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Anti-virulence as a Novel Strategy for Combating Multi-drug Resistant *Escherichia coli*

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ABSTRACT

Escherichia coli is a Gram-negative bacterium that has the potential to be either harmful or beneficial as a foodborne pathogen, nosocomial organism, or part of the normal microbiota. It is one of the most prevalent nosocomial pathogens in the human population and is a life-threatening risk. *Escherichia coli* is the primary causative agent of urinary tract infections, gastrointestinal tract infections, pneumonia, surgical site infections, hemolytic uremic syndrome, sepsis, and neonatal meningitis. The production of virulence factors such as biofilm, motility, serum resistance, and protease enzymes by *Escherichia coli* contributes to its pathogenicity. The emergence of multi-drug resistant strains of *Escherichia coli*, due to the inappropriate use of antimicrobial agents, has made treatment challenging. Infectious diseases stemming from antimicrobial-resistant *Escherichia coli* represent a global health risk, with significant social and economic implications. Anti-virulence therapy presents a promising approach to combat microbial infections. This strategy revolves around disarming microbial pathogens by attenuating the production of virulence factors and eradicating their ability to harm the host. It is predicted that anti-virulence agents could address infections caused by multi-drug resistant *Escherichia coli*, either as supplementary or as individuals for traditional antibiotics.

1. Introduction

Escherichia coli (*E. coli*) was first documented in 1885 by a German pediatrician named Theodor Escherich, who identified it in the feces of a child affected by diarrhea. In 1893, a Danish veterinarian proposed that *E. coli* consists of various strains, with some having pathogenic properties while others do not, as shown in Figure 1. In the present days, the *E. coli* species is categorized into numerous pathogenic strains that are responsible for various infections and diseases affecting the intestines, urinary tract, and internal organs in both animals and humans [1]. *E. coli* is a typical resident of the gastrointestinal microbiota of humans and various warm-blooded animals. The primary habitat of this bacterium is the intestine of animals and humans, while its secondary habitat includes the environment (biphasic existence). The microorganism gets excreted through fecal matter and it can either die or endure for a certain period in soil, water, or sediments, before infecting a new host [3]. While most *E. coli* strains are non-pathogenic commensal microorganisms, they can act as reservoirs

for antimicrobial resistance (AMR) genes. This is attributed to *E. coli's* capability of horizontal gene transfer, specifically through conjugation for adaptation in the niche. The spread of multidrug-resistant (MDR) *E. coli* is a significant public health issue on a global scale, no longer limited to medical facility environments. *E. coli* has acquired various forms of resistance, such as biofilm, β-lactamases, total protease development, motility, efflux pumps, serum resistance, and porin mutations. In 2017, the World Health Organization (WHO) released a comprehensive list of antibiotic-resistant bacteria of global concern to direct efforts in research, exploration, and advancement of novel antibiotics. Within this compilation, *E. coli* strains demonstrating resistance are identified as a priority [3–6].

2. Diseases caused by *E. coli*

Escherichia coli is the main causative agent of urinary tract infections (UTI), gastrointestinal tract infections (GIT), pneumonia, surgical site infections (SSI), bacteremia, sepsis, neonatal meningitis (NM), hemolytic uremic syndrome (HUS) [6].

E. coli stands as the primary cause of 50% of hospital-acquired UTIs and 85% of community-acquired UTIs. Annually, more than 150 million individuals worldwide endure UTIs. Complications stemming from UTIs impact various human organs such as the bladder, ureters, and urethra, establishing it as a severe disease on a global scale. It is anticipated that a minimum of 50-60% of adult females will experience UTIs at least once in their lifetime. Additionally, *E. coli* ranks among the leading causes of bacteremia among hospitalized patients, posing a significant challenge, particularly for the elderly above the age of 55 to 60. *E. coli* bacteremia cases are expected to escalate alongside the aging global population to rise from 617 million in 2015 to 1 billion by 2030 and further to 1.6 billion by 2050. UTIs are the primary causative in over 50% of *E. coli* bacteremia cases [7–11]. Moreover, *E. coli* can cause pneumonia, impacting elderly individuals with underlying medical conditions that compromise host defenses and result in increased morbidity rates. *E. coli*-induced pneumonia commonly arises in hospital environments as the pathogen enters the respiratory system through dispersion from a primary locus in the gastrointestinal or genitourinary tracts, or via aspiration of oropharyngeal secretions. Furthermore, in developed nations, *E. coli* emerges as the predominant cause of NM, with 30% of all NM cases attributed to this pathogen. Extensive cohort studies have demonstrated that NM associated with *E. coli* continues to play a crucial role in sepsis-related morbidity and mortality rates in infants [8,12,13].

Additionally, it is recognized to induce bloody diarrhea, a medical condition referred to as hemorrhagic colitis, with around 4% of instances progressing to HUS. Furthermore, it is commonly detected in surgical wounds and is acknowledged as the most frequently identified bacterium in cultures of SSIs showing a resistance rate ranging from 13.3% to 15.3%. It has been confirmed that there is an increased risk of SSIs following non-sterile surgical procedures [14,15].

3. Pathogenicity determinants of *E. coli*

The Pathogenicity determinants (Virulence factors) are defined as the capacity of an organism to invade the host and induce a disease. They are the substances that support the bacterium in establishing itself within the host on a cellular level. These determinants can be categorized as either secreted, associated with the cell membrane, or located within the cytosol. The cytosolic determinants enable the bacterium to rapidly undergo adaptive changes in metabolism, physiology, and morphology. Cell membrane-associated determinants assist the bacterium in attaching to host cells and evading their defenses. The secreted determinants play a crucial role in the bacterial arsenal, aiding the bacterium in overcoming the immune responses of the host. In the case of extracellular pathogens, secreted virulence determinants work together to eliminate host cells [16].

The Pathogenicity determinants of *E. coli* are either linked to the surface of the bacterial cell, cytosol or are secreted and transported to their site of activity. The key virulence factors generated by *E. coli* encompass the ability to form biofilms, the polysaccharide capsule, toxins, adhesins, proteases, motility, type 3 secretion system, and iron acquisition mechanisms [17].

3.1. Biofilms

Biofilms represent highly structured communities of microorganisms aggregated in a self-produced extracellular polymeric substance (EPS) matrix and adhering to organic or inorganic surfaces. The biofilm phenomenon involves phases including reversible adhesion, irreversible adhesion, maturation, and then dispersion as shown in Figure 2. These biofilm communities exhibit distinct characteristics absent in freely living cells, notably the capability to shield themselves from external pressures, thus bolstering AMR and facilitating the exchange of antibiotic-resistance genes among different bacterial strains. As per the National Institutes of Health, 80% of all human body infections are linked to biofilms. Microorganisms forming biofilms can inhabit various medical tools like urinary catheters, thereby elevating mortality and morbidity rates, and converting infections into

complications. Moreover, biofilms assume a pivotal role in the majority of persistent infections, like UTIs and chronic wound infections [1,17,18]. The formation of a biofilm occurs through the production of matrix into a protective framework that shields bacteria from external pressures while impeding the entry of antimicrobial agents. Furthermore, the matrix structure obstructs the inner regions of the biofilm, limiting the absorption of oxygen and nutrients, and leading certain cells, termed persisters, to transition into a vegetative state, rendering them resistant to antimicrobial treatments. The matrix plays a crucial role in ensuring biofilm stability and

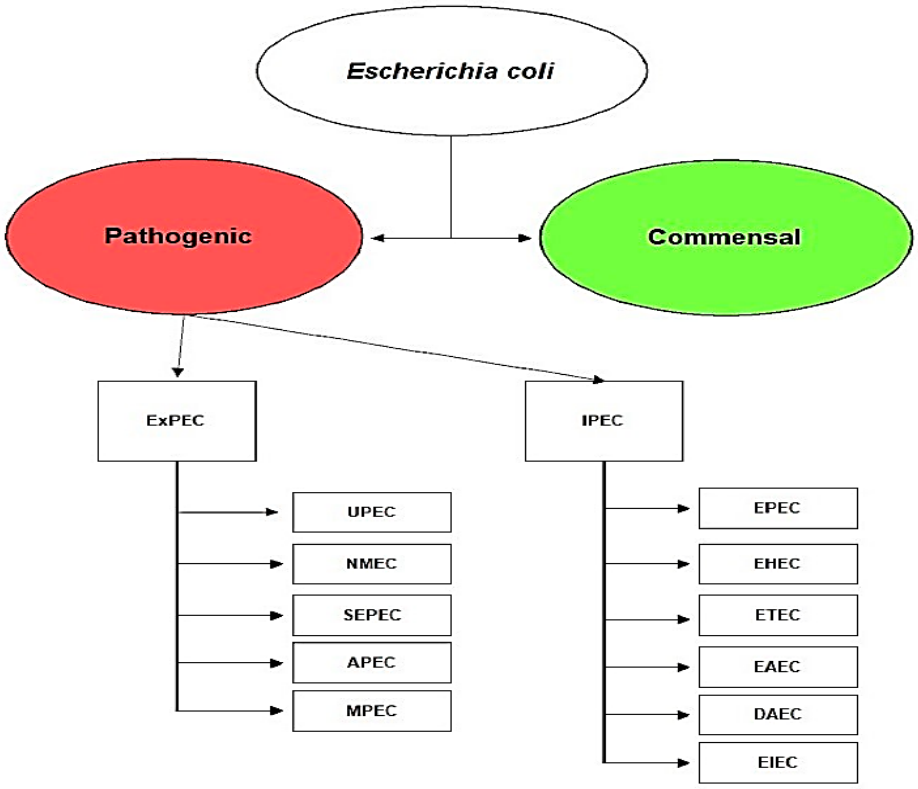

Figure (1). The categorization of extraintestinal and intestinal pathogenic variants of *E. coli* [2]

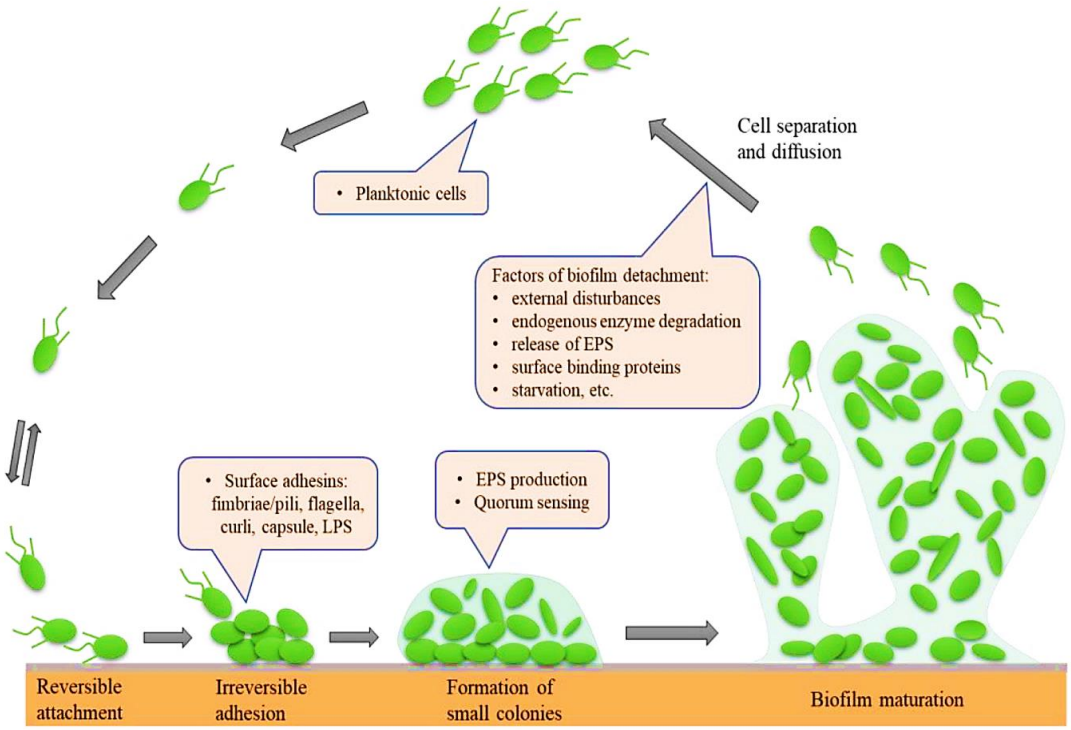

Figure (2). Biofilm formation steps [19]

promoting intercellular interaction, while also facilitating the transport of nutrients and waste throughout the biofilm. Furthermore, the biofilm matrix antibodies, and host immune responses such as complement action and phagocytosis [19,20]. Conventional antibiotic regimens face significant challenges in eliminating a biofilm that has established itself on either an abiotic or biological surface. Despite these constraints, one potential resolution to this dilemma involves utilizing antibiotics in conjunction with compounds possessing anti-biofilm properties [21]. According to previous research [6,22], the anti-virulence effects of some virulence inhibitors can be attributed to their disturbance of the polymeric biofilm matrix and reduction in associated virulence factors. Consequently, the bacteria become powerless and defenseless, thereby reducing the likelihood of resistance. This is due to a decreased selective pressure exerted on the bacteria.

3.2. Serum resistance (Capsule Production)

The spread of Gram-negative bacteria in the bloodstream can be attributed to their capability to develop resistance against the complement system. This essential component of the innate immune system that functions as the primary defense mechanism against microbial infections, consists of a biochemical cascade found in the blood serum, including a sequence of proteins that interact with foreign antigens. When activated, a transmembrane pore is generated in the target cell, resulting in the lysis of the bacterium. The resistance of bacteria to the complement system, which aids in evading the host defense system and promoting infection, is due to their capacity to obstruct the entry of complement proteins to crucial bacterial target sites, such as surface elements like lipopolysaccharide. The development of a surface-bound polysaccharide layer (capsule) is a common feature of *E. coli* strains responsible for causing bacteremia. This capsule expression acts as a protective barrier against host immune responses, like complement factor deposition, by shielding the outer membrane [20].

3.3. Toxins

Toxins are another threatening virulent factor. One of the most well-known toxins produced by certain strains of *E. coli* is called Shiga toxin, also known as verotoxin. This toxin is primarily associated with strains such as *E. coli* O157:H7, which are responsible for foodborne illnesses and can lead to severe complications like hemolytic uremic syndrome (HUS) [23].

Shiga toxin works by inhibiting protein synthesis in the cells it affects, leading to cell death and tissue damage, particularly in the lining of the intestines. This can result in symptoms ranging from mild gastrointestinal discomfort to bloody diarrhea and, in severe cases, kidney failure. Other toxins produced by *E. coli* include heat-stable and heat-labile enterotoxins, which are responsible for symptoms of traveler's diarrhea and other gastrointestinal diseases. These toxins can cause diarrhea and abdominal cramps by affecting the lining of the intestines and altering fluid balance [23,24]. The α-hemolysin (HlyA) pore-forming toxin found in *E. coli* is a distinct category of bacterial toxins that necessitates a posttranslational modification involving a covalent amide linkage of fatty acids to two internal lysine residues for activation. Within a broad range of host cell-specific toxins, the pore-forming HlyA belongs to one class. Its action is directed towards diverse cell types from various species, such as red blood cells, embryo and adult fibroblasts, granulocytes, lymphocytes, and macrophages [25].

3.4. Proteases

The regulation of proteins induced by proteases in bacterial cells has a significant relationship with antibiotic resistance, environmental flexibility, and bacterial infectivity. Manipulating bacterial growth environments by adjusting factors such as temperature, osmotic pressure, nutrient levels, ultraviolet irradiation, or introducing antibiotics or other chemicals has the potential to cause abnormal protein synthesis and accumulation in bacterial cells. In such circumstances, external stimuli can activate bacterial response systems, which in turn can induce the biosynthesis of corresponding proteases. This process may aid bacteria in overcoming unfavorable growth conditions. Most intracellular proteolysis is initiated by energy-dependent proteases, such as *OmpT* (outer membrane protease capable of cleaving T7 RNA polymerase), *StcE* (Secreted protease derived from C1-esterase inhibitor), *Lon* (product of the *lon* gene), and *FtsH* (Filamentous temperature-sensitive H). The ATPase domains of these proteases are accountable for facilitating substrate recognition. Numerous specific proteases found in *E. coli* are highly conserved in both prokaryotes and eukaryotes, and they perform vital functions in the developmental process [26,27].

3.5. Adhesion factors

Adhesion is a critical initial step in the process of bacterial colonization and infection. For *E. coli*, adhesion

factors play a significant role in facilitating attachment to host cells and surfaces. These adhesion factors are often proteins located on the surface of the bacterial cell. Several adhesion factors have been identified in *E. coli*, each contributing to the bacterium's ability to adhere to specific host tissues or surfaces. One well-known adhesion factor in certain pathogenic strains of *E. coli* is called intimin encoded by the *eae* gene and is essential for the bacterium's ability to adhere tightly to intestinal epithelial cells. This adhesion is relevant in *E. coli* strains associated with attaching and eradicating (A/E) lesions, such as Enteropathogenic *E. coli* (EPEC) and Enterohaemorrhagic *E. coli* (EHEC). Other adhesion factors in *E. coli* include fimbriae (pili), which are hair-like appendages on the bacterial surface that mediate attachment to specific receptors on host cells [28].

3.6. Motility

The role of flagella-mediated motility extends to reach a favored site of infection, as it is also implicated in other functions associated with pathogenicity. Specifically, it plays a crucial part in promoting adherence, facilitating biofilm formation, and modulating the immune system. The significance of flagella and their interaction with surface structures is the construction of biofilms and in sustaining microcolony structures through physical interactions [29].

3.7. Type III secretion systems (T3SS)

Gram-negative bacteria utilize type III secretion systems (T3SS) to facilitate the injection of effector proteins into host cells' cytoplasm, thereby inciting virulence. Notably, *E. coli* employs a T3SS to enable the delivery of effector proteins that culminate in the formation of attaching and wiping out lesions. The genomic sequencing of the *E. coli* pathotype O157:H7 has uncovered a gene cluster that encodes constituents of the *E. coli* type III secretion system 2 [25].

3.8. Iron Uptake Systems

E. coli employs mechanisms to acquire iron, an essential nutrient for its growth and survival, particularly in host environments where iron availability is limited due to reservation by host proteins such as transferrin and lactoferrin. The iron uptake systems in *E. coli* are crucial for its pathogenesis and commensal survival. One of the primary iron uptake systems in *E. coli* is mediated by siderophores, small molecules secreted by the bacteria that bind ferric iron (Fe^{3+}) with high affinity. *E. coli* produces several siderophores, including enterobactin, aerobactin, and salmochelin, each with varying affinities for iron and modes of synthesis and transport. Once bound to siderophores, iron is transported into the bacterial cell through specific outer membrane receptors and inner membrane transporters [30,31].

4. Antimicrobial resistance of *E. coli*

E. coli develops resistance to multiple antimicrobial agents, posing significant challenges in clinical settings. The emergence and spread of antimicrobial resistance in *E. coli* can occur through mechanisms such as horizontal gene transfer, mutation, and selective pressure from antimicrobial use. One of the major concerns is the acquisition of genes encoding resistance mechanisms, such as beta-lactamases, which give resistance to beta-lactam, limiting treatment and leading to infections that are difficult to manage [32,33].

Moreover, *E. coli* can develop resistance to other classes of antibiotics, including fluoroquinolones, aminoglycosides, and sulfonamides, through various genetic mechanisms such as target site mutations, efflux pumps, and enzymatic inactivation [5]. The widespread use of antimicrobials in human medicine, agriculture, and veterinary practices has contributed to the selection and dissemination of antimicrobial-resistant *E. coli* strains. Efforts to combat antimicrobial resistance in *E. coli* require an innovative approach, including controlled antimicrobial use, infection prevention and control measures, surveillance of resistant strains, and the development of novel antimicrobial agents and alternative treatment strategies [5,34]. The diversity of antimicrobials and antimicrobial resistance (AMR) genes has arisen because of antagonistic co-evolution among distinct species of environmental bacteria over vast periods. The inappropriate utilization of antimicrobials has led to a selective pressure that facilitates the spread and transfer of these genes among bacteria linked to both humans and other animals. After the initial discovery of antimicrobials, genes encoding mechanisms that degrade, modify, or expel antimicrobials from the cell emerged in *E. coli*. Over time, AMR genes have proliferated across numerous *E. coli* lineages, with their prevalence experiencing a significant rise over the past half-century [3]. The term MDR denotes acquired resistance to a minimum of one agent from three or more antimicrobial classes [35]. MDR clones that thrive are recognized as high-risk, super, or epidemic clones. Bacterial MDR high-risk clones serve as vehicles for the acquisition and dissemination of AMR genes through both horizontal and vertical transmission routes. These clones play a pivotal role in the worldwide emergence and escalation of AMR. MDR high-risk clones possess advantageous transmission characteristics that enable them to outcompete other strains within populations. These attributes encompass ecological adaptability, which allows them to effectively compete, collaborate, and establish themselves within ecosystems to guarantee

their spread and predominance. MDR global high-risk clones are discernible in various bacterial pathogens, notably *Staphylococcus aureus, Pseudomonas aeruginosa,* and the Enterobacterales group (e.g., *Klebsiella pneumoniae,* and *E. coli*) [36]. It has been reported that AMR is higher in low-income countries than in high-income countries. Thus, a recent study has reported on the high prevalence of MDR *E. coli* in Egypt [35].

4. Anti-virulence strategy

The effectiveness of anti-virulence therapy in the prevention of MDR bacterial infections has recently been investigated by researchers. With the rapid failure of antibiotics and the increasing global prevalence of MDR, anti-virulence therapy presents a different approach. Rather than suppressing bacterial growth or killing pathogens, anti-virulence therapy targets bacterial virulence factors to disarm them. By inactivating a portion of the bacterial arsenal of virulence factors, pathogens can be weakened and become susceptible to natural host defenses. There are currently several anti-virulence strategies under development, which have been extensively reviewed both collectively and individually in recent years. Therefore, pharmacological inhibition of virulence factors is an attractive alternative to antibiotics [34,37]. Drug repurposing, also known as drug repositioning, drug reprofiling, or drug rescuing, involves the design of new medicinally active agents from existing or old drugs, pro-drugs, or Food and Drug Administration (FDA)-approved clinically used drugs [38]. One of the alternative methodologies is nanotechnology, where tiny nanoparticles can specifically target various structures within bacteria, thereby rendering the emergence of bacterial resistance impossible. Recent research has been dedicated to the exploration of novel approaches for producing efficient and safe formulations of nanoparticles, whether alone or in conjunction [39].

6. Examples of anti-virulence agents in *E. coli*

6.1. Sitagliptin

Sitagliptin, a pharmaceutical drug, has been utilized in the treatment of type 2 diabetes. Given that *E. coli* is a known pathogen responsible for infecting diabetic feet, it is of paramount importance to investigate the potential anti-virulence properties of sitagliptin against *E. coli* [40]. In our previous research, sitagliptin showed a significant decrease in *E. coli* virulence factors including biofilm development, motility behavior, protease production, and serum resistance phenotypically, genotypically, and in vivo [22]. The repositioning of this hypoglycemic drug has been examined in numerous investigations against other Gram-negative bacteria, wherein it has been found to exhibit notable efficacy in limiting the virulence factors of *P. aeruginosa* and *Serratia marcescens* (*S. marcescens*), including but not limited to biofilm formation, bacterial motility, and proteases production. Furthermore, an early report demonstrated that sitagliptin decreased biofilm development in *S. aureus* [41–43].

6.2. Paracetamol

Paracetamol is utilized as both an analgesic and antipyretic agent. In our previous research, paracetamol showed a significant decrease in *E. coli* virulence factors including biofilm development, motility behavior, protease production, and serum resistance phenotypically, genotypically, and in vivo [22].

Prior research has exhibited its efficacy in other Gram-negative bacteria in impeding the growth of biofilm, protease, and multiple virulence factors generated by *P. aeruginosa.* Furthermore, an additional study reported that paracetamol displays anti-virulence activity in *Acinetobacter baumannii* (*A. baumannii*) [44–46].

6.3. Ascorbic acid

Ascorbic acid, a natural agent, serves as an antioxidant that aids the body in eliminating free reactive oxygen species (ROS) that arise during infection. Vitamin C, as an antimicrobial agent, exhibits direct antimicrobial activity and may modify antibiotic sensitivity [37].

In our previous research, ascorbic acid showed a significant decrease in *E. coli* virulence factors including biofilm development, motility behavior, protease production, and serum resistance phenotypically, genotypically, and in vivo [22].

In other Gram-negative bacteria, ascorbic acid showed the potential to diminish the development of biofilm in Methicillin-resistant *Staphylococcus aureus* (MRSA) and impede the virulence factors associated with *P. aeruginosa*. Furthermore, in a prior investigation, it was demonstrated that ascorbic acid effectively mitigated the generation of *S. aureus* virulence factors, encompassing biofilm formation, staphyloxanthin, proteases, and hemolysin production, along with resistance to oxidative stress, both phenotypically and genotypically, as well as in vivo models [37,47].

6.4. Diclofenac Sodium

The utilization of diclofenac sodium, an FDA-approved non-steroidal anti-inflammatory drug, extends beyond its analgesic properties, as it is also effective in treating an array of inflammatory diseases. In our previous research, diclofenac sodium showed a significant decrease in *E. coli* virulence factors including biofilm development, motility behavior, protease production, and serum resistance phenotypically, genotypically, and in vivo [22]. Previous research has

demonstrated the potential of diclofenac in other Gram-negative bacteria as a virulence inhibitor, with the ability to curtail bacterial colonization and diminish biofilm formation by disrupting the virulence in *P. aeruginosa*. Furthermore, a separate investigation has illustrated the capacity of diclofenac sodium to impede the development of biofilm in potent strains of *Klebsiella pneumoniae* (*K. pneumoniae*) and *E. coli*, commonly associated with urinary tract infections [48,49].

6.5. Nitazoxanide

Nitazoxanide is a novel compound that exhibits extensive-spectrum efficacy against various intestinal protozoa, helminths, and anaerobic bacteria. Its approval for the treatment of infections caused by Giardia intestinalis and Cryptosporidium species underscores its therapeutic potential. The drug is known for its high tolerability, low adverse effects, and brief treatment regimens. Nitazoxanide is a synthetic compound of nitrothiazolyl salicylamide nature that finds its application in the clinical treatment of parasites like giardiasis and cryptosporidiosis. Besides, Nitazoxanide was incorporated into a therapeutic regime for the treatment of Helicobacter pylori infection in pediatric patients. In our previous research, nitazoxanide showed a significant decrease in E. coli virulence factors including biofilm development, motility behavior, protease production, and serum resistance phenotypically, genotypically, and in vivo. In prior research, it was discovered that nitazoxanide exerts an inhibitory effect on the assembly of adhesive pili on the surface of E. coli [22,46,50–52].

6.6. Zinc Oxide Nanoparticles

A recent investigation delved into the utilization of metal and metal oxide nanoparticles due to their benefits compared to other varieties, such as their smaller size, precise morphology, expansive antimicrobial range, and toxicity towards multiple bacterial structures. Consequently, the ability of pathogens to develop resistance against metal and metal oxide nanoparticles poses a formidable challenge. This recent research showed the anti-virulence activity against the highly virulent *E. coli* O157:H7 and *P. aeruginosa* [39].

6.7. Cranberry active compound proanthocyanins (PACs)

Cranberry harbors a class of sugars known as proanthocyanidins. Previous research suggests that PACs can downregulate various genes associated with bacterial fitness, virulence, and antibiotic resistance, potentially making it a promising anti-virulence strategy for managing UTIs caused by such *E. coli*. Specifically, PACs were found to impact genes involved in iron acquisition, stress response, DNA recombination, capsule production, and more [53].

7. Conclusions

The virulence factors found in *E. coli* play a crucial role in the development of AMR. The widespread misuse and overuse of antibiotics has exacerbated the issue of MDR *E. coli* against a wide range of available antibiotics, resulting in limiting the treatment options for numerous infectious diseases; hence, novel strategies to address MDR *E. coli* have become imperative. Anti-virulence agents are a promising avenue for tackling MDR *E. coli*, necessitating further research efforts to manage it.

Ethical consideration: All the participants in this study gave their informed permission.

Conflicts of Interest

No conflicts of interest are disclosed.

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