024 standing at 3.51 per 100 000 population (range across EU countries: 0.02-20.31) and was 61.0% higher compared to the baseline year of 2019 (EARS-Net 2024; ECDC 2026). This increase highlights the need to rapidly strengthen prevention and control measures in the EU, as emphasized in the Council Recommendation on stepping up EU action to combat antibiotic resistance through a One Health approach (2023/C 220/01) (Council of the European Union. Council Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach (2023/C 220/01). Brussels: Council of the European Union; 2023. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/ PDF/?uri=OJ:JOC_2023_220_R_0001).

At the EU/EEA level for the period 2020-2024, as in previous years, significant differences were observed in the rates of AMR to specific antibiotic classes among invasive *K. pneumoniae* isolates, with higher resistance rates in this bacterial species reported in countries in Southern, Central, and Eastern Europe, such as Bulgaria, Croatia, Cyprus, Greece, Poland, Romania, and Italy (EARS-Net 2024; ECDC 2026). In this region, more than one-third (39.1%) of invasive *K. pneumoniae* isolates reported to the EARS-Net network in 2024 were resistant to at least one of the antimicrobial classes under surveillance, namely: fluoroquinolones, third-generation cephalosporins, aminoglycosides, and carbapenems. In 2024, the highest EU/EEA population-weighted mean AMR percentage was reported for third-generation cephalosporins (32.9%), followed by fluoroquinolones (31.4%), aminoglycosides (21.5%) and carbapenems (11.3%). Between 2020 and 2021, the emergence of antimicrobial-resistant (AMR) pathogenic *K. pneumoniae* coincided with the SARS-CoV-2 pandemic, which placed an enormous strain on the healthcare system and significantly impacted infection prevention and control programs. During this period, a significant increase in reported cases of invasive infections caused by all monitored bacterial pathogens was observed compared to 2020 (EARS-Net 2024; ECDC 2026).

In Poland in 2024, EARS-Net laboratories recorded a significant 63% increase in the number of invasive *K. pneumoniae* isolates (n=2066) under surveillance compared to 2019 (n=1172). In the same year, 2024, the incidence of invasive infections caused by *K. pneumoniae* in the country was 26.53 per 100 000 population, compared to 2019, when the rate was 17.96 per 100 000 population (EARS-Net 2024; ECDC 2026).

Compared to the rest of Europe, Poland ranks among the countries with a high percentage of invasive *K. pneumoniae* isolates resistant to third-generation cephalosporins, which stood at 60.6% in 2024. Between 2019 and 2024, the incidence rate per 100 000 population for infections caused by third-generation cephalosporin-resistant *K. pneumoniae* in Poland increased from 10.53 to 16.90 cases. This is confirmed by an analysis of epidemiological data from 2019-2021, which showed a statistically significant increase in the percentage of isolated invasive *K. pneumoniae* resistant to third-generation cephalosporins, from 58.3% in 2019, 63% in 2020, to 70% in 2021, representing 13.11 per 100 000 population. In the subsequent years 2022-2023, this percentage was 61.9% and 64.3%, respectively. In other European countries, the highest proportions of invasive *K. pneumoniae* isolates resistant to third-generation cephalosporins in 2024 were report-

ed in Bulgaria (84.3%), Greece (70.1%), and Cyprus (68.1%). The lowest prevalence of third–generation cephalosporin resistance among invasive *K. pneumoniae* isolates in Europe in 2024 was recorded in: Norway (7.0%), Denmark (5.9%), and Iceland (4.9%). A statistically significant decrease in the population–weighted average for EU/EEA countries in the percentage of invasive *K. pneumoniae* isolates resistant to third–generation cephalosporins was observed, from 34.8% to 32.9% in 2019 and 2024, respectively (EARS–Net 2024; ECDC 2026).

As in Europe, a steady increase in the proportion of carbapenem–resistant invasive *K. pneumoniae* isolates was also observed in Poland between 2019 and 2024. In Poland in 2019, the incidence of carbapenem–resistant invasive *K. pneumoniae* isolates was 1.38 per 100 000 population; in 2020–1.45 per 100 000 population, in 2021 – 3.69 per 100 000 population, in 2022–3.30 per 100 000 population, in 2023–3.69 per 100 000 population, and in 2024–5.15 per 100 000 population (representing a 273.2% increase compared to 2019). In Poland, an analysis of data from 2019–2021 showed a statistically significant increase in the percentage of carbapenem resistance among isolated *K. pneumoniae*, from 7.7% in 2019, 8.2% in 2020 to 19.5% in 2021, which is more than double the rates observed in the preceding years of 2019–2020. In the subsequent years 2022–2024, the percentage of isolated invasive *K. pneumoniae* resistant to carbapenems was 16.8%, 18.1%, and 18.9%, respectively. In Europe during the 2022–2024 period, carbapenem resistance among isolated invasive *K. pneumoniae* varied widely, ranging from 0% (Finland and Iceland) to 72% in Greece, depending on the country. In 2024, the highest rates of carbapenem resistance among invasive *K. pneumoniae* isolates in Europe were recorded in countries such as Bulgaria (67.6%), Greece (60.2%), and Romania (50.3%), while the lowest rates of these isolates were recorded in Ireland (0.2%), Finland (0.2%), and Iceland (0%) (EARS–Net 2024; ECDC 2026). The results indicate the uncontrolled spread of invasive *K. pneumoniae* strains producing various carbapenemases (CPE) across Europe; consequently, immediate action is needed in the areas of infection control and prevention to halt this trend (EARS-Net 2024; WHO GLASS 2025; ECDC 2026). The emergence of invasive *K. pneumoniae* (CPE) strains has radically altered the map of Poland showing the distribution of strains of this species with dangerous resistance mechanisms. Current data from recent years, both in Europe and in Poland, indicate that the epidemiological situation regarding *K. pneumoniae* strains producing NDM-type carbapenemases persists, as well as strains of this species producing KPC-type carbapenemases, OXA-48-type carbapenemases, or ESBL-type beta-lactamases. This situation in Poland is confirmed by successive new isolations of *K. pneumoniae* KPC (+), NDM-1 (+), and OXA-48 (+) strains reported in various regions of the country (Ochońska *et al.* 2021; Wysocka *et al.* 2021; Biedrzycka *et al.* 2022; Brauncajs *et al.* 2022; Sarowska *et al.* 2022; Sękowska *et al.* 2024).

Poland also ranks among the leading European countries in terms of *K. pneumoniae* resistance to fluoroquinolones, where in 2024 the percentage of invasive isolates of this species resistant to this class of antibiotics was 57.4% (EARS-Net 2024; ECDC 2026). In the preceding years (2019–2023), the percentage of invasive *K. pneumoniae* isolates resistant to fluoroquinolones was 61.3% in 2019, 65.2% in 2020, 70.4% in 2021, 60.6% in 2022, and 63.6% in 2023. Since 2019, a statistically significant increase in the frequency of isolation of these fluoroquinolone–resistant bacteria has been observed. Between 2019 and 2024, the incidence rate per 100 000 population of infections caused by fluoroquinolone–resistant *K. pneumoniae* isolates in Poland increased from 11.00 to 15.82 cases. Furthermore, between 2019 and 2024, a statistically significant decrease was observed in the population–weighted average percentage of fluoroquinolone–resistant *K. pneumoniae* isolates across EU/EEA countries, from 34.6% to 31.4%, respectively. In 2024, a higher percentage of invasive *K. pneumoniae* isolates resistant to fluoroquinolones than in Poland was recorded only in Bulgaria (80.3%), Greece (69.9%), Cyprus (69.9%), and Romania (64.5%). Conversely, the lowest percentages of invasive *K. pneumoniae* isolates resistant to fluoroquinolones in Europe in 2024 were recorded in Ireland (9.7%), Denmark (8.2%), and Finland (7.1%) (EARS–Net 2024; ECDC 2026).

Poland is also among the European countries with a high-though, since 2019, stable-rate of aminoglycoside resistance among invasive *K. pneumoniae* isolates, which stood at 47.5% in 2019, 50% in 2020, 55.1% in 2021, 47.4% in 2022, 49.9% in 2023, and 45.5% in 2024 (EARS-Net 2024; ECDC 2026). At the same time, between 2019 and 2024, a statistically significant decrease was observed in the population-weighted average for EU/EEA countries in the percentage of aminoglycoside resistance in the studied bacterial species, from 24.7% to 21.5%. During the 2019-2024 period, the incidence

rate per 100 000 population for infections caused by aminoglycoside-resistant *K. pneumoniae* in Poland increased from 8.30 to 11.84 cases. In Europe, the highest rates of *K. pneumoniae* resistance to aminoglycosides in 2024 were recorded in countries such as Bulgaria (73.8%), Greece (59.6%), and Romania (53.8%). The lowest rates of aminoglycoside resistance among invasive *K. pneumoniae* isolates in Europe in the same year 2024 were recorded in Germany (3.5%), Finland (3.1%), and Denmark (3.0%) (EARS-Net 2024; ECDC 2026).

In Poland in 2024, the percentage of invasive *K. pneumoniae* isolates with MDR strains phenotype (resistant to third-generation cephalosporins, fluoroquinolones, and aminoglycosides, simultaneously) was 42.8%, whereas, in the preceding years 2019-2023, the percentage of invasive *K. pneumoniae* isolates with this MDR phenotype reached the following values: 45% in 2019, 47.4% in 2020, 53.5% in 2021, 44.3% in 2022, and 47.4% in 2023 (EARS-Net 2024; WHO GLASS 2025; ECDC 2026). A statistically significant decrease in the population-weighted average for EU/EEA countries in the proportion of MDR phenotype in *K. pneumoniae* was observed, from 21.8% to 18.8% in 2019 and 2024, respectively. During the 2019–2024 period, the incidence rate per 100 000 inhabitants of infections caused by MDR *K. pneumoniae* in Poland increased from 7.75 to 10.81 cases (EARS–Net 2024; ECDC 2026). In the EU/EEA region in 2024, the highest proportion of *K. pneumoniae* with MDR phenotype was reported in Bulgaria (71.5%), in Greece (57.5%), and in Romania (50.4%), while the lowest proportion of invasive *K. pneumoniae* isolates with this MDR phenotype was recorded in Finland (2.1%), Denmark (1.4%), and Iceland (0%) (EARS-Net 2024; ECDC 2026).

In Poland, information on the antibiotic resistance of invasive *K. pneumoniae* isolates is also provided in the annual reports on the country's sanitary conditions published by the Chief Sanitary Inspectorate (Report on the Activities of the State Sanitary Inspection in the Field of Public Health for 2024, Poland, https://www.gov.pl/web/gis/raport–––stan–sanitarny–kraju). The data contained in the reports indicate the occurrence of epidemic outbreaks in healthcare facilities in Poland. The latest report on the activities of the State Sanitary Inspection in the field of public health for 2024, containing data as of December 31, 2024, indicates that in 2024, as in previous years, a high incidence of outbreaks caused by *K. pneumoniae* was reported in Poland. According to this report, *K. pneumoniae* was the second most common bacterial pathogen causing outbreaks in Polish hospitals in 2024. In total, 249 outbreaks of *K. pneumoniae* were reported to state district health inspectors in 2024, accounting for 23.5% of all recorded infection outbreaks among the 1 060 outbreaks in which a biological agent was identified. In these outbreaks, 1 157 patients were infected, of whom 1 378 were found to have gastrointestinal colonization. In 209 outbreaks, *K. pneumoniae* bacteria producing carbapenemases: KPC, NDM, OXA-48, and OXA-181 were identified, while in 25 outbreaks, the presence of *K. pneumoniae* producing ESBL-type beta-lactamases was confirmed. Since 2015, there has been a noticeable upward trend in the reporting of outbreaks caused by *K. pneumoniae* (CPE) in Poland, with 59 outbreaks in 2019, 75 in 2020, 158 in 2021, 143 in 2022, and 167 in 2023. In 2024, *K. pneumoniae* outbreaks were most frequently reported from adult and pediatric anesthesiology and intensive care units, as well as from general medical wards, i.e., internal medicine, general medicine, and geriatrics and infectious disease wards in both level I, II, and III, as well as in oncology and rehabilitation hospitals (Report on the Activities of the State Sanitary Inspection in the Field of Public Health for 2024, Poland, https://www.gov.pl/web/gis/raport–––stan–sanitarny–kraju).

5. Summary

The constantly growing number of *K. pneumoniae* isolates resistant to antibiotics and/or other antimicrobial agents is currently one of the greatest challenges for modern medicine. Overuse of antibiotics and/or chemotherapeutics creates selective pressure for microorganisms, which accelerates the emergence and spread of *K. pneumoniae* strains with resistance mechanisms in the hospital environment and among outpatients. Due to the above, knowledge about the antibacterial spectrum of antibiotics and/or chemotherapeutics, mechanisms of acquiring resistance to antibiotics and/or other antimicrobial agents among *K. pneumoniae* and constant deepening of knowledge about the epidemiological occurrence of these mechanisms in Poland is crucial. It should be emphasized that only rational antibiotic therapy adapted to the resistance profile of a given strain creates real chances for the effective use of antibiotics and/or other antimicrobial agents in future generations. To sum up, studies on the molecular epidemiology of *K. pneumoniae* are important due to the need to organize epidemiological surveillance in the

hospital environment and to monitor the transmission routes of the pathogen in the situation of the increase in isolation and spread of multidrug–resistant isolates observed in our country.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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TRYPSIN-ACTIVATED PARASPORAL PROTEINS FROM NATIVE *BACILLUS THURINGIENSIS* ISOLATES REDUCE *STAPHYLOCOCCUS AUREUS* VIRULENCE AND BIOFILM FORMATION *IN VITRO*

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Abstract: Antibiotics are pivotal for treating *Staphylococcus aureus* infections, but overuse has led to multidrug-resistant (MDR) strains, necessitating alternative strategies. Targeting virulence factors, rather than bacterial survival, offers a promising approach to combat resistance. This study evaluated the in vitro effects of native, non-insecticidal *Bacillus thuringiensis* (*Bt*) parasporal crystal proteins (PCPs), in protoxin (PT) and trypsin-activated (AT) forms, on *S. aureus* virulence gene expression and biofilm formation. Fourteen *Bt* PCPs were isolated and prepared as PT and AT forms. Their impact was assessed using LacZ promoter fusion assays for *hla* (alpha-hemolysin), *spa* (protein A), and RNAIII (a key virulence regulator), supplemented by quantitative β-galactosidase assays. Biofilm formation was quantified and normalized to planktonic growth (Normalized Biofilm Index, NBI) to distinguish specific antibiofilm activity from growth inhibition. AT-PCPs from *Bt* strains M11, M78, M91, M154t1, and A11 significantly downregulated *hla*, *spa*, and RNAIII expression ($\log_2$ fold changes up to –4.88, $p < 0.001$) and reduced biofilm formation (NBI reduction up to 57%, $p < 0.001$). AT proteins exhibited no hemolytic activity and had MIC values >100 μg/mL, confirming antivirulence effects at sub-inhibitory concentrations. PCR screening revealed the absence of cyt1 genes in four of five active isolates, supporting Cry-mediated targeting. These in vitro findings suggest that trypsin-activated PCPs can disrupt critical virulence pathways in *S. aureus*. Trypsin-activated *Bt* PCPs attenuate virulence and biofilm formation in *S. aureus* in vitro, highlighting their potential as leads for novel antivirulence strategies. These proteins show potential as candidates for standalone or adjuvant strategies alongside conventional antibiotics in the fight against MDR infections, pending further validation. Further research is needed to elucidate their mechanisms, assess their efficacy in vivo, and evaluate enzymatic activation by host-derived proteases. These in vitro results highlight the potential of *Bt*-derived PCPs as novel virulence-targeting leads, though further research is needed to identify the active component(s), elucidate mechanisms, and evaluate efficacy *in vivo*.

Keywords: bacilli; *Bacillus thuringiensis*; biofilm; multidrug resistance; virulence genes; complex multicellular structure

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1. Introduction

Staphylococcus aureus (*S. aureus*) is a prominent opportunistic, multidrug-resistant (MDR) pathogen that causes nosocomial and community-related illnesses with considerable morbidity and mortality in both humans and animals (Felden et al. 2011; O'Neill 2014; Cheung et al. 2021). Because it carries a diverse set of virulence factors, this aggressive Gram-positive bacterium can circumvent all host defense barriers (Bien et al. 2011). *S. aureus* infections range from simple skin and soft tissue infections to potentially fatal invasive diseases such as osteomyelitis, endocarditis, pneumonia, and septicemia (Tong et al. 2015; Turner et al. 2019). Owing to biofilm formation, *S. aureus* is becoming a common contaminant of indwelling medical devices, increasing the economic burden by billions of dollars annually in the United States of America (USA) (Al-Mebairik et al. 2016). The mortality rate attributable to MRSA (Methicillin-resistant Staphylococcus aureus) infections in the USA is higher than that of HIV/AIDS (Bien et al. 2011). By 2050, annual mortality caused by MDR microbes (superbugs) is projected to reach 10 million, matching that of cancer, with an economic burden of about $100 trillion (O'Neill 2014; Ahmad-Mansour et al. 2021; Cassini et al. 2019; de Kraker et al. 2016). Fortunately, there have been considerable advances in our understanding of the molecular mechanisms underlying virulence factors in pathogenic bacteria, including *S. aureus* (Cheung et al. 2021; Ahmad-Mansour et al. 2021; Allen et al. 2014). *S. aureus* is penicillin-resistant due to production of β-lactamases. Moreover, resistance to methicillin (an altered penicillin-binding protein, PBP2a) has become a major public health concern, giving rise to the notorious MRSA strains, which are resistant to almost all β-lactam antibiotics. While MRSA infections have reached epidemic levels of up to 50% in some parts of the USA, they have been reported to be highly prevalent, up to 30%, in Saudi Arabia (Deurenberg and Stobberingh 2008). Additionally, *S. aureus* is part of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter*) group of pathogens that are commonly resistant to multiple antimicrobial agents (O'Neill 2014). *Bacillus thuringiensis* (*Bt*) is an entomopathogenic, Gram-positive, spore-forming bacterium that produces Cry and/or Cyt (δ-Endotoxins) parasporal crystal proteins (PCPs) during sporulation. For more than seven decades, *Bt* has been widely recognized as the most successful eco-friendly bioinsecticide available to humanity due to its selective toxicity against a wide range of crop pests and human disease vectors, including orders such as Lepidoptera, Coleoptera, and Diptera, as well as other invertebrates such as nematodes, mites, snails, and ticks (Bravo et al. 2007). Both the Cyt protein families (Cyt1 to Cyt3) and the Cry protein families (Cry 1 to Cry 74) (Crickmore et al. 2021) belong to a class of pore-forming toxins (PFTs), yet only the Cry proteins are target host-specific insect proteins, presumably due to the requirement for specific host receptors; hence, adverse effects on target vertebrate or invertebrate species are almost null or negligible. Such outstanding specificity prompted several investigators to elucidate the three-domain Cry protein structure, the required receptors, and the mode of action. In the target host insect, larvae ingest the crystalline Cry *Bt*-PCP, which is solubilized under the alkaline pH of the midgut. The released protoxins are activated by host-specific proteases in the lumen, which sequentially split the protoxin into the C-terminus and N-terminus halves. Proteolytic activity continues along the N-terminus half until it reaches a peptide region resistant to the acting protease; the resulting activated toxin (AT) is competent to bind to and insert into receptors in midgut microvilli; finally, the inserted AT oligomerizes, creating pores (cation-selective channels) that lead to cell membrane lysis and death (Palma et al. 2014, Das et al. 2021). The C-terminal half of the PCP Cry protoxin is not involved in specific killing activity, yet it is required for Cry protoxin crystallization during sporulation, thereby protecting PCPs from intrinsic *Bt*-producer proteases as a crystallization strategy (Li et al. 2013). By contrast, Cyt toxins are hydrophobic, show no homology to Cry proteins, do not require a specific receptor but bind directly to membrane lipids, and, even at low concentrations, they synergize with or bypass resistance to mosquito-larvicidal Cry proteins by acting as a membrane-bound receptor for Cry. These Cyt proteins are cytocidal to cells of both invertebrates and vertebrates, including mammalian red blood cells (RBCs) (Thomas and Ellar 1983). This explains why the non-insecticidal *Bt*-PCP-Cry protein exhibiting preferential cytocidal activity for human cancer cells (parasporins; P1 to P6 families) is devoid of such general (PFT)-hemolytic Cyt proteins (Aboul-Soud et al. 2019, Ohba et al. 2009). Additionally, the activated form of Cyt1Aa (23–24 kDa) from *B. thuringensis* subsp. *israelensis* (*Bti*) inhibited the growth of *Escherichia*

coli (bactericidal) and *S. aureus* (bacteriostatic) with minimal inhibitory concentrations (MICs) of 1.25 and 5 μg/ml, respectively (Promdonkoy and Ellar 2003). Within *B. thuringiensis* serovar *israelensis* de Barjac (*Bti*), the genes that encode Cry and Cyt proteins are typically found on a large plasmid (128 kb) known as pBtoxis (Schnepf et al. 1998). In other *Bt* strains, however, the number of plasmids coding for Cry proteins is highly variable, ranging from 1 to 13 plasmids (Bravo et al. 2007), and these plasmids may harbor more than 700 genes coding Cry proteins (Palma et al. 2014). The number of genes in studied *Bt* strains has reached up to 7227 (Bravo et al. 2007), underscoring their enormous genetic diversity. Therefore, discovering more native *Bt* strains, which typically vary from one country to another and even from one region to another within the same country (Li et al. 2013), will likely yield novel Cry toxins and proteins with novel biological activity. In previous reports from our lab (Aboul-Soud et al. 2019; Ahmed et al. 2017; Ahmed et al. 2021), native *Bt* strains isolated from Saudi Arabian habitats exhibited significantly broader and more potent larvicidal activity than the reference *Bti* H14 against *Anopheles gambiae, Culex pipiens* and *Aedes caspius*. Moreover, we demonstrated preferential cytotoxic effects of activated PCPs (ATs) derived from native non-insecticidal *B. thuringiensis* strains against cervical cancer cells (Aboul-Soud et al. 2019). This study built on this work to examine the potential effects of trypsin-activated PCPs (ATs) isolated from 14 documented local non-mosquito-larvicidal *B. thuringiensis* isolates as novel natural inhibitors of expression of the virulence-associated *hla*, *spa*, and RNAIII genes and of biofilm development in *S. aureus*. It is anticipated that the antivirulence activities of PCPs may contribute, either alone or as an adjunct, to strategies for disarming *S. aureus* infections as well as other MDR bacterial pathogens. To the best of our knowledge, this is the first report on the use of activated PCPs (ATs) from native non-mosquito-larvicidal *B. thuringiensis* isolates to silence *S. aureus* gene(s) associated with virulence factors and inhibit biofilm formation.

2. Materials and methods

2.1. Isolation, culturing, and identification of native *B. thuringiensis* isolates

The 14 local *Bt* isolates were obtained from environmental samples collected across 14 regions in Saudi Arabia, as previously described by research group (El-Kersh et al. 2016; Aboul-Soudet al. 2019; Ahmedet al. 2021; El-kersh et al. 2014). Of the original 14 isolates, five (M11, M78, M91, M154t1, A11) were retained for detailed study due to long-term storage constraints. The remaining isolates were unavailable for further analysis. The samples were processed for *Bt* isolation, and colonies resembling the reference *Bti* H14 - white, large, with irregular margins, glossy or scalloped, or nonglossy (Figure 1A and 1B) were purified and further processed for identification using morphological and biochemical characterization (API 20E and API CH50 systems, BioMerieux, Marcyle Etoile, France) and by examining parasporal crystal shapes under both phase-contrast and scanning electron microscopes. The biochemical profiles of recovered *Bt* isolates, including lecithinase, motility, and hemolytic activities, as well as molecular typing via 16S rDNA gene sequencing for nineteen insecticidal *Bt* isolates and four non-insecticidal *Bt* isolates, namely: M78, GenBank: KF026328.1; M154t1, GenBank: KC960017.1; M242, GenBank: KC527056.1; M300 (KF971833.1); and *B. cereus* (ATCC1177, GenBank: KC84954.1), were compared with the *Bti* H14, (GenBank: KJ722438.1) reference strain and have also been previously published (Ahmed et al. 2017). In this study, 14 documented local non-insecticidal *Bt* isolates were included and maintained as stock cultures at -20°C in sterile liquid nutrient broth (NB; Oxoid, UK) containing 20% glycerol, as well as freeze-dried (lyophilized) cultures at 4 °C. None of these 14 selected native *Bt* isolates exhibited larvicidal activity against *Aedes caspius*, even at remarkably high concentrations (El-Kersh et al. 2016; Ahmed et al. 2021).

2.2. Preparation of *Bt*-parasporal crystal proteins (PCP)

From revived lyophilized cultures or glycerol-revived stalk subcultures, a pure colony of each of 14 native non-insecticidal *Bt* isolates was streaked onto fresh Luria Broth (LB) agar supplemented with manganese (II) chloride tetrahydrate ($MnCl_2$) (Loba Chemie) and incubated at 37 °C for 72 h (Palma et al. 2014; El-Kershet al. 2014). After incubation, *Bt* colonies of each isolate were suspended in sterile distilled water (SDW) as a wet mount and examined under a phase-contrast microscope (100X oil-immersion objective) to confirm the presence of spores and crystals (Figures 2A to 2H). Next, *Bt* growth was scraped from each plate, resuspended in 5 mL of SDW, vortexed,

and 5 mL of 1 M NaCl was added, then centrifuged at 7000 rpm for 15 min. The pellet was resuspended in SDW with 1 M NaCl, then washed twice in SDW by centrifugation. A 6% sterile lactose solution was added to the washed pellet to reach approximately 2 McFarland tubes (Priest et al. 2004). The suspension was stirred for 30 min on a magnetic stirrer, and four volumes of acetone (Win Lab) were slowly added. Stirring continued for an additional 45 min. The mixture was allowed to stand at room temperature (RT) for 10 min, then filtered through Whatman No. 1 filter paper. The residues on the filter paper were left to aseptically dry overnight in an incubator at 37 °C. The white crystal-spore mixture pellets obtained after drying were suspended in SDW and washed twice with SDW by centrifugation. The washed pellets were then dissolved in an alkaline buffer (Na_2CO_3, 0.256 g) and dithiothreitol (DTT) (Loba Chemie PVT. LTD., India), 0.08 g, in 50 ml SDW with a few drops of 1 N NaOH (pH 12) for 3 h in a shaking incubator (Shel Lab) at 37 °C, followed by centrifugation at 7000 rpm for 20 min. The pH of the resultant supernatant (containing protoxin) was adjusted to 7.0–8.0 with 1N HCl, followed by membrane sterilization (Merck, Millipore, 0.45 µm), thereby removing spores and/or sporangium fragments. The protein content was then assayed using the Lowry method (El-Kersh et al. 2012) with a calibration curve constructed with bovine serum albumin (BSA) (Sigma-Aldrich; A9647). The protein concentrations for each of the 14 non-insecticidal *Bt* isolate PCPs were also adjusted to 1 mg/ml with SDW to minimize the influence of possible solubilized spore-protein impurities, given that the maximum protein concentration used in all experiments of this study did not exceed 50 to 100 µg, with corresponding volumes of 50 to 100 µl. The obtained solutions were then aliquoted into small volumes (0.5 ml) in sterile Eppendorf tubes and stored at -20 °C. Some aliquots were subjected to trypsin activation (AT), while others remained unactivated as protoxin samples (PT). For trypsin activation, 1 µg of trypsin (Type I; Sigma) was mixed with 20 µg of protoxin, and the mixture was digested with trypsin for 3 h at 37 °C. Trypsin was then inactivated by adding 0.5 mg of trypsin inhibitor (Type II; Sigma) per mg of trypsin. The trypsin-proteolysis fragments (trypsin-activated toxins, AT) were sterilized by bacterial membrane filtration and stored at -20 °C until testing. The effects of non-trypsin-activated protoxins (PT) on S. aureus virulence factor expression were also examined (Aboul-Soud et al. 2019; Ammouneh et al. 2011; Nethravathi et al. 2010; Naimov et al. 2008). To minimize contamination and remove spores and cellular debris, all AT and PT protein preparations were filtered through a 0.22 µm membrane filter before use in subsequent assays. This step effectively removes intact spores, sporangial fragments, and insoluble debris, ensuring that the tested solutions contain primarily soluble proteins.

2.3. Hemolytic activity

To assess the inherent hemolytic activity of native *Bt* strains, fresh overnight cultures were streaked onto sheep blood agar plates. Plates were incubated aerobically at 30°C for 48 hours. Hemolytic zones and their types were recorded. *S. aureus* (MSSA, methicillin-sensitive *Staphylococcus aureus*) ATCC 29213 was used as the positive control.

2.4. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard procedures. Protein bands of activated (AT) and unactivated PCP (PT) proteins were resolved on a 12% SDS-PAGE gel and subsequently visualized by silver staining (Aboul-Soud et al., 2019).

2.5. *S. aureus hla* and *spa* expression reporter assay and stock cultures of *S. aureus* strains

The reporter assay was conducted using *S. aureus* PC203 (spa::lacZ) and *S. aureus* PC322 (hla::lacZ) strains, as previously described and kindly provided by Nielsen and co-workers (Porcar and Juárez-Pérez 2003). These 2 fusion strains were derived from the *S. aureus* wild-type strain (8325-4), which had been cured of known prophages and was successfully used to screen compounds that influence virulence gene expression in *S. aureus* (Porcar and Juárez-Pérez 2003). The two *S. aureus* strains were grown on Tryptic Soy Agar (TSA; Mast Laboratories, Merseyside, UK) containing erythromycin (5 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (150 µg/ml). The PC203 (spa::lacZ) strain appears light blue, while the PC322 (hla::lacZ) strain displays blue colonies. For the *hla* and *spa* expression assay, each of the two *S. aureus* strains was grown over-

night on TSA, and a pure colony of each strain was suspended in sterile saline to a 0.5 McFarland standard. An aliquot of 125 µl of the suspension was placed in a Petri dish and mixed with 25 mL of LB agar at 50 °C, supplemented with erythromycin (5 µg/mL) and X-gal (150 µg/mL). After solidification, wells were punched with a sterile drill (5 mm). *Bt* AT and PT isolates at concentrations of 10, 50, and 100 µg/mL were added to the wells. As controls, equal volumes of saline, alkaline buffer (Na_2CO_3 and DTT), and trypsin (at the specified concentration) were used. The *Bti* H14 reference strain contains Cyt1Aa, Cyt1Ab, and Cyt2Aa proteins (Ahmed et al. 2017), and the trypsin-activated form exhibits strong antibacterial effects (Promdonkoy and Ellar 2003), precluding its use as a control in this study. Plates were incubated at 37 °C, and results (bleaching zones in mm) were recorded after 24 h. The appearance of a white zone surrounding the well indicates downregulation of virulence gene expression (Porcar and Juárez-Pérez 2003). The two *S. aureus* strains were maintained as stock cultures at 4 °C in TSA supplemented with 5 µg/mL erythromycin and stored, as well as at -20 °C in tryptone soya broth (TSB; SPML, Saudi Arabia) containing 15% glycerol, and sub-cultured on blood agar plates at 37 °C before testing. Additionally, MSSA strain (ATCC 29213) and MRSA strain (ATCC 43300) were obtained from King Fahad Medical City (KFMC) and maintained as stock cultures at -20 °C in tryptone soya broth (TSB) containing 15% glycerol (SPML, Saudi Arabia) and sub-cultured on blood agar plates at 37 °C before testing.

2.6. Quantitative β-galactosidase activity

A quantitative β-galactosidase assay was conducted in liquid culture with the two *S. aureus* reporter strains PC203 (spa::lacZ) and PC322 (hla::lacZ) to evaluate the impact of *Bt*-derived PCPs on virulence gene expression and to compare the findings with those of the zone-inhibition assay. We diluted the overnight cultures 1:100 in fresh TSB containing 5 µg/ml erythromycin and allowed them to grow to the early log phase (OD600 = 0.3). Aliquots (1 mL) were treated with *Bt*-derived AT proteins (10, 25, and 50 µg/ml) or an equivalent volume of alkaline buffer (control) and incubated at 37°C with shaking for 3 h. We collected the cells, rinsed them, and then lysed them using both lysozyme (1 mg/ml) and lysostaphin (10 µg/ml) in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). We used o-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/mL) as the substrate to assess β-galactosidase activity. Reactions were terminated by adding 1 M Na_2CO_3, and absorbance was measured at 420 nm and 550 nm. β-galactosidase activity was expressed as Miller units (MU). Three replicates were performed, and the experiment was repeated twice.

2.7. Biofilm assay

The biofilm assay was performed using a 96-well flat-bottom microtiter plate. *S. aureus* PC203 (spa::lacZ), PC322 (hla::lacZ), and methicillin-resistant *S. aureus* (MRSA) ATCC 43300 were cultured overnight in TSB (SPML, Saudi Arabia) at 37°C and then diluted 1:200 in sterile TSB. To assess the effect of diluted (50 µg/mL) and undiluted (UD, 100 µg/mL) *Bt* AT and PT from the five selected (M11, M78, M91, M154t1, and A11) *Bt* isolates (based on data on bleaching zones, differences in geographical origin, bio-typing, and crystal shape) on biofilm formation, wells of 96-well microtiter plates were filled with two hundred µl aliquots of the diluted cultures with AT, PT, or saline in triplicate. Two parallel plates were prepared: one for growth assessment and the other for the biofilm assay. Both plates were incubated overnight at 37 °C. For planktonic biomass growth assessment, absorbance at 600 nm (OD600) was measured. To assess biofilm formation, planktonic cells were aspirated, and the wells were washed three times with sterile saline. The plate was inverted on filter paper and allowed to dry for 1 h at RT. Biofilms attached to the plate were stained with 200 µL of crystal violet (0.1% in SDW) for 15 min at RT. Excess stain was removed by washing three times with sterile saline, and the remaining crystal violet bound to biofilms was dissolved in 230 µL of 95% ethanol. Finally, absorbance at 550 nm (A550) was measured as an indicator of biofilm formation (Head et al. 2004; Kaur et al. 2009; Upadhyaya et al. 2010). To distinguish between growth inhibition and specific antibiofilm activity, the Normalized Biofilm Index (NBI) was calculated using the following formula:

$$\mathrm{NBI} = \frac{\mathrm{A550\ (Biofilm)}}{\mathrm{OD600\ (Growth)}}$$

Calculated NBI values were analyzed using one-way ANOVA with Tukey's post hoc test in GraphPad Prism v10.4.2. Data are presented as mean ± SEM.

2.8. Minimum Inhibitory Concentration (MIC)

According to Clinical and Laboratory Standards Institute (CLSI) recommendations, broth microdilution assays were used to determine MIC values. We tested two-fold serial dilutions of PCPs (100, 50, and 25 µg/mL) against MSSA (ATCC 29213), using *Bti* H14 AT as the positive control (Promdonkoy and Ellar 2003) at concentrations of 10, 5, 2.5, and 1.25 µg/mL.

2.9. PCR screening for *Cyt1* gene

Bacterial plasmid DNA was extracted using an alkaline lysis protocol as previously described. Plasmid DNA was obtained from five native B. thuringiensis isolates (i.e., M11, M78, M154t, M91, and A11), and the reference strain H14 served as a positive control for gene amplification. Universal (UN) primers [Forward: 5′-CCT CAA TCA ACA GCA AGG GTT ATT-3′; Reverse: 5′-TGC AAA CAG GAC ATT GTA TGT GTA ATT-3′] were used to amplify the Cyt1 genes (Cyt1Aa, Cyt1Ab, and Cyt1Ba) by PCR (Aboul-Soud et al. 2019). The PCR reaction mixture (25 µL) comprised the following final concentrations: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1 U of Taq DNA polymerase, and 50 ng of template DNA. Amplification conditions were as follows: an initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 40 seconds, 52°C for 60 seconds, and 72°C for 2 minutes, followed by a final extension at 72°C for 5 minutes. A 1.5% agarose gel stained with ethidium bromide was prepared to visualize the PCR results. *Bt*i H14 was used as a positive control for *Cyt1* amplification.

2.10. MTT cytotoxicity assay

The cervical cancer cell line HeLa was used to assess the toxicity of AT toxins. Cells were routinely grown in standard DMEM supplemented with glucose, pyruvate, and 10% fetal bovine serum (FBS). Cells were grown in T75 tissue culture flasks to confluence, then trypsinized and plated in 96-well plates at 1x104 cells per well in DMEM supplemented with 5% FBS for 24 hours. Next, cells were treated with two-fold serial dilutions of AT toxins at concentrations of 200, 100, 50, 25, 12.5, and 6.25 µg/mL overnight. The next day, the medium was replaced with 150 µL of MTT solution (5 mg/mL), and the plates were washed with isopropanol to solubilize the formazan crystals. Plates were read at 570 nm, and cell viability (%) was calculated after subtracting the background level measured at 630 nm. DMSO-treated cells at the highest AT concentration were set to 100% viability (basal growth control). The final DMSO concentration was less than 2%, which is safe for the cells and does not cause toxicity.

2.11. RT-qPCR

Overnight cultures of MSSA (ATCC 29213) were diluted 1:200 in fresh, sterile Todd-Hewitt broth with 2% yeast extract (THY) containing 0.5% glucose. One-mL aliquots of the diluted cultures were mixed with 100 µg/mL of PCPs *Bt* AT toxins or DMSO and then cultured at 37 °C in triplicate. Samples were collected by centrifugation at OD600 nm ~0.5 (corresponding to the ML growth phase) and after an overnight culture (S phase). Next, RNA-protect Cell Reagent (Qiagen, Hilden, Germany) was added, and lysostaphin (Sigma) was used to lyse the cells. RNA was extracted using Trizol® (Schnelldorf) according to the manufacturer's protocol, followed by a TURBO DNA-free kit (Sigma) to remove residual DNA contamination in the RNA samples (Al-Mebairik et al. 2016). The purified RNA was reverse transcribed to cDNA, and RT-qPCR was performed using the Light Cycler 2.0 System and the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). Predesigned, validated primers were purchased from TIB Molbiol (Berlin, Germany), and their sequences are shown in Table 1.

Table 1. Oligonucleotide primers in the RT-qPCR assay (Al-Mebairik et al. 2016).

Gene	Sequence (5`–3`)
spa	F: GCGCAACACGATGAAGCTCAACAA R: ACGTTAGCACTTTGGCTTGGATCA
hla	F: CTGAAGGCCAGGCTAAACCACTTT R: GAACGAAAGGTACCATTGCTGGTCA
16S rRNA	F: CTGGTAGTCCACGCCGTAAAC R: CAGGCGGAGTGCTTAATGC
RNAIII	F: GCACTGAGTCCAAGGAAACTAACTCT R: AGCCATCCCAACTTAATAACCATGT

The expression levels of the tested genes were normalized using 16S rRNA expression as an internal standard (Al-Mebairik et al. 2016). A positive reaction

was indicated by the accumulation of fluorescent SYBR Green I signal. In this experiment, four *Bt* isolates: M11, M78 (GenBank: KF026328.1), M91, and M154t1 (GenBank: KC960017.1) were used to study the effect of their AT on the expression of *S. aureus hla, spa,* and *RNAIII* genes.

2.12. Statistical Analysis

Data were analyzed using SPSS PC+ version 21.0. Normality was assessed using the Shapiro–Wilks test, and the assumption of homogeneity of variance was evaluated using Levene's test. One-way analysis of variance (ANOVA) was used to analyze biofilm formation in control versus experimental treatments with PCPs. Tukey's non-parametric post hoc test was used for multiple comparisons. Student's t-test was used for RT-qPCR experiments to profile expression of virulence genes. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Morphological descriptions of *Bt* colonies and crystal shapes

In this work, the colony development of the 14 non-insecticidal Bt isolates on LB agar/$MnCl_2$ showed strong resemblance to that of the reference Bt*i* H14, appearing as raised, large, white, almost circular colonies with fine, irregular margins and a glistening or non-glistening scalloped appearance (Figures 1A and 1B). Moreover, strains and crystals were examined morphologically and compared to the reference Bt*i* H14 strain using both scanning electron and phase-contrast microscopy (Figures 1C and 1D).

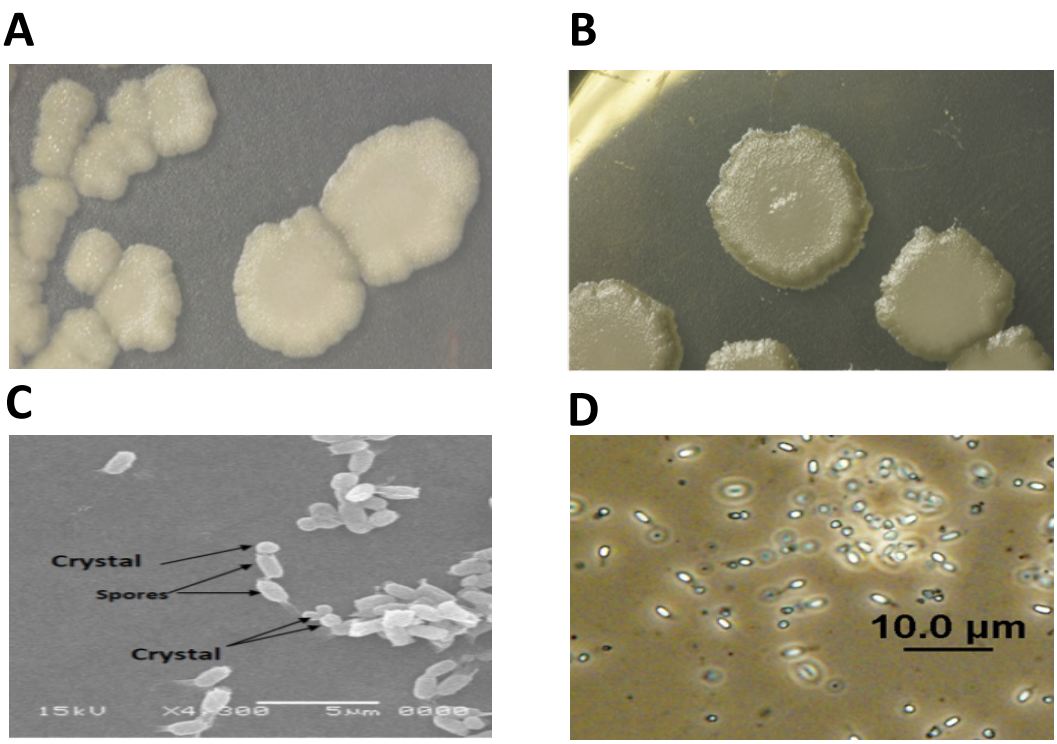

Figure 1: Microscopic examination *of the Bti* H14 reference strain and *Bt* isolate M91. Photomicrographs showing colony morphology of *Bti*-H14 (A), whereas (B) shows colony morphology of native *Bt* M91. Colonies were seeded onto Luria Broth (LB) agar medium supplemented with $MnCl_2$, exhibiting large, white, nearly circular colonies with fine, irregular margins and a glistening or non-glistening appearance. *Bti* H14 scanning electron microscopy (SEM) (C) and phase-contrast (D) at magnifications of X4, 300, and X1000, respectively.

Wet-mount phase-contrast microscopy enabled differentiation of *Bt* colonies from those of B. cereus, which are closely related, except for the presence of parasporal crystals within the sporangia of only *Bt* isolates, which display shapes and sizes unique to each native *Bt* strain (Figures 2A to 2H).

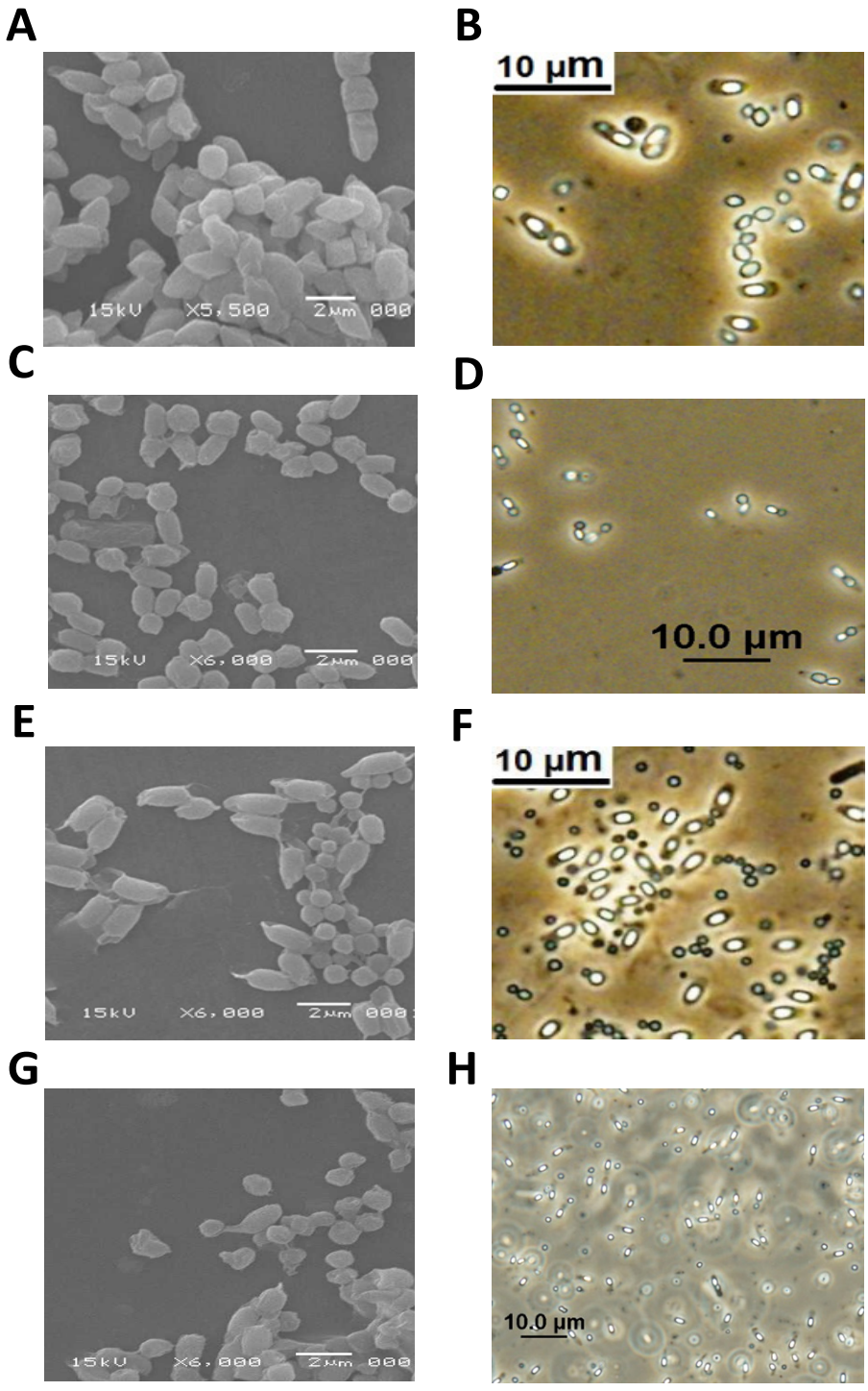

Figure 2. Scanning electron microscopy and phase-contrast micrographs of native, non-insecticidal *Bt* isolates illustrating spores and the diversity of crystal shapes. (A and B) Bt-78 (bipyramidal-oval-square, non-spore-attached). (C and D) Bt-M91 (hexagonal, spore-attached). (E and F) Bt-M154t1 (spherical, non-spore-attached). (G and H) Bt-M11 (bipyramidal-square, spore-attached). In all cases, spores appear non-swollen, bright, and cylindrical, resembling those of the reference *Bti* H14 in Figure 1. 16S rRNA sequences, with reference accession numbers in NCBI GenBank® (https://www.ncbi.nlm.nih.gov/nuccore), when applicable, showed 99% similarity to our native *Bt* isolates (Li et al. 2013).

Like the Bt*i* H14 reference strain, native Bt isolates exhibited many shared morphological features, including cell chain arrangements and cylindrical, non-swollen spores, but showed substantial variation in the shapes of the parasporal crystals formed. None of the 14 recruited *Bt* isolates exhibited mosquito-larvicidal activity against *Aedes aegypti*, even at remarkably high concentrations (data not shown) (El-Kersh et al. 2016; Ahmed et al. 2021). As presented in Table 2, most of the 14 native Bt isolates exhibited crystals attached to spores, with shapes ranging from small to large spherical, cubic, square, or bipyramidal, as clearly visualized by scanning electron microscopy of the examined Bt isolates (i.e., Bt*i* H14, Bt M11, Bt 78, Bt 91, and *Bt* 154t1) (Figure 2A to 2H). By contrast, the characteristics of Bt*i* H14 parasporal crystals, i.e., amorphous irregular, bisected spherical, conical-budding, or merged triangular, not attached to spores (Figure 1E and 1F), were not observed among the investigated 14 non-insecticidal native Bt isolates.

Table 2: Sampling type-of the 14 non-insecticidal *B. thuringiensis* isolates (lab code), their crystal shapes, and the qualitative and quantitative quenching effects of different AT protein concentrations on the expression of *S. aureus spa and hla* strains.

***Bt.* Isolate (Code)/ sample**	***Crystal-Shape***	**Protein Conc. ug/ml**	***Spa***			***hla***		
			Effect	***Bleaching zone (mm)***	***β-galactosidase Activity (MU)***	***Effect***	***Bleaching zone (mm)***	***β-galactosidase Activity (MU)***
(M11) soil	Oval-spherical Nonattached to spores	100	+	20±0.5	90±2.3	+	20±1.2	92±5.5
		50	+	16±1	163±7.2	+	19±0.7	108±4.3
		10	-	0	452±0	-	0	338±0
(M13m) soil	Small spherical, not attached	100	+	18±1	126±7.1	+	19±1.2	108±7.4
		50	+	15.3±1.2	176.13.9	+	15±0.5	155±5.2
		10	-	0	452±0	-	0	388±0
(M67t) Soil	Small spherical	100	-	0	452±0	-	0	388±0
		50	+	25±1.2	0±0	+	20±.2	92±1.9
		10	-	0	452±0	-	0	388±0
(M 78) soil	Irregular, bi- pyramidal/cubic like crystals	100	+	19.6±.3	100±1.4	+	21±1.2	62±3.6
		50	+	16±0.5	163±5.1	+	14.3±1.2	167±14.1
		10	-	0	452±0	-	0	388±0
(M91) soil	Nearly cubic, attached to spore	100	+	20±1.5	90±6.8	+	19.3±1.3	89±6.1
		50	-	16.3±0.8	158±7.8	-	15.6±0.7	147±6.6
		10	-	0	452±0	-	0	388±0
(M154t1) Leaves/soil	Small, spherical non-attached to spore	100	+	21.6±0.8	63±2.3	+	18.6±1.4	101±7.6
		50	+	17±1.5	145±12.8	+	16.3±1.8	136±15.1
		10	-	0	452±0	-	0	388±0
(M157) Dried leaves	Bipyramidal, attached to spore	100	+	19±1	109±5.8	+	19.6±0.8	85±3.5
		50	+	15.3±0.8	176±9.2	+	17±1.2	124±8.7
		10	-	0	452±0	-	0	388±0
(M160) sheep manure	Blunt end bipyramidal attached to spore	100	+	20.6±0.8	81±3.2	+	21±1	62±30
		50	+	19±0.5	109±2.9	+	20±1.2	92±5.5
		10	-	0	452±0	-	0	388±0
(M224) soil	Big spherical and hexagonal with inclined spore	100	+	18.3±1.2	122±8.0	+	20.6±1.5	70±5.1
		50	+	15±1.2	181±14.5	+	16.6±1.2	132±9,6
		10	-	0	452±0	-	0	388
(M242) soil	bipyramidal, with blunt edges	100	+	21.3±0.8	68±2.6	+	22.3±0.8	43±1.5
		50	-	0	452±0	-	0	388±0
		10	-	0	452±0	-	0	388±0

(M268P) Sewage W	Hexagonal attached to spores in long chains)	100 50 10	- - -	0 0 0	452±0 452±0 452±0	- - -	0 0 0	388±0 388±0 388±0
(M296) soil	Large and small bipyramidal	100 50 10	- + -	0 20.3±0.8 0	452±0 68±3.4 452±0	- + -	0 18.6±0.8 0	388±0 101±4.4 388±0
(M300) Rain W	Dark and bright ovoid (attached to spore)	100 50 10	+ - -	24±0.5 0 0	18±0.4 452±0 452±0	+ - -	23.3±0.8 0 0	27±0.9 388±0 388±0
A11 soil	Spherical non-spore attached	100 50 10	+ + -	16.6±1.2 13±1.7 0	154±11.2 217±29.7 452±0	+ + -	19±0.5 15.6±0.8 0	93±2.5 147±7.6 388±0

+: Positive quenching effect, -: Negative quenching effect, β-Galactosidase (MU): enzyme activity calculated as Miller units (MU), AT = trypsin-activated toxin.

3.2. PCR amplification of *Cyt1* gene

To assess potential Cyt toxin production, we conducted PCR amplification of the universal *Cyt1*UN genes to screen for their presence in the five strains under investigation, namely: M11, M78, M154t, M91, and A11. Results indicated that while four out of five strains were negative for *Cyt1*, isolate M91 exhibited a PCR product of approximately 400 bp, indicating the presence of *Cyt1* genes. The product size for *Cyt1* was confirmed by an identical amplicon of the same size as the *Bt*i H14 positive control, which confirms the validity of the assay (Figure 3).

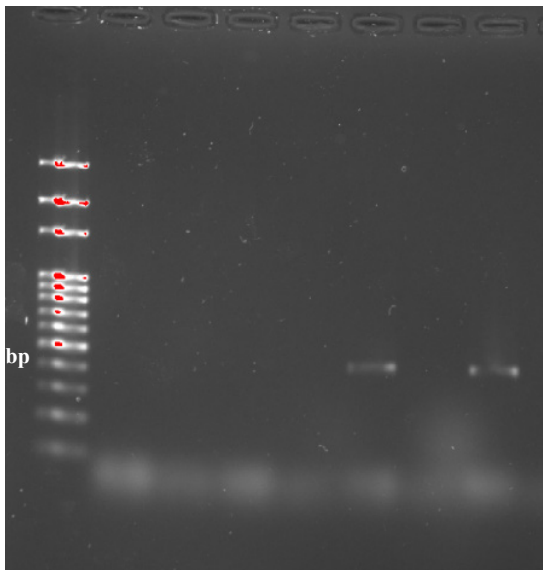

Figure 3. PCR screening of *Bt* isolates for *Cyt1* genes. Amplicons were resolved on 1.5% agarose gels containing ethidium bromide at 80 V for 40 min. Lane (L) contains the 1500 bp DNA standard marker for band size identification. Four *Bt* strains were negative for *Cyt1* genes, except for isolate M91. The reference strain *Bacillus thuringiensis* subsp. *israelensis* (*Bti* H14) and deionized water were included as positive and negative controls, respectively.

3.3. SDS-PAGE of parasporal crystal proteins (PCPs)

SDS-PAGE analysis of activated and unactivated parasporal crystal proteins was conducted to highlight differences in solubilized PC proteins derived from the *Bt* isolates under investigation. As expected, a clear protein band profile was obtained, showing a distinct pattern for the unactivated PC proteins (PT) compared with the activated PC form (AT). Two strains, M11 and M78, were investigated by SDS-PAGE as representa-

tives of all *Bt* strains under investigation (Figure 4). A protein band of about 28 kDa was consistently observed in the AT lanes of all five isolates exhibiting antivirulence activity (*i.e.*, M11, M78, M91, M154t1, and A11; Figure 4). This suggests that it may be an active toxin fragment that should be purified and identified in the future.

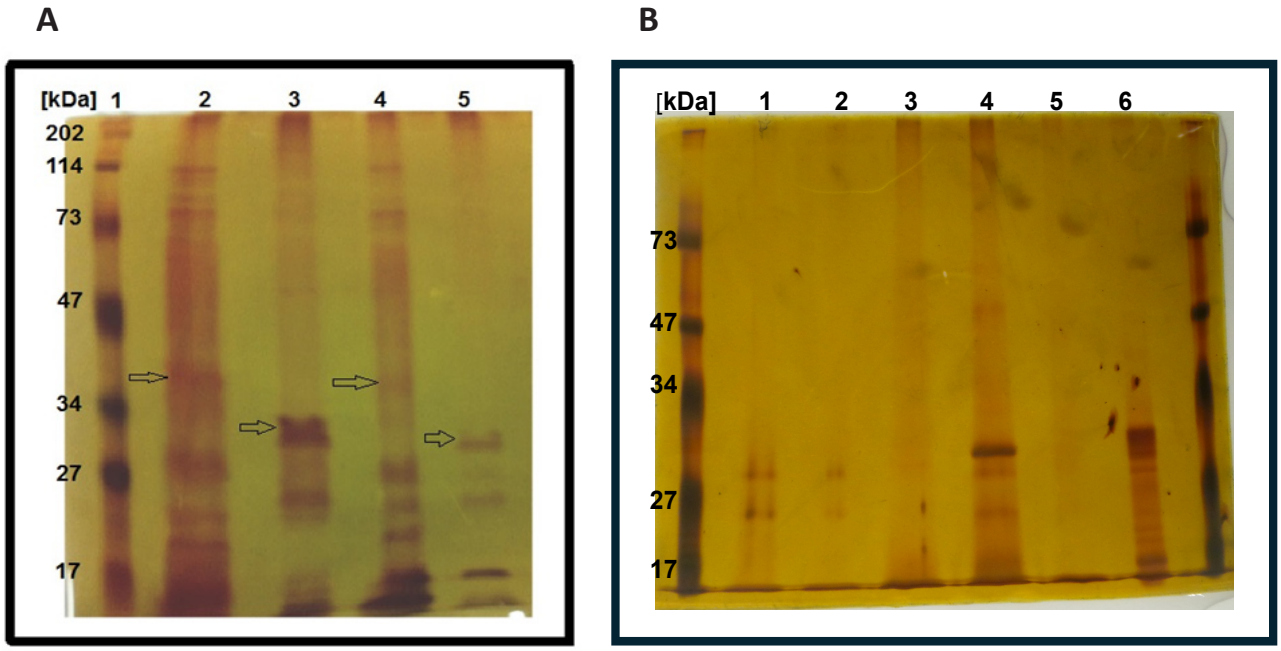

Figure 4. Protein profiles of unactivated (PT) and activated (AT) parasporal crystal proteins (PCPs). Samples from strains M11, M78, M91, M154t, and A11 were run on a 12% SDS-PAGE gel and silver-stained to visualize protein bands. (A): M11 and M78. Lane 1, molecular marker; Lane 2: M11 strain, unactivated PC proteins loaded 100µl (100µg), bands at 111 kDa, 82 kDa, 58 kDa, 35 kDa, 27 kDa, 24 kDa, and 21 kDa; Lane 3, M11 strain, activated PC proteins (trypsinized) loaded 100µl (60µg/ml), bands at 28 kDa, 24 kDa, and 17 kDa; Lane 4: M78, unactivated PC proteins loaded 100µl (100µg), bands at 111 kDa, 82 kDa, 58 kDa, 35 kDa, 27 kDa, and 21 kDa; Lane 5, M78 strain, activated PC proteins (trypsinized) loaded 100µl (30µg), bands at 28 kDa, 24 kDa, and 17 kDa. (B): M91, M154t, and A11. Lane 1: M91 strain, unactivated PC protein, loaded 100µl (20µg), bands at 28 and 26 kDa; Lane 2: M91 activated PC proteins (trypsinized) loaded 100µl (20µg/ml), very faint bands at 28 and 26 kDa; Lane 3: M154t activated PC proteins (trypsinized) loaded 100µl (50µg/ml), faint bands at 28 and 26 kDa. Lane 4: M154t unactivated PC proteins loaded 100µl (50µg), bands at 30, 28, and 26 kDa; Lane 5: A11 activated PC proteins (trypsinized) loaded 100µl (50µg), faint bands at 28 and 26 kDa. Lane 6: A11 unactivated PC proteins loaded 100µl (50µg), bands at 32, 30, 28, 26, 24, 22, and 17 kDa.

3.4. *Bt*-derived AT proteins modulate the expression of virulence genes of *S. aureus*

To assess the effect of *Bt*-derived AT proteins on the modulation of virulence genes in *S. aureus*, we employed bleaching zone (X-gal) and β-galactosidase assays as qualitative and quantitative methods, respectively. We used two reporter *S. aureus* fusion strains (*i.e.*, PC203, *spa::lacZ* and PC322, *hla::lacZ*) to screen for the dose-dependent transcriptional quenching effect of AT derived from the five non-insecticidal *Bt* isolates profiled by SDS-PAGE (Figure 3) at three concentrations (10, 50, and 100 µg/mL). Representative results of the quenching effect of AT derived from *Bt* isolate M91 at the three concentrations are shown (Figure 5A and 4B). In parallel, three controls, namely C1: saline, C2: alkaline buffer, and C3: trypsin, were used to validate the qualitative and quantitative quenching effects of the tested ATs from the five screened *Bt* isolates.

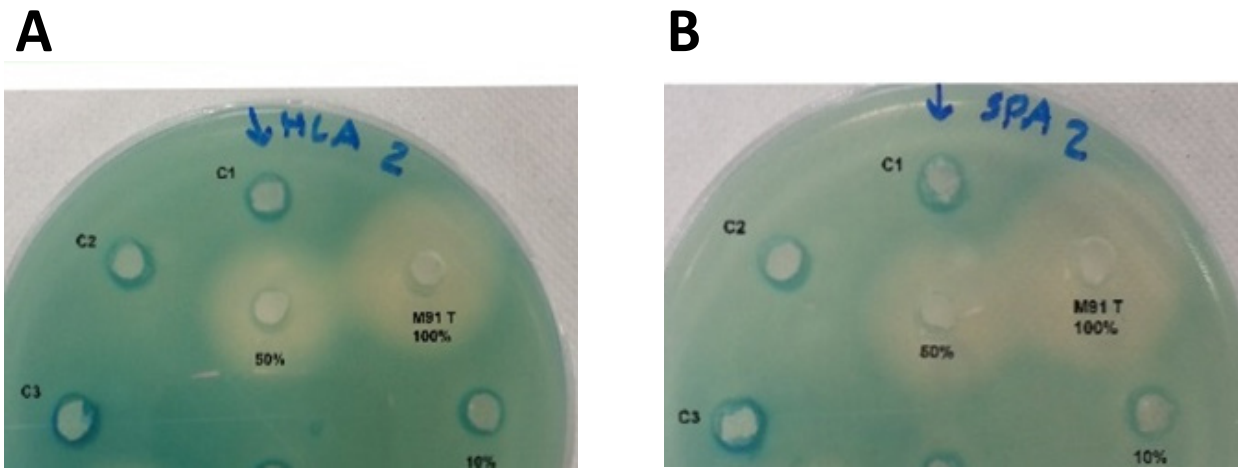

Figure 5. Representative results showing the transcriptional effects of *the Bt* (M91) isolate AT (activated toxin, undiluted; M91 T (100%); and 50%-diluted AT) on *S. aureus* virulence genes (A) *hla* and (B) *spa*. To agar plates containing PC322 (*hla::lacZ*) (A) or PC203 (*spa::lacZ*) (B), 50 µL of *Bt* AT at the indicated concentrations [(100% (100 µg); 50% (50 µg); 10% (10 µg)] was added. Plates were incubated for 24 h at 37 °C. Controls: C1: saline; C2: alkaline buffer; C3: trypsin.

Data in Table 2 indicate that the AT toxins of nine Bt isolates (M11, M13m, M78, M91, M154t1, M157, M160, M224, and A11 at 100 and 50 µg/mL) produced dose-dependent reductions in transcription of the hla and spa genes in both strains. In contrast, AT toxins from Bt isolate M268P, at the three concentrations used (100, 50, and 10 µg/mL) (Table 2), showed no suppression of transcription of both hla and spa genes, reflecting the specificity of AT toxins. Likewise, none of the 14 Bt isolates tested exhibited changes in transcription of the two S. aureus genes (hla and spa) at the lowest concentration of 10 µg/mL, again reflecting a dose-dependent response. AT toxins from the M67t and M296 isolates, however, exhibited reduced mRNA transcription only at the second-highest dose of 50 µg/mL, but not at the lowest dose of 10 µg/mL, as expected (Table 2). These findings indicate that certain trypsin-activated Bt PCP (AT) toxins suppress transcription of both hla and spa genes; this suppression is dose-dependent and specific to the PCS protein type of each respective Bt isolate. This hypothesis remains to be further verified.

To confirm the qualitative findings from the plate-based reporter assay and mitigate potential issues related to diffusion and precipitation artifacts, we conducted quantitative β-galactosidase assays in liquid culture using sub-inhibitory concentrations (10, 50, and 100 µg/mL) of AT PCPs derived from all 14 native *B. thuringiensis* isolates. Table 2 shows β-galactosidase activity (in Miller units) for the five active antivirulence isolates: M11, M78, M91, M154t1, and A11. All five isolates dramatically decreased *spa* and *hla* expression in a dose-dependent manner. For instance, M78 AT at 100 µg/mL decreased *hla* and *spa* expression by 82.3% and 84.0%, respectively. M11 and M91 ATs also exhibited substantial repression at 100 µg/mL, lowering *hla* expression by 73.1% and 73.5%, respectively, and *spa* expression by 78.9% and 73.8%. For the other nine isolates, which showed different or no activity in the plate assay, the quantitative β-galactosidase results matched their qualitative profiles. Isolates M242 and M300, which had large bleaching zones in the plate test, also exhibited substantial suppression in liquid culture (M300: 93% reduction in *hla*; 96% in *spa* at 100 µg/mL). Isolates M268P and M296, which had no zones at 100 µg/mL, did not show a substantial drop in β-galactosidase activity.

3.5. Normalized biofilm index reveals genuine anti-biofilm activity *of Bt*-derived AT proteins

To test the effects of diluted (D=50 µg/mL) and undiluted (UD=100 µg/mL) *Bt* AT and PT on biofilm formation in three *S. aureus* strains, including a MRSA strain, wells of 96-well microtiter plates were filled with 200 µl of diluted cultures containing AT, PT, or saline, in triplicate. Two plates were prepared: one for growth assessment and the other for biofilm formation. *S. aureus* PC203 (spa::lacZ) and PC322 (hla::lacZ), as well as MRSA (ATCC 43300), were randomly assigned to the

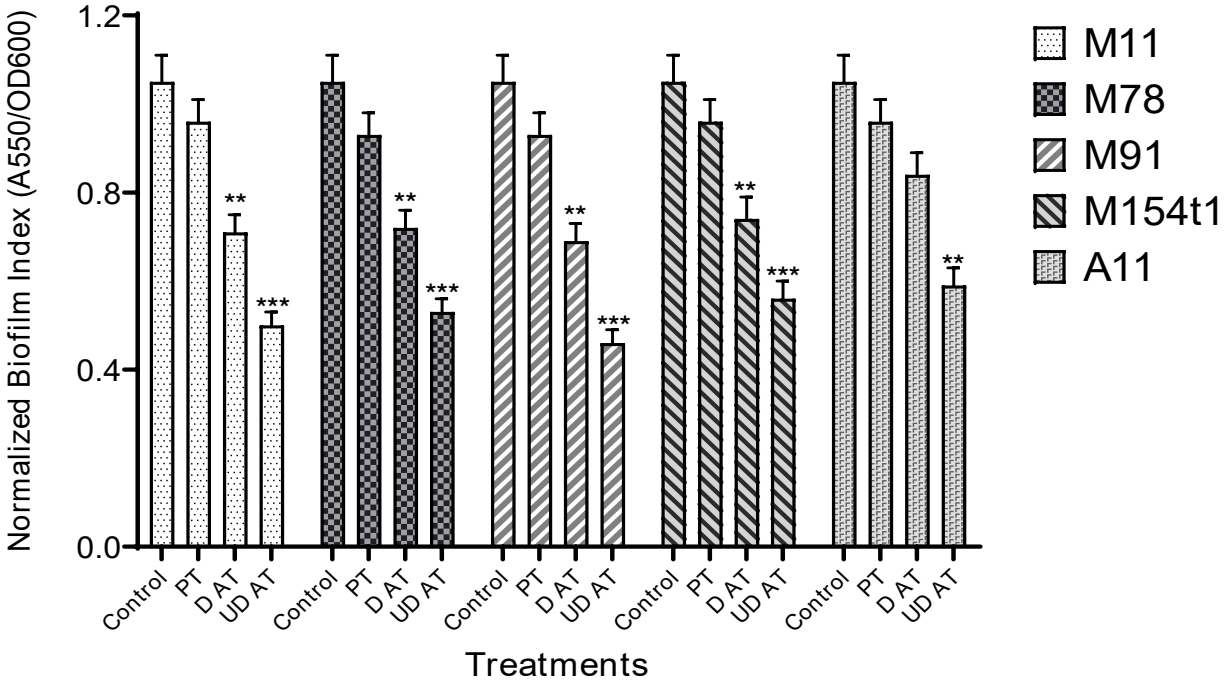

Figure 6. The effect of *Bt*-derived PCPs either AT (diluted, D or undiluted, UD) or PT on *S. aureus* normalized biofilm index (NBI), compared to saline control. Biofilm formation was determined by an OD550 nm reading of crystal violet stain solubilized by ethanol with saline treatment as a control. NBI was calculated by dividing A550 over OD600. NBI for the isolate M11 was tested on PC322 (*hla::lacZ*). Data are presented as the mean ± standard error of means (SEMs). One-way ANOVA was conducted followed by Tukey's multiple comparison test to compare the mean of each treatment with the control. *p<0.05, **p<0.01, ***p<0.001. NBI for the isolate M78 was tested on PC322 (*hla::lacZ*). NBI for the isolate M91 was tested on PC203 (*spa::lacZ*) biofilm formation. NBI for the isolate M154t1 was tested on PC203 (*spa::lacZ*). NBI for the of *Bt* A11 isolate was tested on MRSA (ATCC 43300). Data points are the average of two independent growth experiments and three technical replicates.

five selected *Bt* isolates (M11, M78, M91, M154t1, and A11) to highlight a wide spectrum of AT and PT effects on biofilm formation across different *S. aureus* strains. To account for possible growth-inhibitory effects, biofilm data were standardized to planktonic growth (OD600) and expressed as the Normalized Biofilm Index (NBI). Figure 6 shows that the NBI values for all PT-treated samples were highly similar to those of controls (NBI = 0.93–0.96, $p > 0.05$). This indicates that the protoxin itself had no specific antibiofilm activity. Conversely, UD AT treatments markedly reduced NBI in all *S. aureus* strains. For example, M91 UD AT lowered NBI from 1.05 ± 0.06 (control) to 0.46 ± 0.03 ($p < 0.001$). Similarly, M11 and M78 UD AT lowered NBI to 0.50 ± 0.03 and 0.53 ± 0.03, respectively ($p < 0.001$). Moreover, D AT treatments showed a moderate yet statistically significant reduction in NBI (e.g., M154t1 D AT: 0.74 ± 0.05, $p < 0.01$). These results corroborate that activated PCPs (AT) interfere with biofilm formation independently of bacterial growth suppression.

3.6. The hemolytic activity of native *Bt* isolates

To assess potential Cyt toxin production, the five *Bt* strains selected for antiviral evaluation were streaked onto sheep blood agar. Notably, none of the tested strains exhibited α- or β-hemolysis after 48 hours of incubation (Table 3), indicating that either they lack the capacity to produce cytolytic toxins or the conditions used did not permit their generation.

Table 3. Hemolytic activity of native *Bt* isolates on sheep blood agar.

***Bt* isolate**	**Type of hemolysis**	**Hemolytic activity**
Positive Control (*S. aureus* ATCC 25923)	β	+
M11	γ	-
M78	γ	-
M91	γ	-
M154t1	γ	-
A11	γ	-

+ = hemolytic, - = non-hemolytic

3.7. MIC results

MIC tests showed that none of the *Bt* PCP formulations were bactericidal at levels up to 100 µg/mL. This demonstrates that the antivirulence and antibiofilm activities reported at ≤100 µg/mL are not attributable to growth inhibition.

3.8. MTT toxicity against HeLa cells

The cytotoxicity of AT PCPs on immortal mammalian cells was evaluated using HeLa cells. The colorimetric MTT viability assay was used to assess the toxic potential of AT PCPs derived from four *Bt* strains: M11, M78, M91, M154t1, and A11. AT PCPs were serially diluted twofold, with concentrations ranging from 200 to 6.25 µg/mL. The results were then compared with cell viability in the DMSO control (Figure 7). The cell viability graph indicated that AT PCPs exhibited no significant cytotoxicity at concentrations up to 100 µg/mL. However, at the highest concentration of 200 µM, they exhibited significant cytotoxicity toward HeLa cells, reducing cell viability by approximately 30% across all five strains. These results clearly support the safety profile of the concentrations used, up to 100 µg/mL, in mammalian cells.

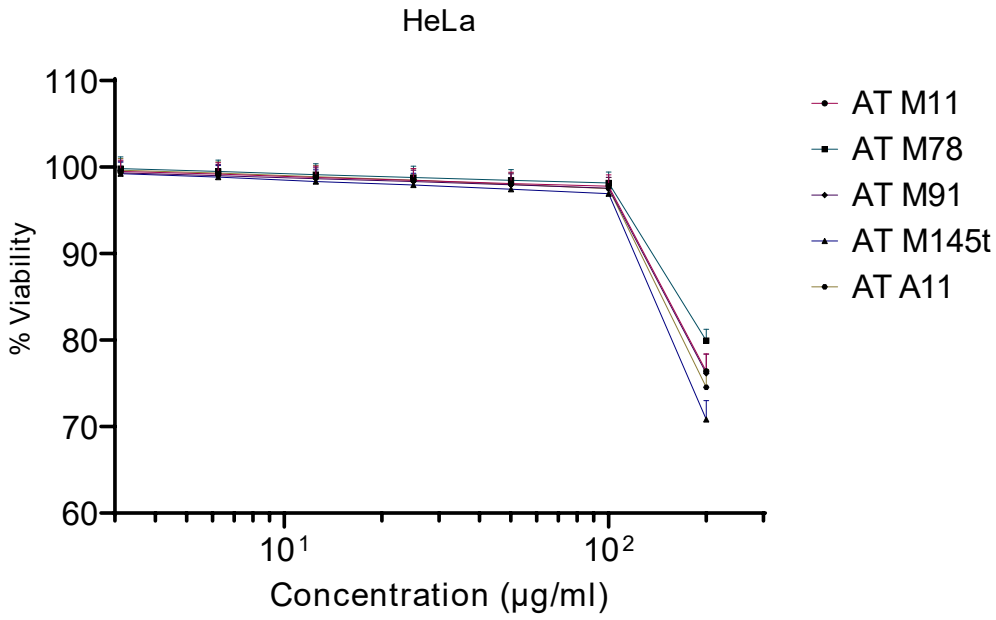

Figure 7. MTT toxicity evaluation of AT PCPs derived from five *Bt* isolates against HeLa cervical cancer cells. Cell viability was normalized to the DMSO-treated control, whose final concentration didn't exceed 2%. Yellow MTT is enzymatically reduced by cellular dehydrogenases to form purple formazan crystals. Absorbance was measured at 570 nm, and background was subtracted by deducting the absorbance at 630 nm. Data represent means±SD of two experiments with 8 replicates for each concentration.

3.9. Bt AT inhibits the transcription of *spa*, *hla* and *RNAIII* in *S. aureus*

The virulence of *S. aureus* is governed by cell wall-associated proteins and secreted toxins, which are regulated and expressed in accordance with its growth environment and/or growth phase. *Bt* A11 was not included in qPCR due to sample limitations but showed strong antibiofilm activity in normalized assays (Figure 5). Therefore, the effect of a dose of 100 µg/ml of PCPs AT toxins derived from four *Bt* isolates (M11, M78, M91 and M154t1) on the transcriptional expression of *hla, spa and RNAIII* genes in *S. aureus* was investigated at mid-logarithmic phase and at stationary phase of growth by quantitative reverse transcription PCR (RT-qPCR). The expression levels of these genes were normalized using 16S rRNA for these *Bt* isolates as an internal standard. A positive reaction was detected by the accumulation of SYBR Green I fluorescence. For statistical analysis to obtain the mean ± standard error, the mean was calculated after the $2^{-\Delta\Delta Ct}$ transformation was performed, and data were represented in the form of the $\log_2$ fold change ($\log_2$FC), relative to the DMSO control (Figure 8).

3.9.1. *spa* gene expression

During the mid-log phase, all four *Bt* isolates markedly reduced *spa* gene expression in *S. aureus* MSSA (ATCC 29213), which encodes surface and secreted proteins involved in aggregation. The greatest suppression was observed with M154t1 ($log_2FC = -3.94 \pm 0.26$, $p < 0.001$), followed by M11 and M78 ($log_2FC = -3.52$, $p < 0.01$), and then M91 ($log_2FC = -2.35 \pm 0.22$, $p = 0.005$). In the stationary phase, however, regulatory effects differed: M78 markedly up-regulated spa ($log_2FC = +2.91 \pm 0.20$, $p = 0.003$), whereas M91 and M154t1 maintained significant down-regulation ($log_2FC = -2.00$ and -1.00, respectively, $p < 0.05$). M11 showed a substantial, non-significant decrease ($log_2FC = -0.68 \pm 0.17$, $p = 0.081$). These phase-dependent changes suggest that *Bt* PCPs might interact with the *spa* regulatory network differently across growth phases.

3.9.2. *hla* gene expression

Modulation of *hla* expression was significantly strain- and phase-specific. At the midpoint of the log phase, M91 caused a large increase in *hla* ($log_2FC = +3.17 \pm 0.22$, $p = 0.004$), whereas M78 and M154t1 caused decreases ($log_2FC = -1.74 \pm 0.67$, $p = 0.029$ and -0.32 ± 0.28, $p = 0.420$, respectively). M11 also increased *hla* ($log_2FC = +1.93 \pm 0.21$, $p = 0.002$). During the stationary phase, M11 and M78 significantly down-regulated *hla* ($log_2FC = -2.00 \pm 0.37$, $p = 0.015$ and -1.59 ± 0.29, $p = 0.010$), whereas M91 and M154t1 showed no significant change. These findings suggest that specific *Bt* PCPs can either activate or inhibit *hla*, potentially circumventing canonical *agr*-mediated control.

3.9.3. *RNAIII* gene expression

Consistent with the *spa* gene expression pattern, all four isolates significantly inhibited *RNAIII* expression during the mid-log phase with log_2FC values ranging from −4.11 (M154t1) to −4.88 (M91) ($p < 0.001$). At the stationary phase, M91 and M154t1 exhibited significant down-regulation ($log_2FC = -1.18 \pm 0.17$, $p = 0.003$ and -1.74 ± 0.41, $p < 0.001$, respectively), whereas M78 showed a modest, non-significant up-regulation ($log_2FC = +0.36 \pm 0.16$, $p = 0.160$). M11 showed no significant difference relative to the control ($log_2FC = -0.06 \pm 0.16$, $p = 0.850$). The consistent reduction in *RNAIII* across isolates during log-phase growth suggests that *Bt* PCPs may disrupt the *agr* quorum-sensing network.

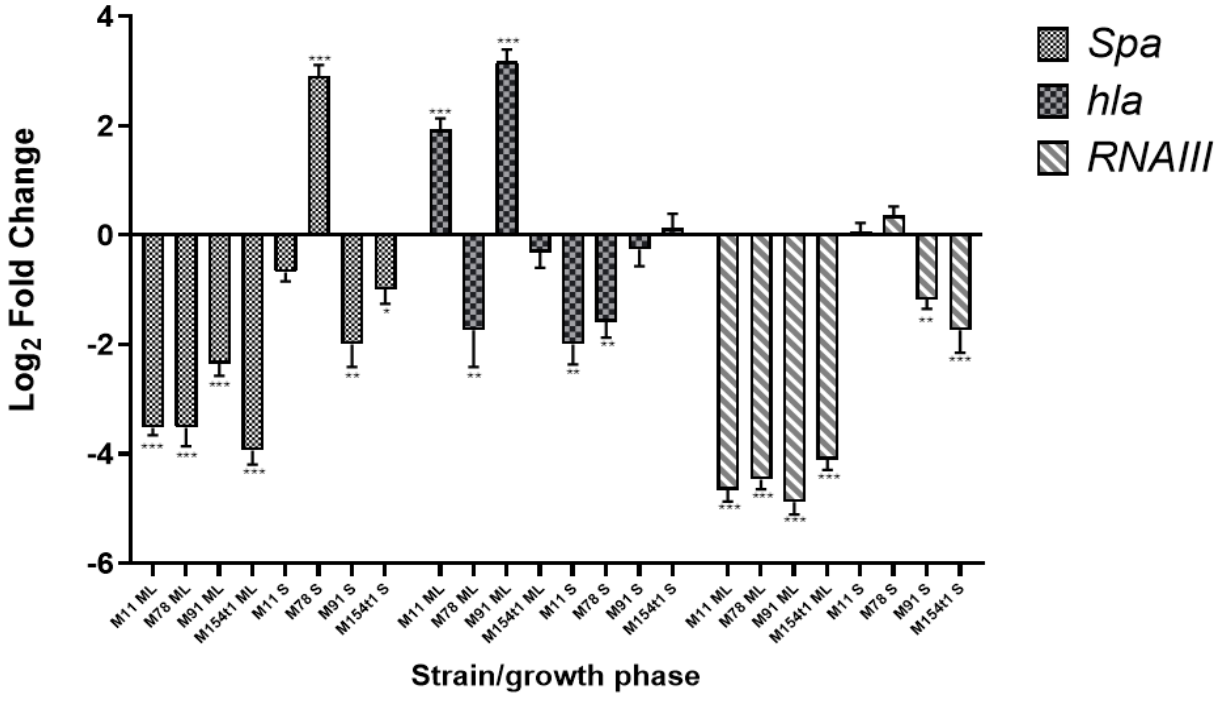

Figure 8. Quantitative RT-PCR expression profiling of S. aureus MSSA (ATCC 29213) spa, hla, and RNAIII genes. S. aureus was treated with a single concentration of 100 μg/ml of PCPs AT toxins derived from M11, M78, M91, and M154t1Bt isolates during the mid-log phase (ML-) or stationary phase (S). DMSO served as the negative control (basal expression level). Data are presented as log2 fold change, with mean ± SEMs from two independent experiments and three technical replicates, calculated by 2-ΔΔCT. *p<0.05, **p<0.01, and ***p<0.001.

4. Discussion

In recent years, antibiotic resistance has become a major threat to public health (Turner et al. 2019). The overuse of conventional antibiotics has led to the increased emergence of multidrug-resistant bacteria (O'Neill et al. 2014; Kim 2019; Ahmad-Mansour et al. 2021; Allen et al. 2014; Lin et al. 2015; Ivanova et al. 2013). Specifically, for S. aureus, which is responsible for worldwide outbreaks of nosocomial infections, the emergence of multidrug-resistant strains poses a threat to the global population; therefore, new strategies are required to disarm pathogenic organisms and expand our existing arsenal of drugs against infectious disease. As an alternative strategy, targeting virulence factors without killing the pathogens has gained increasing attention in recent literature (Cheung et al. 2021; Kurlenda et al. 2012; Kim 2019; Ahmad-Mansour et al. 2021; Gill et al. 2015; Khodaverdian et al. 2013; Nielsen et al. 2012; Wang et al. 2015). The main objective of this study was to screen and analyze the in vitro effects of natural δ-endotoxins (PCPs) isolated from Saudi Arabian native non-insecticidal *Bt* isolates, with (AT) and without prior trypsin activation (PT), on the production of selected *S. aureus* virulence factors. To the best of our knowledge, this is the first study to report the anti-virulence properties of alkaline-solubilized and trypsin-activated PCPs derived from native non-insecticidal Saudi Arabian *Bt* isolates against *S. aureus* strains. In the present study, 14 documented non-insecticidal *Bt* isolates were selected, and their PCP toxins were acetone/lactose co-precipitated (Priest et al. 2004), then alkaline-solubilized and trypsin-activated *in vitro*. This was followed by qualitative and quantitative profiling of *hla* (α-hemolysin) and *spa* (protein A) gene expression using a reporter fusion assay, as detailed in the methods section. In an X-gal-based bleaching zone inhibition assay, ATs from the 14 tested *Bt* strains showed significant, dose-dependent down-regulation of *hla* and spa mRNA expression (Table 2). In addition to the plate-based reporter assay, quantitative β-galactosidase measurements in liquid culture confirmed that *Bt*-derived AT proteins repress *spa* and *hla* transcription in a dose-dependent manner (Table 2). These results mitigate concerns about potential artifacts in the agar diffusion assay and reinforce the conclusion that *Bt*-derived PCPs modulate virulence gene expression independently of bacterial killing. Notably, the effect of *Bt* PCPs on *S. aureus hla* and *spa* mRNA expression is further complicated by the fact that both genes are regulated by a complex network that includes *agr* and additional elements such as *sar*, *sea*, and *srr* (LaSarre and Federle 2013). Because *agr* decreases spa expression and increases *hla* expression during the transition from late-exponential growth to stationary phase, the observed decrease in both *spa* and *hla* suggests that our activated *Bt* AT (PCPs) affect virulence gene expression independently of *agr* (Nielsen et al. 2014; Nielsen et al. 2012a; Yarwood et al. 2003). The present findings resembled those previously reported by Nielsen and co-workers (Porcar and Juárez-Pérez 2003), who found that glucose (a fast-utilizable sugar, a catabolite) markedly reduced the expression of both hla and spa genes. Recently, it has been demonstrated that the catabolite control protein A (CcpA) regulates *S. aureus* biofilm formation by directly repressing staphylokinase expression, and the multifaceted roles and multiple networks involving CcpA have been postulated (Kalia 2013). The authors suggested using staphylokinase for both vascular occlusion and *S. aureus*-associated biofilm infections. Additionally, agrA, which positively regulates the expression of hla, was significantly inhibited in *S. aureus* after treatment with a sub-MIC of allicin (a derivative of garlic). Consequently, the mode of action (MOA) by which allicin reduces α-toxin production may, in part, involve inhibition of the agr regulatory system (Tong et al. 2015). In a similar study, nigribactin, a novel siderophore from *Vibrio nigripulchritudo*, and Solonamide B, a cyclo-depsipeptide isolated from *Photobacterium halotolerans*, reduced hla expression while increasing spa expression, indicating that they interfere with activation of the agr quorum sensing (QS) system (Nielsen et al. 2014; Nielsen et al. 2012a).

Bacterial biofilms are complex microenvironments composed of single or mixed species. These species adhere to one another on biotic or abiotic surfaces, adopting a multicellular lifestyle encased in extracellular polymeric substances. Key characteristics of biofilm-forming bacteria include resistance to host defenses and tolerance of antimicrobials. It has been estimated that 65% to 80% of bacterial infections in humans are due to biofilm formation (Kalia 2013). The present results revealed that, unlike ATs, biofilm formation by the tested *S. aureus* strains was unaffected by the non-activated PTs of all five *Bt* isolates tested (M11, M78, M91, M154t1, and A11). By contrast, these PTs exhibited increased *S. aureus* growth. This enhancement of bacterial growth is presumed to result from the auto-proteolysis of PT proteins by *S. aureus* intrin-

sic proteases, which in turn further enhances *S. aureus* growth. These findings are consistent with those previously reported by Beena and co-workers (Koo et al. 2017) for activated parasporins derived from Bt. The authors found that none of the activated PCPs (PT) of the non-insecticidal *Bt* KAU59 was toxic to Jurkat cells, a human lymphocytic leukemic cancer cell line, and also increased cell viability. The authors considered this finding evidence for the effectiveness of proteolytic activation of *Bt*-KAU59 PCPs by proteinase K, which thereby resulted in cytotoxicity against Jurkat cells at the selected dose (Koo et al. 2017). The absence of NBI decrease in PT-treated samples, even when growth increased in some cases, underscores the essential role of trypsin activation in antibiofilm efficacy. Notably, when biofilm data were normalized to planktonic growth (NBI), the antibiofilm action of AT proteins remained substantial, indicating that biofilm breakdown was not merely a consequence of growth suppression. This aligns with earlier studies showing that natural products, such as honey, at low concentrations can target biofilm matrix components without killing bacteria (Paharik and Horswill 2016). Additionally, in the present study, M91 was PCR profiled and confirmed to contain the Cyt1 gene. However, reductions in biofilm formation by *S. aureus* spa (PC203) were observed that were independent of growth inhibition, suggesting that this isolate, despite having the Cyt1 gene, might not be active under our current experimental conditions. The absence of the Cyt1 gene in four of five active isolates (M11, M78, M154t1, and A11) suggests that Cyt toxins do not contribute to their antivirulence efficacy. Although M91 tested positive for Cyt1, it did not show hemolytic activity on sheep blood agar. This indicates that the gene may be nonfunctional or that the Cyt protein it encodes does not function under the tested conditions. These findings suggest that future studies on the antivirulence of Bt PCPs (ATs) should begin with fully pure toxins. The cyt toxins are well known as non-receptor-specific, general pore-forming cytolytic (hemolytic) toxins that act in vertebrate and invertebrate species as well as in Gram-positive and Gram-negative bacteria (Palma et al. 2014; Cahan 2008). In contrast, 50 µg/mL ATs from both Bt-M78 and *Bt* M154t1 reduced biofilm formation and increased the growth yield of *S. aureus spa* (PC203). In a similar study, Norwegian forest honey (Medihoney™), Manuka honey, and one Yemeni honey reduced the growth rate of planktonic cultures of MRSA, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* in a concentration-dependent manner and inhibited both biofilm formation and established biofilms (Paharik and Horswill 2016). As an explanation for *P. aeruginosa* biofilm inhibition, it has been proposed that fructose in honey samples blocks PA-IIL lectin-mediated binding (Conlon et al. 2013). In the current study, the AT of Bt strains M78, M91, and M154t1 showed reduced biofilm formation and increased growth yield. Such growth enhancement suggests that, although AT decreased biofilm formation, many complex substances (proteins, DNA, and polysaccharides) were retained to support better growth (García-Contreras et al. 2016). This suggestion is well-substantiated, as sub-inhibitory antibiotic concentrations that inhibit protein synthesis usually decrease biofilm formation, whereas those that inhibit (wane) cell wall synthesis augment biofilm formation (Hoiby et al. 2010). However, several of the transcriptional patterns observed here, such as the simultaneous downregulation of *RNAIII* and biofilm formation, or the overexpression of *hla* with repression of *RNAIII*, don't align with the typical *S. aureus* regulatory logic. Although we hypothesize that these effects may engage other pathways (e.g., SaeRS activation or SarA/SarS inhibition), these ideas have not been verified in the current investigation. The intricate relationship between virulence regulation and biofilm dynamics in *S. aureus* suggests that *Bt* PCPs may interact with multiple regulatory nodes, a hypothesis that warrants focused examination in subsequent research. Interestingly, AT of Bt isolate A11 demonstrated a reduction in biofilm formation while S. aureus growth was not affected. Therefore, it is concluded that AT in Bt isolate A11 is considered a realistic model for biofilm inhibition. Our native *Bt* isolates did not exhibit hemolytic activity on blood agar, indicating that our PCP preparations do not contain significant quantities of Cyt toxins. This corroborates the assertion that the antiviral and antibiofilm effects are mediated by Cry-like proteins rather than by nonspecific cytolysis. In a similar study, two chemical compounds, CCG-203592 and CCG-205363, were reported to consistently inhibit *S. aureus* biofilm formation without affecting bacterial growth (Al-Mebairik et al. 2016). The authors stated that these inhibitions result from interference by the chemical compounds (CCG-203592 and CCG-205363) with the expression of genes involved in biofilm formation, including icaADBC, dltABCD, spa, sdrD, hla, and RNAIII. Additionally, researchers found that the fungal derivative apicidin attenuates MRSA

virulence by inhibiting QS and enhancing host defense (Jamal et al. 2018). The same authors also proposed the clinical application of apicidin to promote immune clearance of the pathogen and block disease progression. It was demonstrated that virulence gene expression in *S. aureus* is controlled and coordinated by specific, sensitive cascade devices, primarily at the transcriptional level, rather than by translational and/or post-translational controls (Qvortrup et al. 2019). In the current study, three genes (*spa, hla*, and *RNAIII*) were selected to evaluate their susceptibility to inhibition of gene expression by a single dose of 100 µg/mL ATs from four Bt isolates, namely M11, M78, M91, and M154t1, using RT-qPCR. We observed profound alterations in the expression of virulence genes under investigation. To rule out the possibility that the observed antivirulence effect is merely due to a bactericidal effect, we conducted an MIC assay. AT proteins from the selected four strains exhibited no antibacterial activity (MIC >100 µg/mL) against MRSA (ATCC 43300) at the concentrations used in this study (25, 50, and 100 µg/mL), confirming that the profound alterations in virulence gene expression are a direct result of anti-virulence targeting and not a secondary effect of growth inhibition or cellular toxicity. In *S. aureus*, the *spa* gene encodes protein A, which is known for its ability to bind to the FC region of human Igs and inhibit opsono-phagocytosis. In addition, SPA protein A promotes cell-to-cell interactions and biofilm formation (Rabin et al. 2015). This gene is expressed during the logarithmic phase of growth and is downregulated as cells progress into the post-exponential phase. Regulation of *spa* expression has been found to be complex, involving multiple factors, including the positive regulators SarA and SarS, as well as the *agr* QS systems (Cheung et al. 2008; Gao et al. 2004). The present results showed that, except for AT Bt-M78, all tested ATs from *Bt* M11, M91, and M154t1 isolates at a concentration of 100 µg/mL downregulated spa gene expression in both growth phases evaluated. By contrast, 100 µg/mL AT from the Bt-M78 isolate decreased spa expression during the ML growth phase and increased it during the S phase. Thus, all tested *Bt* isolates are efficient at inhibiting spa expression, and these findings confirm our present results from both the reporter fusion assay and the biofilm assay). Nevertheless, with the current data, it remains unknown whether *agr, sar*, or other regulators are involved. Therefore, we presume that the reduction in spa expression may, in part, depend on inhibition of *sarA* and *sarS,* which are induced by ATs in tested *Bt* PCPs. Among the virulence factors contributing to the success of *S. aureus* infection is the expression of α-hemolysin (hla gene). *hla* gene expression is promoted by the *agr* QS system. On the contrary, the *agr* QS system down-regulates *hla* expression during the logarithmic phase of growth and up-regulates gene expression during the post-exponential phase of growth (Vestergaard et al. 2016). This reciprocal regulation facilitates the progression of an infection from the preliminary stages, when staphylococcal surface proteins are required to promote host tissue colonization, to the later stages, when exotoxins are required to combat host immune defenses (Qvortrup et al. 2019). In the present study, ATs from the *Bt* M78 and *Bt* M154t1 isolates both showed downregulation of *hla* gene expression across all tested growth phases. ATs from the Bt-M11 and Bt-M91 isolates showed upregulation of *hla* expression during the ML phase and downregulation during the S phase. However, inhibition of both *spa* and *hla* genes suggests that activated crystal proteins (ATs) affect virulence gene expression independently of *agr* (Cheung et al. 2021). Notably, M91 increased *hla* expression while suppressing *spa* and *RNAIII*, suggesting activation of the SaeRS system independent of *agr*. Similarly, M78 up-regulated *spa* in stationary phase despite *RNAIII* suppression, possibly *via agr*-independent enhancement of SarA/SarS. These patterns highlight the ability of *Bt* PCPs, such as those found in M91 and M78, to selectively perturb distinct nodes within the complex *S. aureus* regulatory network by bypassing or perhaps partially uncoupling *agr*-mediated control. In this context, and in support of our hypothesis, it has been reported that *hla* can be triggered independently of *agr* through the SaeRS and SigB systems in *S. aureus*, particularly under stressful conditions or in response to unfavorable stimuli, such as antimicrobial peptides (Montgomery et al. 2010).

The significant inhibition of *RNAIII* (the agr effector) alongside the down-regulation of *spa* suggests that M91 may predominantly affect the SarA/SarS regulators, which positively regulate *spa*, while preserving *hla* induction through SaeRS. Furthermore, *RNAIII* is the intracellular effector of the *agr* QS system that controls many virulence factors, including exoproteins and cell wall-associated proteins (Cheung et al. 2021; Chevalier et al. 2010). Therefore, any reduction in *RNAIII* transcription provides an additional route to block *S. aureus* virulence. In the present study, *RNAIII* was downregulated by *Bt* M78 and *Bt* M154t1

across the two growth phases evaluated, whereas isolate M91 showed reduced gene expression during the ML phase and induced expression during the S phase. The AT of *Bt* M11 downregulated *RNAIII* expression during the ML phase and showed no effect during the S phase. According to Cheung and co-workers (Cheung et al. 2021), *RNAIII*-dependent gene regulation occurs mostly via inhibition of a DNA-binding repressor protein (Rot). The extracellular QS signal of the *agr* system is a post-translationally modified, short thiolactone-containing autoinducing peptide (AIP) that requires prior cleavage for activation and recognition as a modification type. The blockage of such AIP activation has been shown to prevent *S. aureus*-induced abscess formation in mice (Baraniak et al. 2011). Likewise, the majority of non-self AIPs, such as those from other staphylococcal species or other bacteria, are inhibitory (Cheung et al. 2021). This would explain the observed inhibitory effects on the transcription of *S. aureus hla, spa*, and *RNAIII* at a concentration of 100 ug/ml of ATs isolated from the *Bt* PCPs tested. In Gram-negative bacteria, however, the most common QS auto-inducer is acyl-homoserine lactone (AHL), which is generated from S-adenosyl methionine by signaling pathway proteins analogous to LuxI or LuxR of *Vibrio fischeri*. Therefore, whole culture extracts of *Bacillus* species, including *B. thuringiensis* (Dong et al. 2004; El Aichar et al. 2022), which contains the enzyme lactonase, were proposed as anti-virulence agents for *Pseudomonas aeruginosa* and the tobacco plant pathogen *Erwinia carotovora*. This is attributed to the fact that such enzymes specifically inactivate AHL molecules, thereby inhibiting biofilm formation in the respective organisms. Our *in vitro* findings indicate that AT-PCPs from local Bt isolates attenuate virulence in *S. aureus*, suggesting they may serve as natural candidates for further exploration as antivirulence agents. Moreover, Bt is well recognized for its eco-friendliness and safety. Of interest are the enormous anti-virulence factors of natural origin that are intensely present in garlic extracts, essential oils, forest honey (Medihoney) (Paharik and Horswill 2016), whole culture extracts of various Bacillus species (Gutiérrez et al. 2017), or whole insecticidal *B. thuringiensis* co-cultured with the *E. carotovora* plant pathogen. Indeed, such approaches involve the use of non-specific, multi-active ingredients, including those with antibacterial activity. By contrast, our approach specifically utilizes ATs of Bt PCPs, which are well-recognized, target-specific proteins, are feasible for commercial production, cloning, and simple purification, thereby ultimately permitting effective dose-response assessments (Bravo et al. 2007).

5. Limitations and future directions

It is important to recognize that this study has several limitations. First, not all original isolates were available for complete analysis, which may affect reproducibility. Second, trypsin is commonly used to activate PCPs *in vitro*, but it doesn't fully recapitulate the protease environment present during infection. Insect midgut proteases naturally activate Cry proteins. However, it remains unclear how trypsin activation relates to human infection. Subsequent research should examine whether activation by host proteases, such as neutrophil elastase, cathepsins, or serum proteases, yields analogous antivirulence effects, thereby augmenting the translational significance of these results. Moreover, although trypsin-activated PCPs exhibit strong antiviral efficacy, their stability, immunogenicity, and potential cytotoxicity in mammalian systems need to be thoroughly examined before they can be incorporated into any therapeutic programs. Nonetheless, our results provide a fundamental proof of concept that native *Bt* PCPs, upon proteolytic activation, can interfere with critical virulence pathways in *S. aureus*, thereby warranting further mechanistic and preclinical exploration. Third, this study uses semi-purified PCP preparations, which are protein mixtures rather than purified toxins. While this approach enabled screening of native *Bt* isolates for antibiofilm formation and antivirulence activity against *S. aureus*, it precludes identification of the specific protein(s) responsible for the observed bioactivity. Future studies should include PCP protein purification, LC-MS/MS identification, cry gene sequencing, and recombinant expression to confirm the active moiety. The persistent ~28 kDa band in the SDS-PAGE profiles of active isolates suggests a viable candidate protein for subsequent purification and mechanistic studies. The present investigation uses semi-purified PCP mixtures; however, the consistent band across geographically diverse isolates indicates a conserved bioactive component, potentially an activated Cry or parasporin-like fragment. Fourth, the regulatory mechanisms underlying the observed gene expression patterns remain unresolved and would require transcriptional profiling of key regulators (e.g., *agrA, sarA, saeR)*. Future research

should incorporate qPCR analysis of essential regulators (i.e., *agrA, sarA, sarS, saeR,* and *sigB)* to accurately delineate the regulatory targets of each PCP. Fifth, all experiments were conducted *in vitro*; *in vivo* validation is needed to assess therapeutic potential. Sixth, not all original isolates remained available for complete analysis, which may affect reproducibility. Future investigations should focus on protein purification, mechanism-of-action mapping, and evaluation in infection models.

6. Conclusions

Our findings are promising and highlight the need for further evaluation of native trypsin-activated B. thuringiensis parasporal crystal proteins (AT-PCPs) against other clinically relevant pathogens and for detailed molecular characterization of Cry proteins to identify strains with broad-spectrum antivirulence potential. Results provide proof of concept that AT-PCPs can attenuate virulence and biofilm formation in *S. aureus*, supporting further investigation of their potential as adjuvants to conventional antimicrobials. In conclusion, the present study models the activated ATs of PCPs from the studied native non-insecticidal *B. thuringiensis* isolates (M11, M78, M91, M154t1, and A11) as novel natural biodegradable inhibitors of virulence-associated hla, spa, and RNAIII gene expression and biofilm formation in S. aureus. Although filtration (0.22 µm) removed spores and particulate matter, the PCP preparations used in this study remain semi-purified and likely contain a mixture of solubilized Cry and possibly Cyt proteins. However, the absence of hemolytic activity in our PCP preparations supports the notion that the observed antivirulence effects are not due to nonspecific cytolysis mediated by Cyt toxins. Future studies involving protein purification and LC-MS/MS identification are needed to delineate the exact bioactive component and fully correlate parasporal protein composition with bioactivity. Further study is required to identify the involved Cry-gene(s), determine their sequences, and clone, express, and purify the corresponding PCPs. Our RT-qPCR results indicate that *Bt* derived PCPs can influence virulence gene expression through mechanisms that may circumvent or reconfigure canonical *agr* systems, which may present new avenues for antivirulence therapies. However, it is important to emphasize that these findings are based on *in vitro* experiments with semi-purified protein mixtures, and the specific active component(s) and regulatory mechanisms remain unidentified. Future work should focus on (i) purifying and identifying the specific active component(s), (ii) elucidating the molecular mechanisms using regulator-focused assays, and (iii) evaluating efficacy and safety in relevant in vivo infection models. Despite these necessary next steps, our results provide a proof-of-concept foundation for exploring *Bt* parasporal proteins as a new class of antivirulence leads against antibiotic-resistant *S. aureus* and potentially other priority pathogens.

Data availability statement

The five key *Bt* isolates are archived in our laboratory and will be made available upon request.

Acknowledgment

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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DETECTION OF VIRULENCE GENES AND ABC GENOTYPES IN CANDIDA ALBICANS ISOLATES FROM CANDIDEMIA PATIENTS

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Abstract: Candidemia is the most common form of invasive fungal infection, and is associated with high mortality rates particularly in intensive care patients. In this study, 50 *C. albicans* strains isolated from blood samples were genotyped by 25S intron analysis, and the presence of virulence genes was investigated by PCR. Genotyping revealed that 28 isolates (56%) were genotype A, 14 (28%) genotype B, and eight (16%) genotype C. The *SAP1* gene was detected in 96% of isolates, *SAP2* and *SAP4* in 98%, *ALS1* and *HWP1* in 92%, and *PLB1* in 94%. A comprehensive understanding of *Candida* virulence factors, particularly in *C. albicans* which is the most prevalent *Candida* species acting as both a commensal organism and an opportunistic pathogen, is essential for improving diagnostic methods, and guiding the design of novel antifungal agents targeting virulence mechanisms.

Keywords: *Candida albicans,* Invasive candidiasis, Candidemia, ABC genotyping, Virulence genes.

1. Introduction

Invasive fungal infections pose a significant global health threat due to their high mortality rates and the recent increase in antifungal resistance. Candidemia is the most common form of invasive fungal infection, particularly affecting immunocompromised individuals and patients in intensive care units, where it is associated with high mortality rates (Shrivastava and Whiteway 2025). The high incidence in these patient groups is associated with risk factors such as the use of broad-spectrum antibiotics, chemotherapy, neutropenia, and invasive procedures (Dawoud et al. 2024). The epidemiology of candidemia varies considerably across geographic regions, even among healthcare facilities within the same country. In recent years, there has been a notable rise in cases caused by non-albicans species, including *Nakaseomyces glabratus* (formerly *Candida glabrata*), *Candida tropicalis*, *Candida parapsilosis*, and *Pichia kudriavzevii* (formerly *Candida krusei*). Furthermore, *Candidozyma* (formerly *Candida*) *auris* has emerged as an important cause of candidemia, gaining attention due to outbreaks in healthcare settings (Chavez et al. 2025). Nevertheless, *Candida albicans* remains the most prevalent species, both as a member of the microbiota and as an infectious agent (Shrivastava and Whiteway 2025).

In the development of candidemia, both host immune factors and the virulence attributes of the causative species play a crucial role (Dawoud et al. 2024). *C. albicans* possesses several major virulence factors, including adhesion, phenotypic switching, hyphal formation, biofilm production, and the secretion of extracellular hydrolytic enzymes such as hemolysins, proteases, and phospholipases. These factors contribute to the pathogenicity of *C. albicans* by enabling adhesion and proliferation on host cell surfaces and abiotic materials such as catheters, promoting biofilm formation,

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facilitating the yeast-to-hypha transition, producing tissue-damaging enzymes, and allowing evasion from immune defenses. Through these mechanisms, *C. albicans* can multiply, colonize, invade tissues, and cause extensive damage (Shrivastava and Whiteway 2025; Saiprom et al. 2023).

The first step in the infection process is the attachment of *C. albicans* cells to host cell surfaces, a process in which specific surface proteins called adhesins play a crucial role. Among these adhesins are glycosylphosphatidylinositol (GPI)-anchored cell surface proteins encoded by the eight-member agglutinin-like sequence (ALS) gene family (*ALS1–ALS7* and *ALS9*). Another major adhesin of *C. albicans* is hyphal wall protein 1 (HWP1), a hypha-associated GPI-anchored mannoprotein located on the outer surface and expressed during germ tube and hyphal growth. The *HWP1* and *ALS* gene families are essential for *C. albicans* adhesion to host tissues, invasion, and biofilm formation. Another important virulence factor is secreted aspartyl proteinases (SAP), encoded by ten different genes (*SAP1–SAP10*). Among these, SAP1–SAP3 are primarily associated with mucosal infections, while SAP4–SAP6 are linked to systemic infections (Talapko et al. 2021). Seven genes, *PLA*, *PLB1*, *PLB2*, *PLC1*, *PLC2*, *PLC3*, and *PLD1*, are involved in the synthesis of phospholipase enzymes, with phospholipase B1 (PLB1), PLB2, PLC1, and PLD1 being particularly associated with virulence. PLB1 is a glycoprotein that exhibits both hydrolase and lysophospholipase transacylase activities, disrupting the structural integrity of cell membranes by hydrolyzing the ester bonds of phospholipids. Enzyme activity is especially concentrated at the tips of hyphae, where it contributes to tissue invasion (Shrivastava and Whiteway 2025).

C. albicans is divided into genotypes based on the presence or absence of a transposable group I intron in the ITS1 region of the 25S rDNA. This method, also referred to as 25S intron analysis or ABC genotyping, relies on ribosomal sequencing and classifies *C. albicans* isolates as genotype A (450 bp), genotype B (840 bp), or genotype C (450 and 840 bp) according to the size of the PCR amplification products. Genotyping is a very useful tool for epidemiologic analysis. Studies have shown that genotype A is the most prevalent genotype worldwide in all clinical forms of candidiasis, while the frequencies of genotypes B and C vary according to geographical region and the type of infection involved. Genotypes B and C are predominantly isolated from oral samples (Moron and Cabrera 2019; Kumar and Tejashree 2022; Ali et al. 2024).

The aim of this study was to determine the genotypes of *C. albicans* strains, isolated from blood samples of patients with candidemia, by 25S intron analysis and to investigate the presence of virulence genes by PCR, including *ALS1* and *HWP1*, which are involved in biofilm formation and adhesion; *PLB1*, which is associated with phospholipase activity; *SAP1*, *SAP2*, and *SAP4*, which are related to secreted aspartyl proteinase activity.

2. Material and methods

2.1. Isolates

This study included a total of 50 strains isolated from the blood cultures of patients with candidemia and identified as *C. albicans* using either the API ID 32 C (bioMérieux, France) or the VITEK 2 YST (bioMérieux, France) systems in the Mycology Laboratory of the Department of Medical Microbiology, Istanbul University, Istanbul Faculty of Medicine, between 2021 and 2025. These strains were stored at -80 °C in brain heart infusion broth (BD Difco, USA) supplemented with 3% glycerol. *C. albicans* ATCC 10231 was used as the control strain.

The isolates were inoculated onto Sabouraud dextrose agar (BD Difco, USA) and incubated at 37 °C for 24-48 hours.

DNA isolation was performed with the DNA Extraction Kit (MicroLine, Türkiye) following the manufacturer's instructions.

2.2. ABC genotyping

ABC genotyping was based on the presence or absence of a transposable group I intron in the ITS1 region of rDNA. Amplification of the 25S rDNA region was performed using the primers CABC Forward (5'-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3') and CABC Reverse (5'-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3'). The PCR reaction mixture was prepared according to the manufacturer's instructions and consisted of 12.5 µl 2x qPCRBIO Taq Mix (PCR Biosystems, USA), 1 µl forward primer, 1 µl reverse primer, 5.5 µl sterile ultrapure water, and 5 µl DNA, for a total volume of 25 µl. PCR amplification was carried out using the Labcycler Basic (SensQuest, Germany), with an initial denaturation at 95 °C for one minute, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds, extension at 72 °C for 2 minutes, and a final extension at 72 °C for 5 minutes (Mashaly and Zeid 2022; Fornari et al. 2016).

The amplified products were separated by size using agarose gel electrophoresis. The genotypes of *C. albicans* isolates were determined based on their band sizes by comparison with a DNA ladder as genotype A

(450 bp), genotype B (840 bp), and genotype C (two bands of 450 and 840 bp) (Mashaly and Zeid 2022).

2.3. Detection of the virulence genes

The presence of *SAP1*, *SAP2*, *SAP4*, *ALS1*, *HWP1*, and *PLB1* genes in *C. albicans* isolates was investigated by PCR. The primers used are listed in Table 1. The PCR reaction mixture was prepared according to the manufacturer's instructions and had a total volume of 25 µl, consisting of 12.5 µl 2x qPCRBIO Taq Mix (PCR Biosystems, USA), 1 µl forward primer, 1 µl reverse primer, 5.5 µl sterile ultrapure water, and 5 µl DNA. The same PCR conditions were applied for all genes and amplification was carried out using the Labcycler Basic (SensQuest, Germany), with an initial denaturation at 95 °C for one minute, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds, extension at 72 °C for 2 minutes, and a final extension at 72 °C for 5 minutes. The amplified products were visualized by agarose gel electrophoresis with a UV trans-illuminator using a DNA ladder (Costa et al. 2010; Dikmen et al. 2021).

Table 1. Primers used for the detection of virulence genes

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
SAP1-F	TCAATCAATTTACTCTTCCATTTCTAACA	161	Costa 2010
SAP1-R	CCAGTAGCATTAACAGGAGTTTAATGACA		
SAP2-F	AACAACAACCCACTAGACATCACC	178	
SAP2-R	TGACCATTAGTAACTGGGAATGCTTTAGGA		
SAP4-F	CATTCATTCCTTTAATACCGACTATC	156	
SAP4-R	GGTAACAAACCCTGTAGATCTTTTAA		
ALS1-F	GACTAGTGAACCAACAAATAC	318	Dikmen 2021
ALS1-R	CCAGAAGAAACAGCAGGTGA		
HWP1-F	ATGACTCCAGCTGGTTC	572	
HWP1-R	TAGATCAAGAATGCAGC		
PLB1-F	ATGATTTTGCATCATTTG	751	
PLB1-R	AGTATCTGGAGCTCTAC		

2.4. Statistical analysis

Statistical analyses were performed using SPSS version 30.0 (IBM Corp., Armonk, NY, USA). The presence of virulence genes was compared among different *C. albicans* genotypes using the Freeman-Halton extension of Fisher's exact test. This test was applied because the number of cells with expected values below 3 exceeded 20% of the total number of cells in the n×r contingency tables. A p-value of <0.05 was considered statistically significant.

3. Results

A total of 50 *C. albicans* isolates included in the study were obtained from eight pediatric (0-18 years, 16%) and 42 adult (>18 years, 84%) patients. Of these patients, 28 (56%) were female and 22 (44%) were male. The mean age of the patients was 54.08 years. Among the adult patients, six (12%) were aged 18-44 years, 14 (28%) were aged 45-65 years, and 22 (44%) were over 65 years. Overall, 31 patients (62%) were hospitalized in surgical units, 10 (20%) in intensive care units, and nine (18%) in internal medicine units.

Based on the amplicon sizes observed after gel electrophoresis, 28 (56%) of the 50 *C. albicans* isolates were identified as genotype A, 14 (28%) as genotype B, and eight (16%) as genotype C.

The *SAP1* gene was positive in 48 (96%) and negative in two (4%) isolates; *SAP2* and *SAP4* were positive in 49 (98%) and negative in one (2%) isolate; *ALS1* and *HWP1* were positive in 46 (92%) and negative in four (8%) isolates; and *PLB1* was positive in 47 (94%) and negative in three (6%) isolates (Table 2).

No significant differences were observed in the distribution of virulence genes among the different genotypes of *C. albicans*. As all p-values were greater than 0.05, each gene showed a similar distribution across the groups (Table 2).

Table 2. Distribution and relationship between genotypes and the presence of virulence genes (n, %).

Gene	Total	Genotype A (n=28)	Genotype B (n=14)	Genotype C (n=8)	p value
SAP1	48 (96.0)	26 (92.8)	14 (100)	8 (100)	0.680
SAP2	49 (98.0)	28 (100)	13 (92.8)	8 (100)	0.439
SAP4	49 (98.0)	28 (100)	13 (92.8)	8 (100)	0.439
ALS1	46 (92.0)	26 (92.8)	13 (92.8)	7 (87.5)	0.801
HWP1	46 (92.0)	25 (89.3)	13 (92.8)	8 (100)	0.999
PLB1	47 (94.0)	26 (92.8)	13 (92.8)	8 (100)	0.999

4. Discussion

Candida species cause a variety of clinical conditions, usually of endogenous origin, ranging from mild skin and mucosal infections to severe invasive infections, particularly in individuals with weakened immune systems. In addition to host-related risk factors, strain-specific virulence factors also play an important role in the development of infection. *C. albicans* is the most common *Candida* species, both as a member of the normal microbiota and as a pathogenic agent. It possesses several major virulence factors, including adhesion, phenotypic switching, hyphal formation, biofilm production, and the secretion of hydrolytic enzymes such as hemolysins, proteases, and phospholipases. Through these virulence mechanisms, *C. albicans* multiplies, colonizes, invades host tissues, and causes cellular damage (Shrivastava and Whiteway 2025). Among *Candida* species, *C. albicans* has been reported to exhibit the highest virulence due to its strong biofilm-forming capacity and high levels of proteinase and melanin production. Moreover, virulence genes have been found to be more prevalent in *C. albicans* than in other *Candida* species (Makled et al. 2024). Makled et al. (2024) also reported that the antifungal resistance profiles of *Candida* isolates were associated with the presence of virulence genes, particularly noting a correlation between flucytosine resistance and the presence of *HLP* and *HWP* genes.

Some researchers have reported a correlation between the body sites from which *Candida* spp. are isolated and the expression of virulence factors. *Candida* species isolated from blood have been found to be more virulent and to possess a greater number of virulence factors, likely due to their ability to adhere to catheters and secrete extracellular enzymes that facilitate tissue invasion (Talapko et al. 2021; Makled et al. 2024). Samaranayeke et al. (2013) demonstrated that, in vitro, the presence of serum enhanced *C. albicans* virulence gene expression and promoted the formation of a thicker biofilm layer. However, other researchers reported no significant differences in virulence factor profiles among strains isolated from different clinical samples (Chen et al. 2021; Jabeen et al. 2023).

SAPs, which are hydrolytic enzymes of *C. albicans*, comprise ten members encoded by the *SAP* gene family and are expressed at different stages of infection. These enzymes play crucial roles in host tissue invasion, immune evasion, adhesion, hyphal growth, and biofilm formation, thereby contributing to tissue damage and dissemination (Gerges et al. 2023; Pawar et al. 2022). In vitro studies have shown that *SAP1*, *SAP2*, and *SAP3* are expressed exclusively by yeast cells, whereas *SAP4*, *SAP5*, and *SAP6* are expressed during the hyphal phase. *SAP1-SAP3* are primarily associated with mucosal infections, while *SAP4-SAP6* are linked to systemic infections (Mohammed et al. 2017). Among these, *SAP1* plays a particularly important role in promoting tissue damage and facilitating host invasion (Dikmen et al. 2021).

Makled et al. (2024) reported the presence of the *SAP1* gene in 26 (54.2%) of 48 *C. albicans* strains isolated from various clinical specimens, including 28 from blood. They also found that all isolates showing phenotypic proteinase activity were *SAP1*-positive (100%). Shrief et al. (2019) detected the *SAP1* gene in 65 (65%) of 100 *C. albicans* isolates, 56 of which were isolated from blood samples. Dawoud et al. (2024) identified the *SAP2* gene in nine (52.9%) of 17 *C. albicans* isolates obtained from pediatric and adult patients with candidemia. Bilgin et al. (2020) investigated the presence of the *SAP4* gene, known to play a key role in systemic

infections, in 50 *C. albicans* strains isolated as causative agents of candidemia and found it to be positive in 49 isolates (98%). Similarly, Ardehali et al. (2019) detected the *SAP4* gene in 57 (88%) of 65 *C. albicans* strains isolated from various clinical specimens, including 21 blood samples, obtained from patients in intensive care units. In the present study, among the 50 *C. albicans* isolates examined, the *SAP1* gene was detected in 48 (96%) isolates, while *SAP2* and *SAP4* were detected in 49 (98%) isolates each.

ALS, a family of glycoproteins, constitutes the largest group of adhesins in *C. albicans*. ALS1, one of the members of this family, is a cell surface protein that enhances adhesion to endothelial and epithelial cells and plays a crucial role in adhesion and hyphal development (Dikmen et al. 2021; Obais et al. 2023). Together with *HWP1*, *ALS1* is also important for mature biofilm formation, and the expression of these genes has been shown to increase during biofilm development (Makled et al. 2024; Gerges et al. 2023). Obais et al. (2023) reported that *ALS1* and *ALS3* play a significant role in the development of systemic candidiasis.

Makled et al. (2024) detected the *ALS1* gene in 28 of 48 *C. albicans* strains (58.3%) isolated from various clinical specimens, including 28 from blood. Dawoud et al. (2024) reported that 13 of 17 *C. albicans* isolates (76.5%) from blood cultures were *ALS1*-positive. Shrief et al. (2019) found the *ALS1* gene in 65 of 100 isolates (65%), 56 of which were from blood samples. Ardehali et al. (2019) reported the presence of *ALS1* in 60 of 65 isolates (92%), including 21 from blood. Similarly, İnci et al. (2013) detected this gene in 11 of 13 blood isolates (84.6%). In the present study, 46 of the 50 isolates examined (92%) were *ALS1*-positive.

The β-glucan-bound mannoprotein encoded by the *HWP1* gene, located in the hyphal cell wall, is an important protein that enables *C. albicans* to adhere to various surfaces and host cells, including epithelial cells (Makled et al. 2024; Gerges et al. 2023; Obais et al. 2023). Expression of *HWP1* has been shown to be particularly high during the early stages of biofilm formation (Mohammadi et al. 2023). HWP1 plays a critical role in hyphal development and the induction of tissue damage in the host. In addition, HWP1, together with ALS1 and ALS3, has been reported to contribute significantly to the development of systemic candidiasis (Obais et al. 2023).

Makled et al. (2024) reported that 44 of 48 *C. albicans* isolates (91.7%), including 28 from blood, were *HWP1*-positive. Dawoud et al. (2024) detected *HWP1* in 14 of 17 blood isolates (82.4%), and found that all 12 biofilm-forming isolates were positive (100%). Shrief et al. (2019) examined 100 *C. albicans* isolates, 56 from blood, and found *HWP1* in 77 isolates (77%). Ardehali et al. (2019) reported *HWP1* positivity in 62 of 65 strains (95%), including 21 blood isolates. İnci et al. (2013) detected this gene in only one of 13 blood isolates (7.7%). In the present study, *HWP1* was detected in 46 of 50 isolates (92%) and absent in four (8%).

Phospholipases are enzymes that hydrolyze phospholipids, leading to disruption of host cell membranes. Among them, PLB1 and PLD1 play key roles in the pathogenicity of *C. albicans* (Mohammadi et al. 2023). By cleaving the ester bonds of glycerophospholipids in host cell membranes, phospholipases lyse cells and facilitate tissue penetration and invasion. In addition, they promote adhesion by altering the surface properties of host cells (Mashaly and Zeid 2022). *C. albicans* exhibits stronger phospholipase activity than other *Candida* species (Saiprom et al. 2023).

Tunç et al. (2021) detected *PLB1* in all 30 *C. albicans* strains (100%) isolated from blood, with 26 strains (87.7%) exhibiting phenotypic phospholipase activity. In the same study, 30 *C. albicans* strains isolated from the oral cavity were also 100% *PLB1*-positive, with 80% showing phospholipase activity, and no statistically significant difference was observed between the two groups. Shrief et al. (2019) reported the presence of *PLB1* in 52 of 100 isolates (52%), 56 of which were from blood samples. In the present study, *PLB1* was detected in 47 of 50 isolates (94%) and absent in three (6%).

C. albicans is genotyped by 25S intron analysis based on the presence or absence of a transposable group I intron in the ITS1 region of rDNA. Previous studies have shown that genotype A is the most prevalent genotype worldwide (Moron and Cabrera 2019; Kumar and Tejashree 2022; Ali et al. 2024). Mahmoodi et al. (2023) reported the genotyping results of 20 *C. albicans* strains isolated from blood cultures as genotype A (12/20; 60%), genotype C (6/20; 30%) and genotype B (2/20; 10%). Similarly, Ali et al. (2024) investigated 55 *C. albicans* isolates from blood cultures and found that 28 strains (50.9%) were genotype A, 17 (30.9%) were genotype B, and 10 (18.2%) were genotype C. Karahan et al. (2012) determined the distribution of 121 blood culture isolates as genotype A (77/121; 63.7%), genotype B (24/121; 19.8%), and genotype C (20/121; 16.5%). In the present study, among the 50 *C. albicans* isolates analyzed, genotype A was the most common (28 isolates, 56%), followed by genotype B (14 isolates, 28%) and genotype C (eight isolates, 16%).

There are few studies comparing ABC genotypes of *C. albicans* in terms of virulence factors, and no comparative study has specifically addressed the presence of virulence genes. Ali et al. (2024) determined the genotypes of 55 *C. albicans* isolates from blood cultures and additionally assessed their phospholipase,

protease, esterase, hemolysin, coagulase, and biofilm formation properties using phenotypic methods. They found no statistically significant differences in virulence factors among the genotypes. Mashaly et al. (2022) genotyped 88 *C. albicans* strains isolated from various clinical samples of pediatric patients, including 19 from blood, and evaluated proteinase, phospholipase, and biofilm formation using phenotypic methods. The study reported no significant differences in virulence factors among genotypes or clinical sample types. Similarly, Gharaghani et al. (2021) analyzed *C. albicans* isolates from urine samples of pediatric patients, grouping them only as genotype A (72%) and C (28%) with no genotype B detected. A statistically significant difference was found only in esterase activity, with genotype A strains showing higher activity than genotype C, while no differences were observed for other virulence factors. Moron et al. (2019) examined 26 *C. albicans* isolates from various clinical samples and investigated phospholipase, esterase, hemolysin production, biofilm formation, and phenotypic transformation using phenotypic methods. No significant differences in virulence factors among genotypes were detected. Da Silva-Rocha et al. (2014) grouped 76 *C. albicans* isolates from the oral cavities of kidney transplant patients as colonizers or causative agents according to genotype and evaluated phospholipase activity, phenotypic change, and resistance to phagocytosis. Since only three isolates were identified as genotype B, statistical comparisons were made between genotype A (n=58) and genotype C (n=15). While no differences were found in phospholipase activity or phenotypic change, genotype C strains exhibited greater resistance to phagocytosis. In the present study, no statistically significant differences were observed among genotypes A, B, and C regarding the presence of *SAP1*, *SAP2*, *SAP4*, *ALS1*, *HWP1*, and *PLB1* virulence genes.

As a result, in the present study, a total of 50 *C. albicans* strains isolated from blood samples were genotyped by 25S intron analysis and examined for the presence of virulence genes *SAP1*, *SAP2*, *SAP4*, *ALS1*, *HWP1* and *PLB1*. Genotype A was found to be the most common (56%), consistent with reports from other regions worldwide. The *SAP1* gene was detected in 96% of the isolates, *SAP2* and *SAP4* in 98%, *ALS1* and *HWP1* in 92%, and *PLB1* in 94%. A better understanding of the virulence factors and the pathogenesis of infections caused by *Candida* species, particularly *C. albicans*, is essential for improving diagnostic approaches, developing infection control strategies, guiding antifungal therapy, and discovering novel agents targeting virulence factors that could be used in combination with antifungal drugs. Considering the increasing incidence of antifungal resistance, efforts to design new therapeutic strategies targeting virulence factors have become particularly important. Studies in this field will contribute to a deeper understanding of fungal pathogenesis and help identify potential targets for more effective therapeutic approaches.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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PATHOGENIC BACTERIA VARIATION IN HYDROELECTRIC RESERVOIRS: CASE OF KOSSOU, TAABO AND FAÉ IN CÔTE D'IVOIRE

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Abstract: Recent studies have shown that the Taabo, Kossou, and Faé reservoirs are contaminated by faecal pollutants, which could pose health risks to users due to a potentially high abundance of pathogenic bacteria. Accordingly, a monitoring study was planned to assess variation in pathogen abundance, including *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*), in these reservoirs. Water sampling was conducted between February and October of the same year along a linear transect in each reservoir. Pathogenic bacteria were analyzed using culture and polymerase chain reaction (PCR) methods. The primer pairs PA-SS-F/PA-SS-R were used for *P. aeruginosa*, and 16S rRNA-F/16S rRNA-R were used for *S. aureus*. Overall, the median concentrations of *P. aeruginosa* and *S. aureus* ranged from 0.97 and 2.57 Log CFU/100 mL. The highest concentrations of these bacteria were generally recorded during the rainy periods in June, July, September, and October. Spatially, the highest concentrations were found in the shore areas of the different reservoirs. The results indicated weak correlations between the concentrations of these pathogenic bacteria. To preserve the integrity of these hydroelectric reservoirs, it is advisable to establish safety zones around them and to treat wastewater prior to discharge.

Keywords: Culture methods, PCR, Human pressure, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Water pollution;

1. Introduction

Water pollution threatens aquatic life and human health (Goh et al. 2019; Lin et al. 2022). This pollution is linked to the impact of large quantities of organic and inorganic waste resulting from human activities (Chowdhary et al. 2020; Lin et al. 2022). The immediate consequences are water quality deterioration, creating a favourable environment for numerous microorganisms, including viruses, fungi, parasites, and bacteria (Gamze et al. 2023).

Concerning bacteria, *Pseudomonas aeruginosa* (*P. aeruginosa*), which is commonly found in aquatic environments, and *Staphylococcus aureus* (*S. aureus*), which is generally associated with external contamination, are implicated in many waterborne infections (Fanny et al. 2008; Gretchen et al. 2025). Their abundance in surface waters is most often linked to nutrient and organic pollution resulting from human activities (Esiobu et al. 2013; Adriana et al. 2020), and this may pose health risks to people who use these resources (Fanny et al. 2008; Gretchen et al. 2025). In fact, peo-

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ple can become infected by swimming in contaminated waters, drinking contaminated water, or eating fish from contaminated waters. The body parts most at risk are the skin, mouth, salivary glands, oesophagus, stomach, and intestines (Sharma and Gilbert 2018; Treuting et al. 2018; Soni et al. 2024). *P. aeruginosa* and *S. aureus* are the most common colonizers of the cystic fibrosis lung and frequently overlap to cause chronic and persistent coinfections associated with severe diseases (Gretchen et al. 2025).

Land use in the catchment areas of the Taabo, Kossou, and Faé reservoirs is characterized by intense urbanization, livestock farming, agriculture, and mining activities. Several populations observed near these bodies of water and upstream waterways practice these activities (Groga et al. 2012; Koné 2012). Therefore, discharges from these activities could end up in the reservoirs, altering their quality and causing waterborne diseases, while these reservoirs are commonly used for fishing and fish farming. Recent studies on the quality of these three reservoirs showed an abundant presence of faecal indicator bacteria, in particular *Escherichia coli* and Intestinal Enterococci (Tiémoko et al. 2020) and high concentrations of cultivable heterotrophic bacteria (Tiémoko et al. 2025). This indicates that these water bodies are increasingly exposed to faecal contamination and excessive inputs of other organic matter, which may contribute to a deterioration in water quality. The concentration of pathogenic bacteria could therefore be high in these waters, posing a threat to public health. Furthermore, unlike lakes, there is little knowledge about the distribution of pathogenic bacteria in these reservoirs, which are complex ecotones linking fluvial ecosystems with the terrestrial environments of their catchments. The aim of this study was to assess the variation in pathogen abundance, including *P. aeruginosa* and *S. aureus*, in the Kossou, Taabo, and Faé reservoirs.

2. Materials and Methods

2.1. Study area

The Kossou, Taabo and Faé hydroelectric reservoirs, located in the center, south and south-west of Côte d'Ivoire respectively, constitute the study area (Fig. 1). In brief, the Taabo, Kossou, and Faé reservoirs cover areas of 62 km², 1,700 km², and 16.28 km² with watersheds of 58,700 km², 32,400 km², and 2,424 km², respectively (Da costa and Diétoa 2007; Groga et al. 2012; Koné 2012). These catchment areas are mainly characterized by urban areas, livestock farming, agriculture, and farms.

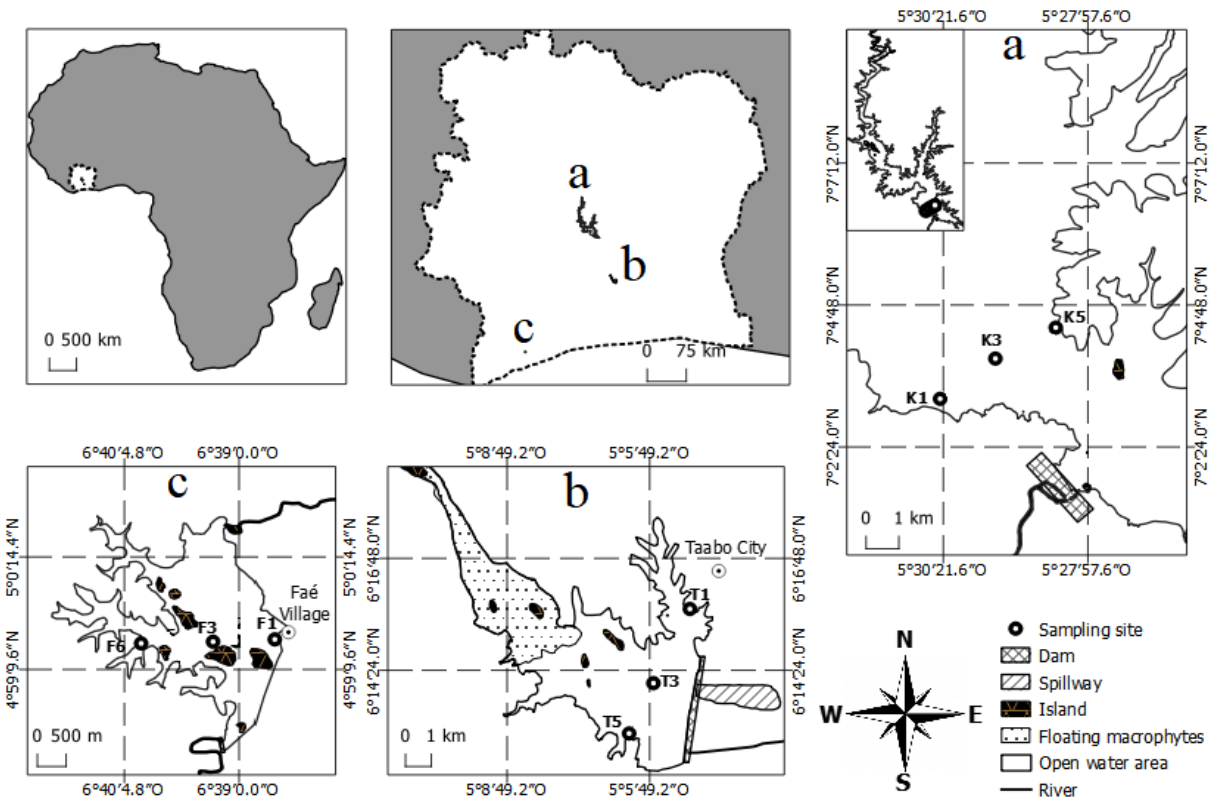

Fig. 1 Location of sampling stations in the studied reservoirs. a: Lake Kossou; b: Lake Taabo; c: Lake Faé (**Tiémoko et al. 2025**)

2.2. Water sampling

Water sampling was conducted from February 2018 to October 2018, specifically in February (Fe), April (Ap), June (Jn), July (Jl), September (Se), and October (Oc). Three sampling points were selected along transects in Lake Kossou (K1, K3, and K5), Lake Taabo (T1, T3, and T5), and Lake Faé (F1, F3, and F6) to compare shore areas and depth areas (Figure 1). Briefly, sterile 1L bottles were filled with water sampled from the surface of all reservoirs at the various sampling points. A total of 54 samples were collected for microbiological analysis. The bottles were labeled and transported to the laboratory on ice in cooler boxes (4 °C), then analyzed within a maximum of 8 h after sampling. The activities observed around the various sampling points are described in Table 1.

Table 1: Coordinates and characteristics of the sampling sites in each man–made reservoir

Reservoirs	Sampling points	Description of land use types	Coordinates	
			Latitude (N)	longitude (W)
Kossou	K1	Small village near of the lake, cattle farming, mining activities in the shore	07°03'12.5"	-05°30'25.1"
	K3	Fishing area	07°03'53.3"	-05°29'30.1"
	K5	of the lake, cattle farming	07°04'24.6"	-05°28'30.2"
Taabo	T1	Urban zone near of lake, cattle farming on the shore, agriculture (hevea, cassava crops)	06°15'46.6»	-05°04'57.4»
	T3	Fish farming into the lake, fishing area	06°13'58.3»	-05°05'45.2»
	T5	Big village close to the lake, fishing area, agriculture	06°12'59.2»	-05°06'14.6»
Fae	F1	Big village very close to the lake, fishing area	04°59'24.2»	-06°38'01.3"
	F3	Fishing area, watercourse pathway, agriculture on the shore (cocoa, hevea)	04°59'26.3»	-06°39'00.8"
	F6	Fishing area, agriculture on the shore (cocoa, hevea crops)	04°59'24.5»	-06°39'55»

2.3. Culture-based enumeration of pathogen bacteria

Pseudomonas aeruginosa (*P. aeruginosa*) was analyzed using Cetrimide agar (Liofilchem: 610041, Italy) supplemented with glycerol and *Staphylococcus aureus* (*S. aureus*) was analyzed using Baird-Parker agar (CONDA Pronodisa: CAT: 1100.00, Spain) supplemented with Egg Yolk Tellurite Enrichment and sulfamethazine. These analyses were carried out in accordance with the manufacturers' instructions. Briefly, filtration of a sample volume (1 mL, 10 mL, or 100 mL) through a sterile filter membrane (porosity 0.22 µm) was carried out, and the membrane was deposited on the agar. The agar plates were incubated for 44 hours at 41°C for *P. aeruginosa* and for 48 hours at 35°C for *S. aureus*. Following incubation, the characteristic blue-green, fluorescent colonies of *P. aeruginosa* and the characteristic convex, black, shiny colonies with a halo of *S. aureus* were isolated for biochemical testing. Biochemical tests were performed on the two bacterial strains, focusing particularly on motility, Gram staining, catalase activity, and oxidase activity. DNAse and coagulase tests were carried out as additional tests for *S. aureus* only. After incubation and biochemical tests, plate counts were expressed as Log Colony Forming Units per 100 milliliters (Log CFU/100 mL). Pure cultures were collected and stored in 20% glycerol at −20°C for PCR analysis.

2.4. Molecular characterization of pathogen bacteria

2.4.1. DNA extraction of colonies

Genomic DNA of *P. aeruginosa* and *S. aureus* was extracted from isolated colonies using a method adapted from Sambrook et al. (2001). The following modifications were introduced: each colony was suspended in 400 µL of PBS buffer, and for *S. aureus*, an enzymatic treatment with lysozyme (1 mg/mL) was performed at 37°C for 30 min to facilitate cell wall lysis. Cells were then lysed by adding SDS (1%) and proteinase K (0.25 mg/mL), followed by incubation at 56°C for 30 min. DNA was purified by phenol-chloroform-isoamyl alcohol extraction (25:24:1), then precipitated with isopropanol in the presence of 0.5 M NaCl. The pellet was washed with 70% ethanol, allowed to air dry, and resuspended in 50 µL of TE buffer. The extract was stored at −20°C. DNA quality and quantity were assessed using a Nanodrop (Mettler Toledo UV5Nano) and electrophoresis on 0.8% agarose gel. The results indicated that the extracted DNA was of good quality, with absorbance ratios and gel profiles consistent with genomic DNA, making it suitable for further molecular analysis.

2.4.2. Polymerase chain reaction (PCR)

The molecular detection of *P. aeruginosa* and *S. aureus* was performed using conventional PCR targeting the gene encoding 16S rRNA. Thus, for *P. aeruginosa*, the primer pair PA-SS-F (5′-GGGGGATCTTCGGACCTCA-3′) and PA-SS-R (5′-TCCTTAGAGTGCCCACCCG-3′) was used to amplify a 956 bp fragment (Spilker et al. 2004). Concerning *S. aureus*, the primer pair 16S rRNA-F (5′-GTAGGTGGCAAGCGTTATCC-3′) and 16S rRNA-R (5′-CGCACATCAGCGTCAG-3′) was used to amplify a fragment of approximately 228 bp (Karmakar et al. 2016). The PCR amplification reaction was performed using the GoTaq Polymerase Flexi 2 enzyme kit (Promega), with a 25 µl reaction mixture consisting of: 1X Gotaq buffer (Promega) of Taq polymerase (Promega) at 0.025 U/µl, 10 µM of each primer, 0.2 mM dNTPs (New England Biolabs), 1 mM $MgCl_2$ (Promega) and 5 µl of DNA (200 ng). The amplification conditions were as follows for the 16S RNA gene of *P. aeruginosa*: a denaturation phase at 94°C for 5 min, followed by 35 cycles (denaturation: 94°C for 30 s, hybridization: 53°C for 30 s and elongation: 72°C for 1 min), with a final elongation at 72°C for 10 min. Concerning *S. aureus,* the amplification conditions were as follows for the 16S rRNA gene: a denaturation phase at 94°C for 5 min, followed by 35 cycles (denaturation: 94°C for 1 min, hybridization: 55°C for 1 min and elongation: 72°C for 1 min), with a final elongation at 72°C for 10 min. PCR products were detected using an automated gel reader (Gel Documentation System BL, Axygen) after electrophoresis in a 1.5% agarose gel containing SybrSafe in TAE buffer (1X).

2.5. Comparison test and representation of pathogen bacteria line plot

Statistical tests were required to analyze the data on pathogen bacteria distribution in hydroelectric reservoirs. This includes the Shapiro-Wilk test, followed by Kruskal-Wallis and U Mann-Whitney tests. RStudio 4.1.2 software was used to perform these tests and the significance level is 0.05. A Spearman test was performed to examine the correlation between pathogen bacteria. Principal Component Analysis (PCA) was used to show the distribution of bacteria according to the sampling sites. The ade4 and factoextra packages were used for PCA implementation. Pathogen bacteria line plots, Spearman correlation analysis, and PCA were all carried out using RStudio software.

3. Results

3.1. Detection of *P. aeruginosa* and *S. aureus*

The genomic DNA extraction from colonies showed good integrity, confirmed by electrophoresis on 0.8% agarose gel, with high molecular weight DNA profiles. Absorbance ratios (A_{260}/A_{280}) ranged from 1.7 to 1.9, indicating sufficient purity for molecular analysis. Amplification of the 16S rRNA gene enabled specific detection of the two bacteria studied. The primer pairs PA-SS-F/PA-SS-R generated a fragment of approximately 956 bp (Fig. 2), specific to *P. aeruginosa*. Similarly, using the primer pairs 16S rRNA-F/16S rRNA-R, a 228 bp fragment (Fig. 3) specific to *S. aureus* was obtained. No non-specific amplification was detected in the negative controls. Among 54 strains of each bacterium, only 18 strains of *P. aeruginosa* (33.3%) and 16 strains of *S. aureus* (29.6%) showed negative results for 16S rRNA gene amplification.

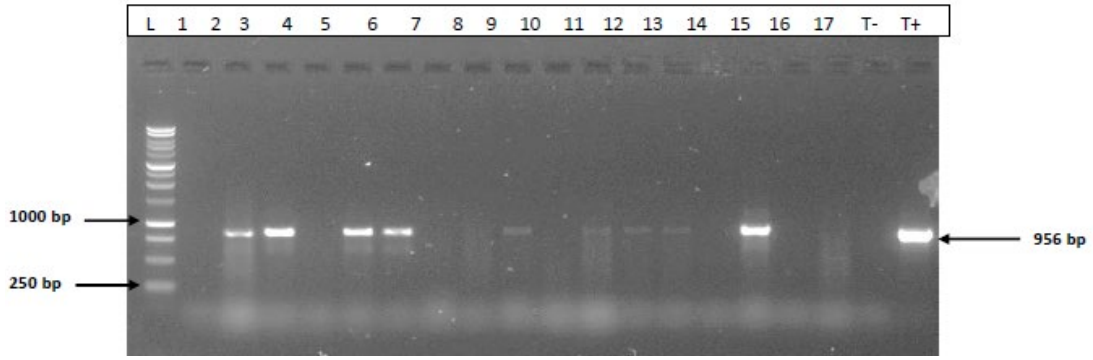

Fig. 2 Gel electrophoresis (1.5%) of amplified 16S rRNA gene products from *P. aeruginosa.* L: molecular weight marker (1kb); 1-17: samples; T-: negative control, T+: positive control (956 bp)

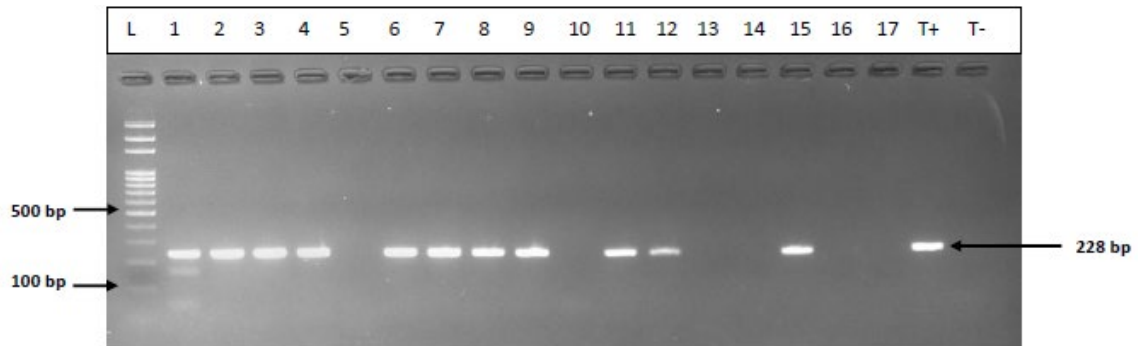

Fig. 3 Gel electrophoresis (1.5%) of amplified 16S rRNA gene products from *S. aureus.* L: molecular weight marker (100 bp); 1-17: samples; T-: negative control, T+: positive control (228 bp)

3.2. Spatial and temporal variation of pathogen bacteria abundance

The temporal variation of pathogen bacteria abundance in different reservoirs was observed to determine periods of high growth. Bacteria median concentrations ranged from 0.97 Log CFU/100 mL to 2.57 Log CFU/100 mL. The highest abundances of *P. aeruginosa* and *S. aureus* were generally recorded during the last four sampling months, namely June, July, September, and October (Fig. 4; Fig. 5).

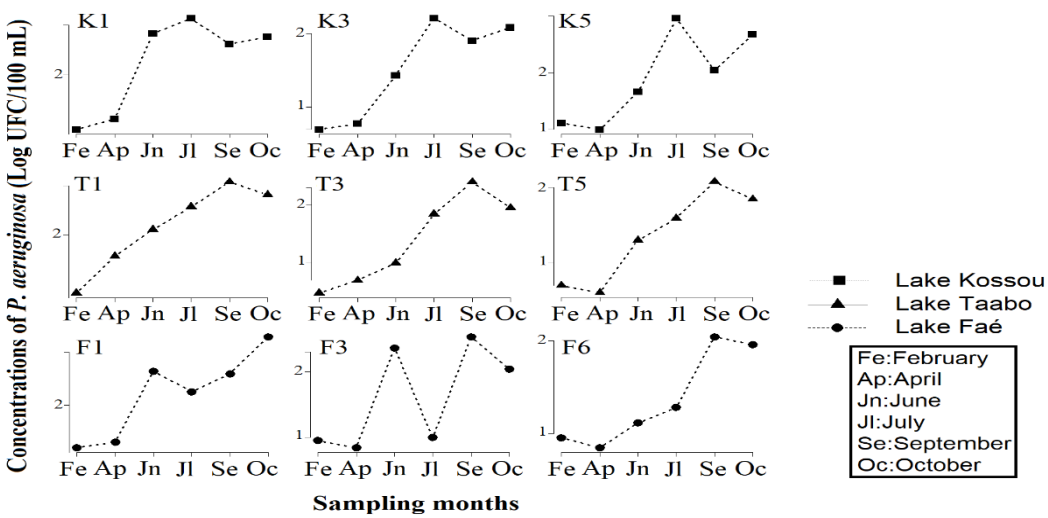

Fig. 4 Spatial and temporal variation of *P. aeruginosa* in hydroelectric reservoirs. Lake Kossou sampling sites: K1, K3, K5; Lake Taabo sampling sites: T1, T3, T5; Lake Faé sampling sites: F1, F3, F5.

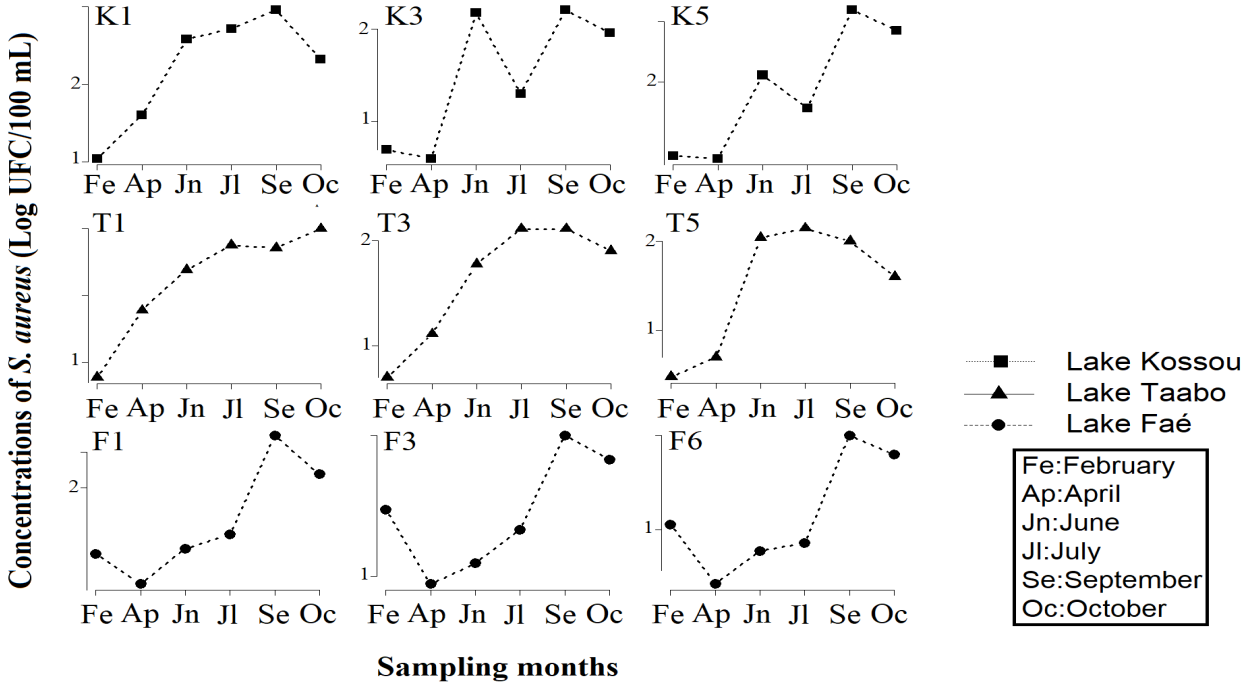

Fig. 5 Spatial and temporal variation of *S. aureus* in hydroelectric reservoirs. Lake Kossou sampling sites: K1, K3, K5; Lake Taabo sampling sites: T1, T3, T5; Lake Faé sampling sites: F1, F3, F5.

PCA revealed that the K1, T1, and F1 sampling sites recorded high overall concentrations of *P. aeruginosa* and *S. aureus*, respectively, for the Kossou, Taabo, and Faé reservoirs (Fig. 6). The highest concentrations of pathogen bacteria were therefore obtained in shore areas in the different reservoirs. The Kruskal-Wallis test revealed no significant differences in the concentrations of *P. aeruginosa* recorded at the different sampling points for each reservoir (Lake Taabo: $p = 0.1171$; Lake Kossou: $p = 0.3123$; Lake Faé: $p = 0.09281$). There were also no significant differences in *S. aureus* concentrations between sampling points in Lake Taabo (Kruskal-Wallis: $p = 0.1528$) and Lake Kossou (Kruskal-Wallis: $p = 0.2636$). However, in Lake Faé, the Mann-Whitney test ($pF1\text{-}F5 = 0.04307$) revealed a significant difference in *S. aureus* concentrations between F1 and F5.

3.3. Relation between pathogen bacteria in reservoirs

Correlation relationships between pathogenic bacteria were studied in all hydroelectric reservoirs (Fig. 7). The results indicated weak correlations between these pathogenic bacteria.

4. Discussion

The primer pairs PA-SS-F/PA-SS-R generated a fragment of approximately 956 bp, specific to *P. aeruginosa*. Similarly, using the primer pairs 16S rRNA-F/16S rRNA-R, a 228 bp fragment specific to *S. aureus* was obtained. No non-specific amplification was detected in the negative controls. These results confirm the accuracy of the applied primers for the rapid and specific detection of *P. aeruginosa* and *S. aureus* from pure bacterial colonies. The molecular detection of *P. aeruginosa* and *S. aureus* from colonies confirmed the results of microbiological tests carried out in the Kossou, Taabo, and Faé reservoirs. These results highlight the usefulness of combining culture methods and PCR for analyzing bacteria, as this increases the reliability of diagnoses and speeds up the identification of pathogens present in the environment. These observations are also consistent with the conclusions of the study conducted by Adingra et al. (2012) on the Grand-Lahou lagoon, which already reported the regular presence of *P. aeruginosa* and *S. aureus*. However, the negative results for 16S rRNA gene amplification could be linked to the quantity of bacterial DNA being below the detection limit of PCR or to the potential presence of PCR inhibitors (Akram et al. 2017).

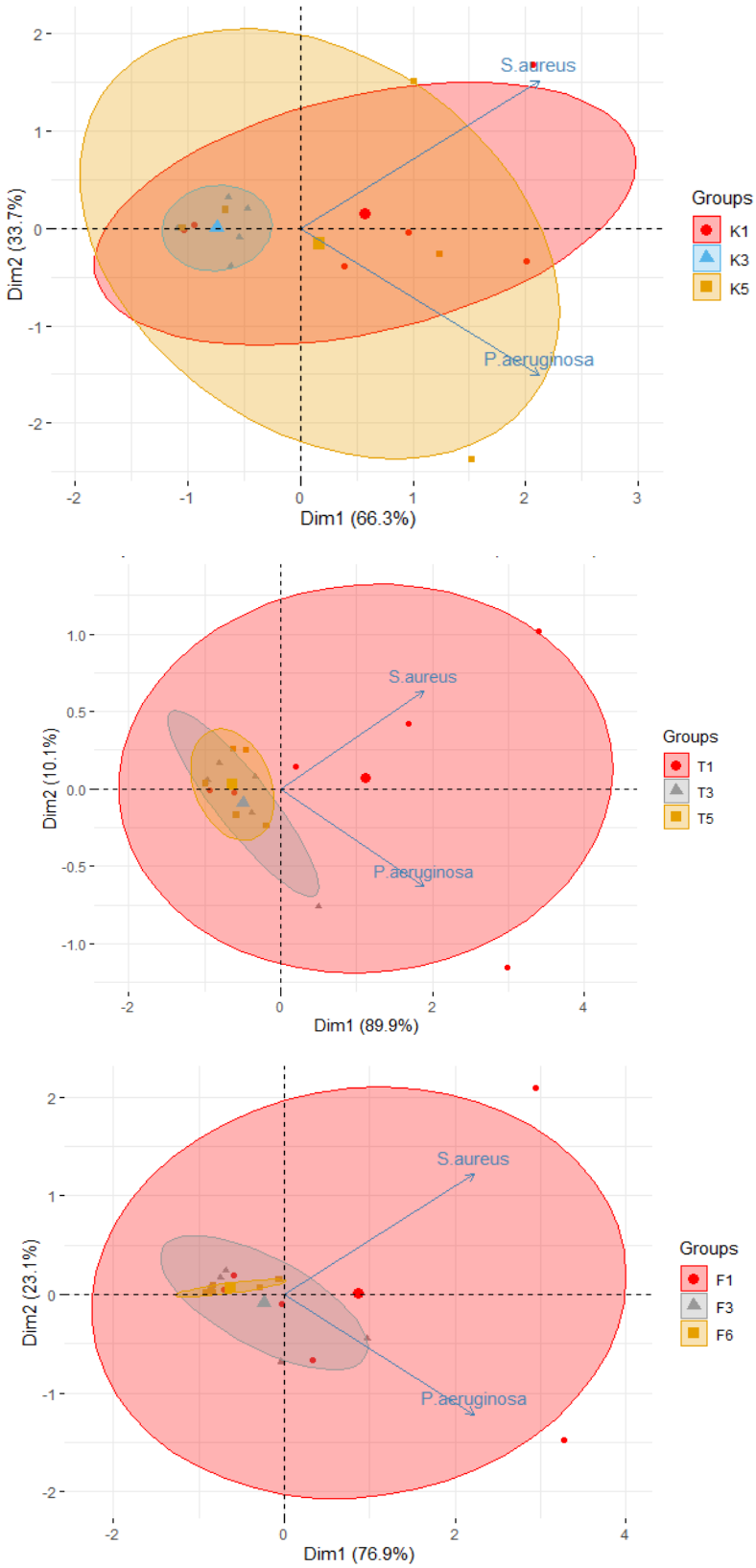

Fig. 6 Principal Component Analysis (PCA) on the distribution of bacteria; *Pseudomonas aeruginosa: P. aeruginosa; Staphylococcus aureus*: *S. aureus;* Lake Kossou sampling sites: K1, K3, K5; Lake Taabo sampling sites: T1, T3, T5; Lake Faé sampling sites: F1, F3, F6

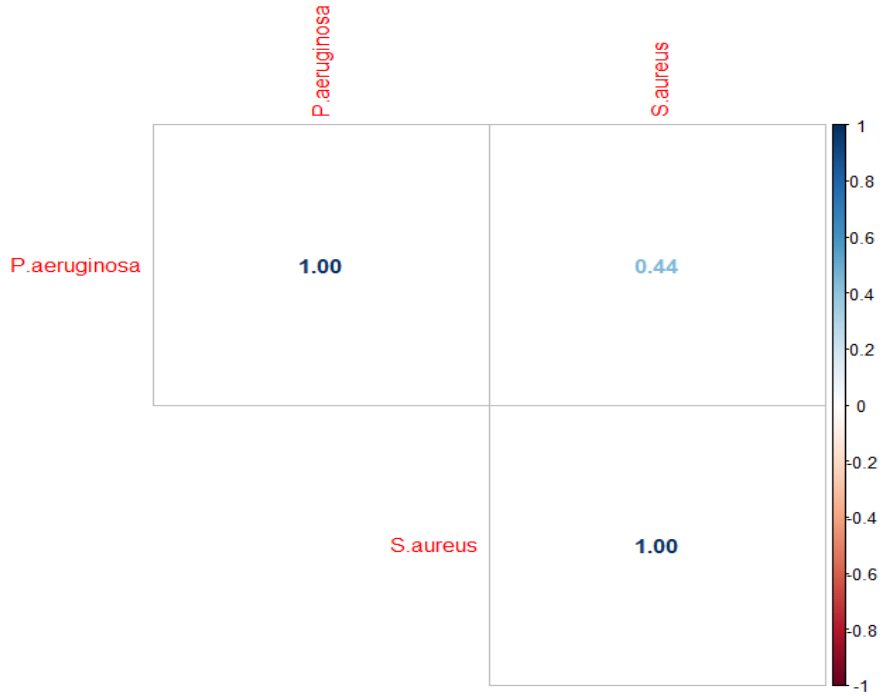

Fig. 7 Relation between pathogen bacteria in all hydroelectric reservoirs

The highest abundances of *P. aeruginosa* and *S. aureus* were generally recorded during the last four sampling months, namely June, July, September, and October. The sampling months with the highest levels of pathogenic bacteria abundance in hydroelectric reservoirs are characterized by intense rainy periods. This could explain the high abundance of bacteria. Indeed, during rainy periods, wastewaters, sewage, and faecal matter are rapidly discharged into surface waters, simultaneously releasing multiple bacterial species, particularly those linked to the activities practiced in the watersheds (Coulibaly-Kalpy et al. 2016; Kiran et al. 2018; Saturday et al. 2021). Several researchers showed that these hydroelectric reservoirs are influenced by human activities in the watersheds and upstream (Da costa and Diétoa 2007; Groga et al. 2012; Koné 2012; Kouao et al. 2012).

The highest concentrations of pathogen bacteria were obtained in shore areas in the different reservoirs. This could be due to the fact that shore areas are the primary receptors of anthropogenic discharges. These different sampling points (T1, K1, K5, and F1) were characterized by household activities and livestock farmland. Faecal pollutants, wastewaters, and sewage resulting from these activities contribute to the degradation of water quality and the discharge of many pathogenic bacteria (Coulibaly-Kalpy et al. 2016; Kouamé et al. 2019; Chowdhary et al. 2020; Lin et al. 2022; Gamze et al. 2023). Esiobu et al. (2013) and Adriana et al. (2020) respectively showed the influence of human discharges on the variation in abundance *of S. aureus* and *P. aeruginosa*. Despite the river inflows, which cause continuous mixing of the reservoirs, the pathogenic bacteria remain concentrated in the littoral zones. This could also be due to the hydrodynamic processes of the reservoirs. Indeed, hydrodynamic conditions are less effective in these shallow areas, leading to reduced water circulation and the formation of retention zones. The lower dilution capacity, combined with higher organic matter availability and favorable environmental conditions, further supports bacterial persistence. Moreover, sediments in littoral zones can act as reservoirs for bacteria, which may be periodically resuspended into the water column (Brookes et al. 2003; Pachepsky and Shelton, 2011). Pathogen bacteria abundance levels show the utilization risk of the dam waters due to diseases that may be induced by these bacteria (Manizan et al. 2009; Coulibaly-Kalpy et al. 2016).

The results indicated also weak correlations between these pathogenic bacteria. These weak correlations could be due to the diversity of anthropogenic activities in catchment areas (Groga et al. 2012; Kouao et al. 2012). Indeed, each anthropogenic input, wheth-

er industrial, domestic or agricultural, could contribute differently to the growth of pathogenic bacteria (Zhang et al. 2021). Goh et al. (2019) also obtained weak correlations between pathogen bacteria present in reservoirs.

5. Conclusion

The aim of this study was to assess the variation in *Pseudomonas aeruginosa* and *Staphylococcus aureus* abundance in the Kossou, Taabo, and Faé reservoirs. Culture and PCR methods showed that both approaches can be used to achieve good results. The highest concentrations of pathogen bacteria were obtained in shore areas in the different reservoirs and during the last four sampling months, namely June, July, September, and October, characterized by intense rainy periods. Human activities continue to exert pressure on the microbial quality of these environments. These results highlight the importance of these reservoirs as areas where pathogens proliferate, exposing local populations to health risks linked to the water used for irrigation, fishing, or household chores. A comprehensive surveillance approach for other pathogenic bacteria, including quantitative techniques (qPCR) and the monitoring of antibiotic resistance, is recommended. However, it is important to establish protection zones around reservoirs and treat wastewaters before discharge.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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