

CRISPR/Cas Systems as Diagnostic and Potential Therapeutic Tools for Enterohemorrhagic *Escherichia coli*

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Abstract

Following its discovery as an adaptive immune system in prokaryotes, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) system has been developed into a multifaceted genome editing tool. This review compiles findings aimed at implementation of this technology for selective elimination or attenuation of enterohemorrhagic *Escherichia coli* (EHEC). EHEC are important zoonotic foodborne pathogens that cause hemorrhagic colitis and can progress to the life-threatening hemolytic uremic syndrome (HUS). Advancements in the application of CRISPR methodology include laboratory detection and identification of EHEC, genotyping, screening for pathogenic potential, and engineering probiotics to reduce microbial shedding by cattle, the primary source of human infection. Genetically engineered phages or conjugative plasmids have been designed to target and inactivate genes whose products are critical for EHEC virulence.

Keywords

EHEC • CRISPR/Cas • Cas9 • Cas12 • Probiotics • O157:H7

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1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), particularly serotype O157 and certain non-O157 strains (collectively known as the “big six”), are significant foodborne pathogens causing severe human illnesses. EHEC infections can lead to complications such as hemorrhagic colitis and the hemolytic uremic syndrome (HUS), the latter being a major cause of acute renal failure in children. EHEC poses a significant public health threat, with complex interactions between the pathogen, its healthy ruminant reservoir hosts, and the environment. In this review, we discuss EHEC virulence mechanisms, transmission pathways, clinical manifestations, microbiome interactions, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) system applications. The CRISPR/Cas system has potential applications for targeting and eliminating EHEC strains from cattle without disrupting the overall microbiome. Understanding EHEC virulence mechanisms and transmission dynamics is crucial for closing the gap between effective prevention and EHEC elimination strategies. Innovative

genetic approaches like the CRISPR/Cas system hold potential for mitigating EHEC risks in livestock and human populations.

2. EHEC Human Infections

EHEC belong to a group of Shiga toxin-producing *E. coli* (STEC). They are zoonotic foodborne pathogens of substantial public health concern (Jiang et al. 2015; Lee et al. 2021; Tahoun et al. 2021). More than 400 STEC serotypes have been identified, with the majority considered as non-pathogenic. While EHEC O157:H7 is the serotype responsible for the most severe human disease, a group of non-O157 STEC strains termed the “big six” (O26:H11, O45:H2, O103:H2, O111:H8(NM), O121:H19, and O145:NM) are responsible for ~64% of the annual STEC illnesses (Beutin et al. 2004; Brooks et al. 2005; Mellmann et al. 2008; Soysal et al. 2016; Kim et al. 2017b; Karmali 2018; Ogura et al. 2018; Fan et al. 2019; Warr et al. 2019; Gardette et al. 2020; Hua et al. 2021; Spano et al. 2021; Bai et al. 2022).

The EHEC infectious dose for humans is estimated to be as low as 10–100 cells (Cameron et al. 2018). Clinical manifestations are broad, ranging from asymptomatic cases to death. The usual outcome involves non-bloody diarrhea and abdominal pain that develops within 3 days after ingestion of contaminated food or water. These symptoms can resolve without further complication or progress to hemorrhagic colitis (bloody diarrhea) in 1–3 days. The bloody diarrhea generally

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subsides after 4–5 days but in some individuals, the disease progresses to life-threatening sequelae, the HUS. HUS is clinically characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury (Lim et al. 2010; Zumbrun et al. 2013; Liu et al. 2019; Gardette et al. 2020; Larzábal et al. 2020; Hua et al. 2021). HUS complicates ~6%–9% of EHEC infections, mostly in the pediatric and geriatric populations (Sauder and Kendall 2018), and has a lethality rate of 3%–5% (Soysal et al. 2016). HUS remains the leading cause of pediatric acute renal failure in the United States and the European Union (Lee et al. 2021).

3. EHEC Virulence

Two cardinal virulence attributes of EHEC intestinal colonization and pathogenesis are Shiga toxin (Stx) production and the ability of bacterial cells to form attaching and effacing (A/E) lesions on host intestinal epithelia (Cameron et al. 2018; Pacheco et al. 2018; Liu et al. 2019; Nawrocki et al. 2020).

Stx is a potent AB₅ cytotoxin with N-glycosidase activity inhibiting protein synthesis. The five B subunits bind the toxin to the surface-expressed receptor, globotriaosylceramide-3. The toxin is internalized and localized to the ribosomes by a retrograde pathway. The enzymic A subunit removes a specific 28S rRNA adenine residue in the 60S ribosomal subunit that results in the inhibition of protein synthesis and induction of ribotoxic stress. Stx can be translocated through the intestinal barrier via transcellular and paracellular mechanisms. This allows the toxin to enter the systemic circulation and induce organ-specific injuries. Kidney glomeruli and small vessel endothelial cells in other organs such as the brain, liver, pancreas, and heart are primary targets (Schuller 2011; Keir et al. 2012; Gardette et al. 2020). Two Stx types (Stx1 and Stx2) with several subtypes are described and individual EHEC strains produce one or both (Hauser et al. 2020; Nawrocki et al. 2020). Stx1 and Stx2 are antigenically distinct but share the same mode of action (Schuller 2011; Hua et al. 2021). The Stx operon is encoded on a lambdoid-like bacteriophage integrated within the bacterial genome. The toxin is induced by bacterial response to stress, including exposure to antibiotics (Gardette et al. 2020; Hauser et al. 2020). Hence, antibiotic therapy during acute EHEC infection is contraindicated (Sauder and Kendall 2018; Hua et al. 2021). Interestingly, Stx1 does not require phage induction to be expressed because it possesses an independent promoter (Karmali 2018). Infections with Stx1-producing STEC are associated with less severe disease than those with Stx2-producing STEC. Moreover, strains producing both Stx1 and Stx2 are often less toxic *in vitro* and *in vivo* than strains producing Stx2 only (Brooks et al. 2005; Lim et al. 2010; Cameron et al. 2018; Nawrocki et al. 2020; Hua et al. 2021).

E. coli O157:H7 and the “big six” non-O157 serotypes use A/E lesions as an adhesion mechanism (Montero et al. 2017). The A/E lesions induce extensive actin cytoskeleton rearrangement in host epithelial cells, culminating in the formation of pedestal-like structures underneath the bacterial cell. This promotes tight bacterial adherence to host cells and effacement of intestinal microvilli. The process is mediated by genes encoded in the locus of enterocyte effacement (LEE) pathogenicity island (Montero et al. 2017; Liu et al. 2019; Jiang et al. 2021). The LEE harbors five major operons that encode a type III secretion system (T3SS), secreted effectors referred to as Esps, the adhesin (intimin), and the receptor for adhesin, Tir. Successful colonization by EHEC is largely dependent on the T3SS—a syringe-like apparatus that injects both LEE-encoded and non-LEE encoded effector molecules into host cells resulting in alteration or disruption of numerous cell processes (Ogura et al. 2009; Cameron et al. 2018; Carlson-Banning and Sperandio 2018; Pacheco et al. 2018; Hua et al. 2021; Jiang et al. 2021). The injected Esps, intimin, and Tir interact with host proteins leading to actin polymerization and formation of A/E lesions on enterocytes (Pacheco et al. 2018). Esps translocated by T3SS also subvert innate immune pathways—specifically those involved in phagocytosis, host cell survival, apoptotic cell death, and inflammatory signaling (Santos and Finlay 2015). Pathogenesis-related functions of *E. coli* O157:H7 Esps have been summarized in our previous review (Kolodziejek et al. 2022).

4. EHEC Transmission

Although many domestic and wild animals can serve as a reservoir of STEC, ruminants, especially cattle, have been recognized as the primary reservoir for *E. coli* O157 and non-O157 serotypes (Caprioli et al. 2005; Jiang et al. 2015; Fan et al. 2019). EHEC colonizes the bovine gastrointestinal tract primarily at the lymphoid follicle-dense mucosa at the terminal rectum, referred to as the rectoanal junction (RAJ) mucosa (Lim et al. 2010). Livestock are silent carriers because they lack Stx Gb3 receptors on intestinal epithelium and the receptors on bovine kidney and brain cells have a lower Stx binding capacity (Vasco et al. 2021).

The healthy cattle carry EHEC transiently and sporadically. The prevalence of STEC in cattle is varied and ranges between 0.4% and 74.0%. This discrepancy reflects the differences in bovine genetics, farm environment, seasonal dependency of colonization, age, and diagnostic methods. Three distinct patterns of animal carriage of *E. coli* O157 are recognized: (1) transient carriage for a short duration (few days) with passive shedding not associated with colonization of the RAJ mucosa, (2) RAJ colonization and shedding for an average of 1 month, and (3) long-term colonization and shedding (>3 months) (Lim et al. 2010). In a feedlot, the majority of cattle are *E. coli* O157 low-level shedders (Kim et al.

2017a). However, a small number of persistently colonized cattle shed large numbers of bacteria ($>10^4$ CFU/g feces). The latter group, referred to as super-shedders, is of great importance due to the increased risk of transmission to other animals, humans, and the environment (Chase-Topping et al. 2008).

Undercooked ground beef is considered the most common vehicle for EHEC outbreaks. Other major outbreaks are linked to consumption of unpasteurized milk, apple cider, fresh vegetables, fruits, and sprouts (Lim et al. 2010) that have been fertilized with ruminant manure or contaminated during harvesting or processing (Caprioli et al. 2005). Additionally, direct contact with EHEC-positive animals (petting zoos, dairy farms) or environmental exposure (campgrounds where cattle have previously grazed) can transmit these pathogens to humans (Lim et al. 2010). Currently, no therapeutics for human EHEC infections are available, antibiotics are contraindicated, and therefore, treatment is limited to supportive care (Cordonnier et al. 2017). Hence, preventive measures to decrease the risk of transmission, including mitigation of EHEC carriage in cattle, have gained increased attention (Sheng et al. 2006; Sperandio and Hovde 2015; Mir et al. 2016; Wells et al. 2017; Compart et al. 2018; Tamminen et al. 2019; Shringi et al. 2021; Kolodziejek et al. 2022; Puligundla and Lim 2022).

5. EHEC and the Bovine Microbiome

Microbial ecology of the bovine gastrointestinal tract is complex, with Firmicutes, Bacteroidetes, and Proteobacteria representing the most abundant bacterial phyla (Zhao et al. 2013; Wells et al. 2014; Mao et al. 2015; Wang et al. 2018; Vasco et al. 2021). These three phyla include certain prevalent genera such as *Faecalibacterium* and *Ruminococcus* (Firmicutes), *Prevotella* and *Bacteroides* (Bacteroidetes), and *Succinivibrio* (Proteobacteria), which are consistently observed in bovine feces and considered a part of the bovine resident microbiota (Mir et al. 2020). Studies on the diversity and composition of the microbial communities are challenging due to the high degree of variability among individual samples, animal-to-animal variation, feed practices, and sample processing (Kim et al. 2014; Wells et al. 2014; Xu et al. 2014; Wang et al. 2018; Larzábal et al. 2020; Mir et al. 2020; Vasco et al. 2021). No consistent difference in the composition of the fecal microbiota of STEC-shedders and non-shedders has been documented (Zhao et al. 2013; Xu et al. 2014; Zaheer et al. 2017; Stenkamp-Strahm et al. 2018; Wang et al. 2018; Salaheen et al. 2019; Vasco et al. 2021).

EHEC cattle colonization requires SdiA, a transcription factor involved in an intercellular signaling process referred to as quorum-sensing (QS). Its proper function and folding are dependent on the interaction with acyl-homoserine lactones (AHLs)—signaling molecules (autoinducers) of the QS

system. Detection and responding to AHLs leads to alteration of the expression of bacterial genes involved in inhabitation of specific niches. It was shown that SdiA harbored by EHEC is required for efficient passage through the bovine gastrointestinal tract. Chemical signaling through SdiA-AHL promoted EHEC survival within the rumen by repressing the LEE expression and activating the glutamate decarboxylase acid-resistance system (*gad*). Following the passage through the acidic bovine intestinal environments, EHEC colonize the RAJ, where, due to the absence of AHLs, the expression of LEE is activated (Sperandio 2010). Since EHEC do not produce AHLs, they are assumed to sense the molecules produced by other, largely unknown, members of the rumen microbiome (Sperandio 2010; Won et al. 2020). On the other hand, microbiota production of bacteriocins, other metabolites, and/or competition for nutrient sources or colonization sites can reduce EHEC numbers (Kim et al. 2017a). The influence of the microbiome on EHEC colonization and vice versa remains an active area of research (Xu et al. 2014; Kim et al. 2017a; Wang et al. 2018; Mir et al. 2020).

The bovine intestinal microbiota is a hotspot for lateral gene transfer due to its high density and diversity of microorganisms (Neil et al. 2020). This genetic transfer across genera drives the rapid evolution and adaptation of bacterial strains by mediating the propagation of various metabolic properties, including symbiotic mechanisms, virulence, biofilm formation, and resistance to heavy metals and antibiotics (Virolle et al. 2020). Conjugative plasmids transfer between different bacterial species residing in the gut microbiota and can be regarded as an efficient DNA delivery system for microbiome editing. DNA mobilization technology could potentially precisely eliminate specific harmful bacteria causing dysbiosis, human disease, or carrying antibiotic resistance genes (Neil et al. 2021). One of the newest research directions aims to selectively eliminate or attenuate EHEC strains from cattle by implementing feed that contains genetically engineered phages or conjugative plasmids carried by bovine commensal *E. coli*. Targeting and inactivating genes critical for EHEC virulence and pathogenicity without causing adverse effects on the bovine microbiome use the CRISPR/Cas system, the most advanced genome-editing tool (Citorik et al. 2014; Kolodziejek et al. 2022).

6. Overview of the CRISPR/Cas System

The CRISPR, along with the Cas, also referred to as the CRISPR/Cas system, is a prokaryotic hereditary and adaptive immune system. It protects bacteria against foreign mobile genetic elements (MGEs) derived from bacteriophages, conjugative plasmids, and transposons (Barrangou et al. 2007). This system occurs naturally in 40% of eubacteria and 90% of *Archaea* (Grissa et al. 2007; Sorek et al. 2008). Importantly, the CRISPR/Cas system can be repurposed as a versatile

genetic editing or regulation tool in a broad spectrum of microorganisms, including *E. coli* (Adiego-Pérez et al. 2019). CRISPR-Cas system protection is based on the incorporation of short DNA sequences called protospacers that have been excised from past invading DNA and placed into the CRISPR array (Figure 1). Following the adaptation process, protospacers become spacers intervening in adjacent, partially palindromic direct DNA repeats (DRs) in the CRISPR locus (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). The DRs are highly conserved regions varying in size between 23 bp and 47 bp, whereas the unique spacer sequence usually remains within the range of 0.6–2.5 × the DR size. Acquisition of a novel spacer occurs in a polarized manner at the proximal end of an AT-rich leader sequence assumed to play the role of a transcriptional promoter (Grissa et al. 2007; García-Gutiérrez et al. 2015). Therefore, positional information of spacers in the CRISPR array reflects a chronological order of exposure to foreign DNA. It represents a sophisticated sequence-specific cellular memory that provides immunity against subsequent attacks (Barrangou et al. 2007; Rath et al. 2015).

If the MGE equipped with regions matching the spacer sequences is encountered again, it will be recognized as foreign and targeted for degradation. Importantly, the foreign protospacer target site can be distinguished from the identical “self” spacer sequence constituting a part of the CRISPR array due to the presence of a short (3–5 bp) specific sequence, referred to as the protospacer-adjacent motif (PAM). PAM is adjacent to the protospacer (target) site that is complementary to the transcribed CRISPR RNA (crRNA spacer) segment (Mojica et al. 2009). Importantly, PAM-dependent target recognition (REC) is one of the crucial factors influencing the accuracy of the CRISPR/Cas-associated genome editing (Lee and Lee 2021). Elimination of foreign genetic entities occurs during the interference stage following annealing of the target invading sequence with its complementary sequence in a processed mono-spacer crRNA molecule. The crRNA molecule, in turn, guides Cas protein to recognize and cleave the complementary sequence (Figure 1) (García-Gutiérrez et al. 2015; Jiang et al. 2015; Pawluk et al. 2018; Long et al. 2019; Ebrahimi and Hashemi 2020).

7. Classification of CRISPR/Cas Systems

The CRISPR/Cas systems are divided into class I and II, which include a complex of multiple Cas proteins and a single large Cas protein, respectively. Further subcategorization denotes six CRISPR/Cas types (I–VI). Class I includes types I, III, and IV, whereas class II comprises types II, V, and VI. Type I, II, and V systems recognize and cleave DNA, whereas type VI can edit RNA, and type III edits both DNA and RNA (Makarova et al. 2020).

Each of these systems utilizes different mechanisms to produce CRISPR components and demonstrates its own characteristics. The CRISPR/Cas systems differ in their PAM regions, Cas protein sizes, and the cleavage sites. The characteristics of the main CRISPR/Cas systems have been extensively reviewed (Liu et al. 2020).

Since the structures of the type II CRISPR/Cas9 and type V CRISPR/Cas12a (Cpf1) systems representing the class II are relatively simple, they have been used for genome engineering in many organisms, including *E. coli* (Cho et al. 2018; Ebrahimi and Hashemi 2020; Dong et al. 2021).

The endogenous type II CRISPR machinery derived from *Streptococcus pyogenes* is used most frequently in gene editing (Cho et al. 2018; Ebrahimi and Hashemi 2020) due to its high efficiency, simple design, and simple operation (Liu et al. 2020). It consists of two principal components: a single-guide RNA (sgRNA) and Cas9 endonuclease (Figure 1). Both components form a complex. As a result, the enzyme is directed to the target site complementary to sgRNA promoting site-specific cleavage (König et al. 2018). The CRISPR locus undergoes transcription mediated by RNA polymerase into a long precursor crRNA (pre-crRNA) subsequently processed into mature crRNAs. The processing mechanism is mediated by *trans*-activating small RNA (tracrRNA) transcribed from a genomic element located upstream of the CRISPR locus (from a repeat region). The tracrRNA, pre-crRNA, and Cas9 protein form a complex in which the tracrRNA activates endoribonuclease III (RNaseIII) to promote the pre-crRNA maturation. The pre-crRNA is cleaved into small crRNAs. Mature crRNA consists of a complete spacer sequence and a partial repeat sequence. Mature crRNA that is fused to tracrRNA is called an sgRNA. This hybrid subsequently combines with Cas9 protein, forming an active ribonucleoprotein complex (Figure 1). Subsequent attacks by the same invader initiate the interference process, during which the crRNA subunit of the effector complex guides Cas9 toward the foreign genetic element via a reciprocal base pairing mechanism (Figure 1). The fact that sgRNA can effectively recognize specific sequences and direct the action of Cas9 protein is the essence of this protective memory system.

The Cas9 endonuclease is a structurally bilobed enzyme, consisting of the REC and nuclease (NUC) sites. The REC lobe is required for the binding of sgRNA and DNA. The NUC lobe contains RuvC and HNH NUC domains (Figure 2). The RuvC domain cleaves the same single strand (non-complementary) as the protospacer sequence, whereas the HNH domain cleaves a single strand with 20-nt fragment homology to the crRNA complexed with tracrRNA in sgRNA. As a result, Cas9 induces site-specific in the target sequence (Figure 2). This is almost always irreversible damage. The PAM region of Cas9 is at the 3' end of the target 5'-NGG-3' sequence (Nishimasu et al. 2014). CRISPR/Cas9-mediated genome cutting kills cells that fail to be edited successfully;

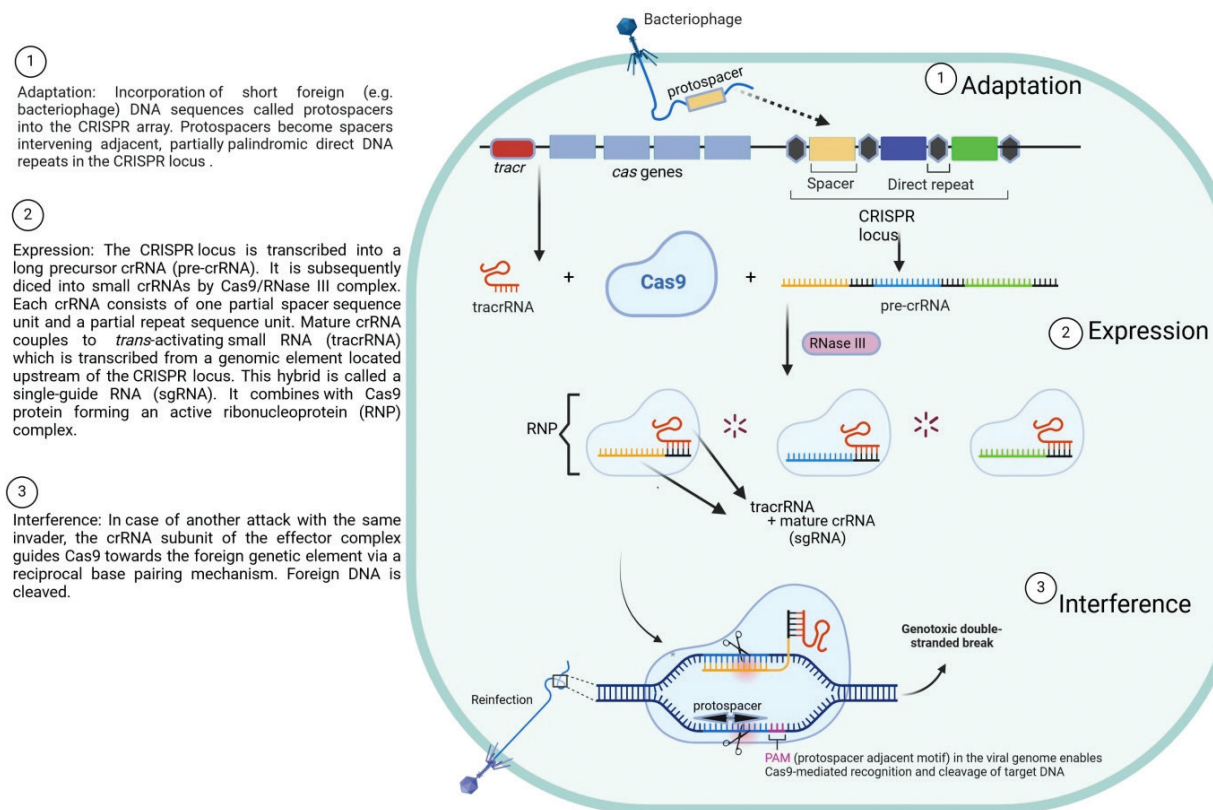


Fig 1. The natural CRISPR/Cas9 antiviral defense system. The figure was prepared using the BioRender.com. Cas, CRISPR-associated proteins; CRISPR, clustered regularly interspaced short palindromic repeats; pre-crRNA, precursor crRNA; RNaseIII, endoribonuclease III; sgRNA, single-guide RNA; tracrRNA, trans-activating small RNA.

hence, markers for the selection of mutants are not required (Dong et al. 2021).

CRISPR/Cas12a (CRISPR-Cpf1) is the archetype of the type V system. Its working principle comprises crRNA and Cas12a protein derived from *Francisella novicida* (Figure 3). There are several important differences between the type Cas9 and Cas12a systems. Double strand breaks (DSBs) the Cas12a enzyme is able to process its crRNA guide autonomously. The Cas12a-associated CRISPR arrays are processed into mature crRNAs without the requirement of tracrRNA. As the Cas12a-crRNA complexes cleave target DNA molecules without the requirement for any additional RNA species, the design and delivery of genome-editing tools are simplified. For example, the shorter (~42 nt) crRNA employed by Cas12a has an advantage over the long (~100 nt) sgRNA in Cas9-based systems, as shorter RNA oligos are easier and cheaper to synthesize (Zetsche et al. 2015; Adiego-Pérez et al. 2019). Cas12a has a potential to implement multiple-gene editing by designing crRNA arrays. Moreover, the enzyme recognizes T-rich (TTTV) (V = A, G, or C) PAM regions, which provides a supplement to Cas9 that requires the NGG (N = A, G, C, T) PAM (Zhu et al. 2022). As a result, the Cas12a-crRNA complexes cleave target DNA preceded by a short T-rich PAM, in contrast to the G-rich PAM following the

target DNA in Cas9 systems (Zetsche et al. 2015). The T-rich PAM of Cas12a can expand the scope of target sites in the organism's genome (Ao et al. 2018). In addition, Cas12a has a single NUC domain, RuvC in contrast to two domains present in Cas9 (Figure 3). As a consequence, it sequentially cleaves the non-targeting strand and the targeting strand to form the DSBs. The enzyme introduces a staggered DNA DSB leaving sticky ends with a 4 or 5-nt 5' overhang (Figure 3) (Zetsche et al. 2015; Liu et al. 2020). Moreover, the smaller size of Cas12a compared with Cas9 (1200–1300 amino acids vs. 1368 amino acids, respectively) decreases the metabolic burden imposed on host cells and facilitates genetic manipulation (Ao et al. 2018; Liu et al. 2020).

8. Delivery of Engineered CRISPR/Cas Systems to Target Bacteria

Plasmid conjugation and viral transduction represent two major mechanisms of horizontal gene transfer in bacteria exploited in the CRISPR/Cas-based technology (Citorik et al. 2014). The advantage of the horizontal gene transfer by conjugation is the availability of plasmids with wide host ranges. Contact between donor and recipient cells contributes to the

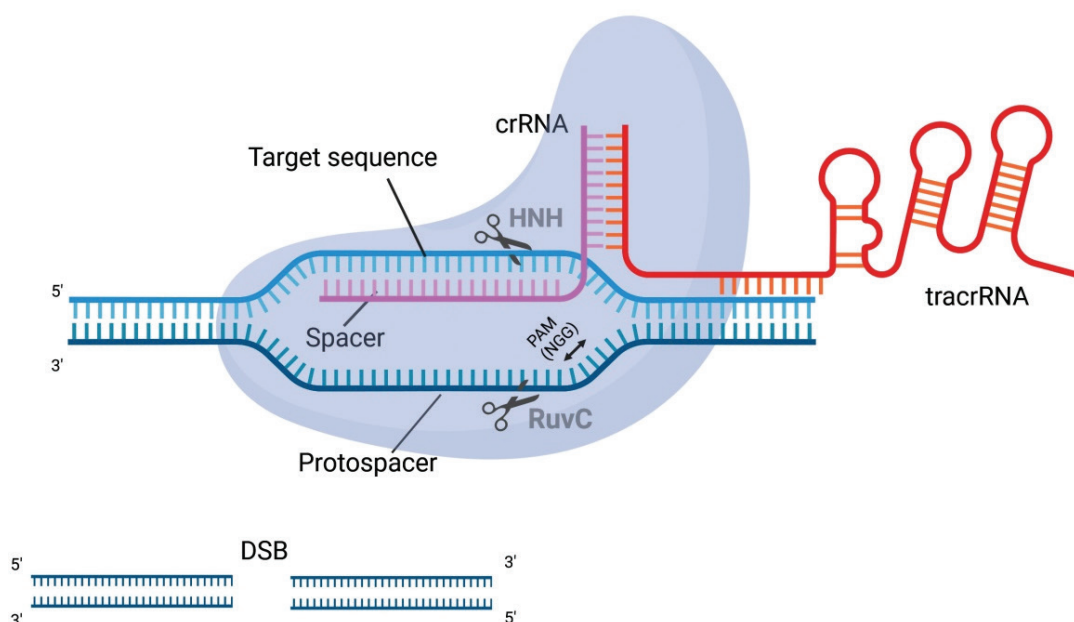


Fig 2. The working principle of Cas9. Active ribonucleoprotein complex is formed by Cas9 protein and sgRNA, a hybrid of crRNA and tracrRNA. Cas9 contains two NUC domains: RuvC and HNH. The RuvC domain cleaves the protospacer sequence on the non-complementary strand. The HNH NUC domain cleaves a single strand containing 20-nt homology to the mature crRNA. G-rich (NGG) PAM follows immediately 3'- of the crRNA complementary sequence and is required for the cleavage. Site-specific DSBs in the target sequence with blunt ends are formed. The figure was prepared using the BioRender.com. Cas, CRISPR-associated proteins; crRNA, CRISPR RNA; DSBs, double strand breaks; NUC, nuclease; PAM, protospacer-adjacent motif; sgRNA, single-guide RNA; tracrRNA, trans-activating small RNA.

efficiency of conjugation and relatively fast delivery (~46,000 base pairs/min). The efficiency of the plasmid transfer through the targeted population is also influenced by the composition and density of microbial communities, nutrient and oxygen availability, colonization niches, and mating pair stabilization (Citorik et al. 2014; Neil et al. 2021; Sheng et al. 2023).

DNA delivery by phage injection is faster than through conjugation and viruses can spread rapidly through a large bacterial population (Puligundla and Lim 2022). The drawbacks include the requirement for threshold numbers of the bacterial targets, a limited host range (phages are bacterial species- or strain-specific), and phage-resistant mutants (Puligundla and Lim 2022). Interestingly, phage-host engineering can be utilized to tackle the host range restriction. One of the studies harnessed the CRISPR/Cas9 methodology to edit the genome of T2 phage and expand its host range to include *E. coli* O157:H7. The chimeric T2 phage had both genes encoding the long and short tail fibers replaced with the corresponding genes of the O157:H7-specific phage. The resultant recombinant phage showed increased adsorption ability to *E. coli* O157:H7 (Hoshiga et al. 2019).

It should be mentioned that MGEs, including phages, can avoid detection by CRISPR-Cas systems through accumulation of mutations in their protospacers or PAM. On the other hand, it was shown that phages are unable to avoid the CRISPR-Cas system by mutation alone if they are targeted

by multiple and diverse CRISPR spacers that naturally evolve upon viral exposure. The observed synergy between spacer diversity and high specificity of infection significantly increases overall resistance of a bacterial population (van Houte et al. 2016; Pawluk et al. 2018). Moreover, several CRISPR/Cas systems (type I) can acquire new spacers that enable them to re-immunize themselves against escape mutants in a process named "primed adaptation" (Pawluk et al. 2018). Resistance to CRISPR/Cas can also emerge through anti-CRISPR (*acr*) genes which encode small-sized proteins presumably able to directly block CRISPR/Cas. CRISPR/Cas inhibitors encoded by phages and other MGEs can be disseminated between bacterial communities. If they become integrated into the bacterial chromosome, stable expression of anti-CRISPR proteins may lead to permanent inactivation of the host CRISPR/Cas system. Anti-CRISPR proteins can be used to regulate genome editing using the CRISPR/Cas system (Pawluk et al. 2018).

9. CRISPR/Cas-Based Assays as an Omnidirectional Strategy Against EHEC

In terms of EHEC, CRISPR methodology has found several applications, including their laboratory detection and identification, genotyping, screening for pathogenic potential,

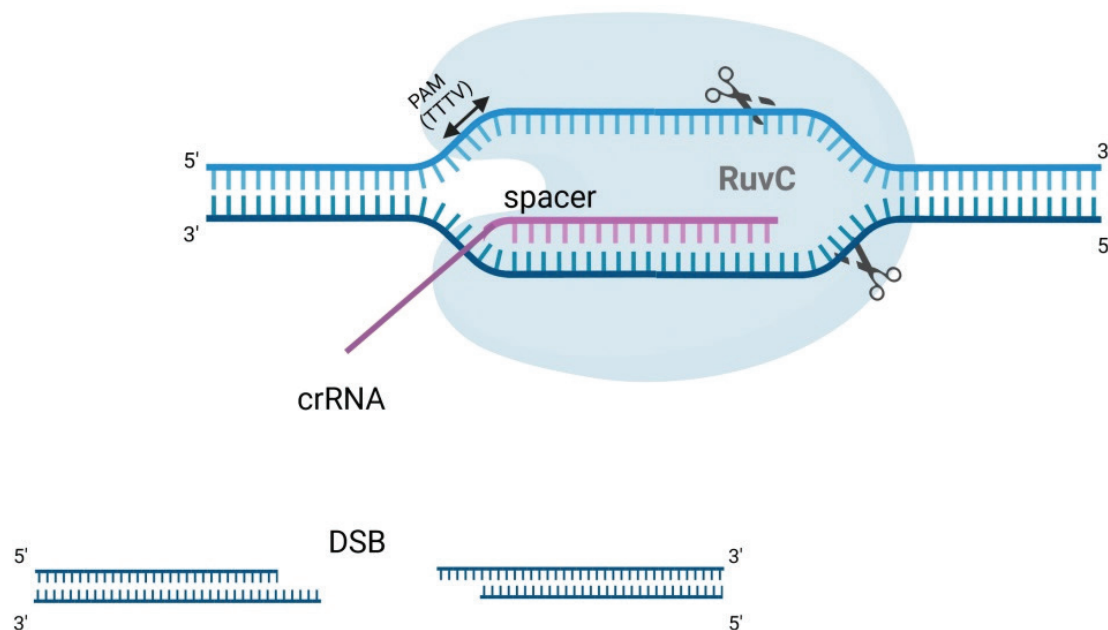


Fig 3. The working principle of Cas12a. Cas12a has a single RuvC NUC domain. TTTV PAM and sequentially cleaves the non-targeting strand. The cleavage site in the targeting strand is defined by the spacer region in the crRNA. The DNA DSBs with sticky ends and a 4 or 5-nt 5' overhang are formed. The figure was prepared using the BioRender.com. Cas, CRISPR-associated proteins; crRNA, CRISPR RNA; DSBs, double strand breaks; NUC, nuclease; PAM, protospacer-adjacent motif; TTTV, The enzyme recognizes T-rich.

as well as engineering probiotics to reduce shedding of *E. coli* O157 by cattle.

9.1. Application of the CRISPR/Cas-based technology in the laboratory detection and identification of EHEC

Recent years have witnessed an increasing interest in the application of the CRISPR/Cas system as a molecular tool for the rapid, sensitive, and specific identification of EHEC in food products of different origins (Table 1). This methodology outweighs important shortcomings of traditional detection strategies, including selective culture and immunoassay approaches which are time-consuming, labor-intensive, and burdened with low sensitivity.

Moreover, CRISPR/Cas-based detection assays have been reported to demonstrate greater sensitivity compared with other DNA-based methods, including the real-time-PCR (RT-PCR) (Wang et al. 2021; Fang et al. 2022; Lee and Oh 2022; Zhu et al. 2023). Due to the high specificity of the target gene detection, in turn, the risk of false-positive results can be significantly reduced. Importantly, the CRISPR/Cas-based assays circumvent the need for complex instruments, which, along with a short reaction time and a nucleic acid detection based on fluorescence (Fang et al. 2022; Lee and Oh 2022; Kim et al. 2023; Song et al. 2023; Zhu et al. 2023; Wang et al. 2024), colorimetric reactions (Jiang et al. 2023), or lateral flow assays (Zhu et al. 2023), provides a potential to implement this technology as an effective point-of-care testing.

Several groups have published encouraging results that hold promise for the future application of CRISPR/Cas-based systems in the food sector as a preventive and control measure for these pathogens.

Pre-amplification of target genes enhances the trans-cleavage activity of the CRISPR/Cas12a that has been used in these studies. Following the crRNA-guided target dsDNA REC and cleavage, *trans*-cleavage activity of Cas12a for the single-stranded DNA (ssDNA) reporter molecules is triggered. Studies reviewed below have used different types of ssDNA reporters, including fluorescence-quencher labeled DNA probes for the fluorescence-based signal readouts or the fluorescein-biotin labeled reporters for the lateral flow detection.

Lee and Oh (2022) developed an assay based on the loop-mediated isothermal amplification (LAMP) of the *stx2* gene combined with the CRISPR/Cas12a system for the rapid (70 min) and sensitive (4.80×10^3 CFU/g) detection of *E. coli* O157:H7 in romaine lettuce. Importantly, this approach enabled omission of the enrichment culture step by using filtration to increase bacterial concentration in the sample material. In one of the most recent studies (Wang et al. 2024), LAMP-CRISPR/Cas12a-based assay was used for the one-pot detection of *E. coli* O157:H7 within an hour. An initial contamination of milk with 7.4×10^3 CFU/mL enabled detection of the microorganism following 3 h of cultivation. Recombinase polymerase amplification combined with the CRISPR/Cas was employed in another study for the rapid (45 min) detection of *E. coli*

Table 1. CRISPR/Cas-based assays used for the identification of EHEC in food products of different origins

Methodology	Target gene	Application	Assay characteristics	Method of detection of the reaction product	References
LAMP-CRISPR/Cas12a (using filtration enrichment)	<i>stx2</i>	Detection of <i>E. coli</i> O157:H7 in spiked romaine lettuce	Turnaround time: 70 min Sensitivity of detection on food products: 4.80×10^2 CFU/g	Fluorescence	Lee and Oh (2022)
LAMP-CRISPR/Cas12a	<i>ecs_2840R</i>	Detection of <i>E. coli</i> O157:H7 in spiked milk	Turnaround time: 60 min Sensitivity of detection in milk: 7.4×10^2 CFU/mL following 3 h of cultivation; 7.4×10^2 CFU/mL without incubation	Fluorescence	Wang et al. (2024)
tHDA-CRISPR/Cas12a (combined with the filter concentration method)	<i>stx2</i>	Detection of <i>E. coli</i> O157:H7 in spiked fresh salad mix	Sensitivity of detection in food products: 10^3 CFU/g	Fluorescence	Kim et al. (2023)
RAA-CRISPR/Cas12a	<i>rfbE</i>	Detection of <i>E. coli</i> O157:H7 in spiked and ground beef samples	Turnaround time: 30 min (after 4 h enrichment in ground beef samples spiked with 9.0 CFU/25 g of <i>E. coli</i>)	Fluorescence	Fang et al. (2022)
CRISPR/Cas12a/RPA	<i>rfbE</i>	Detection of <i>E. coli</i> O157:H7 in spiked romaine lettuce and in natural food products	Turnaround time: 45 min Sensitivity of detection in artificially contaminated samples: $>2.5 \times 10^2$ CFU/mL	Fluorescence and lateral flow chromatography	Luo et al. (2024)
MIRA/CRISPR/Cas12a (combined with the metal organic framework immunomagnetic beads enrichment)	<i>rfbE</i>	Detection of <i>E. coli</i> O157:H7 in ground beef	Sensitivity of target detection in ground beef: 14 CFU/mL (after 4 h of culture through Metal Organic Framework immunomagnetic beads enrichment)	Fluorescence	Wang et al. (2021)
RAA/CRISPR/Cas12a	<i>rfbE</i>	Detection of <i>E. coli</i> O157:H7 in spiked skim milk and drinking water	Turnaround time: 55 min Sensitivity: ~ 1 CFU/mL and 1×10^2 CFU/mL for the fluorescence and the lateral flow assay, respectively	Fluorescence and the lateral flow assay	Zhu et al. (2023)
HCR-CRISPR/Cas12a	<i>rfbE</i>	Detection of <i>E. coli</i> O157:H7 in spiked environmental water samples	Turnaround time: 50 min Sensitivity: 17.4 CFU/mL	Evanescent wave fluorescence biosensor	Song et al. (2023)

CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated proteins; HCR, hybridization chain reaction; LAMP, loop-mediated isothermal amplification; MIRA, multienzyme isothermal rapid amplification; PMNT, cationic-conjugated polythiophene derivative; RAA, recombinase aided amplification; RPA, recombinant polymerase amplification; tHDA, thermophilic helicase-dependent amplification.

O157:H7 and its *rfbE* gene encoding for the lipopolysaccharide. Visualization of the results was based on the CRISPR/Cas12a-derived fluorescence and the lateral flow chromatography (Luo et al. 2024). The authors reported the following limits of detection: 1.8 fg/ μ L and 2.4 CFU/mL for genomic DNA and *E. coli* O157:H7, respectively (fluorescence detection), and 1.8 fg/ μ L and 2.4×10^2 CFU/mL for genomic DNA and *E. coli* O157:H7, respectively (lateral flow detection). Bacterial concentrations $>2.5 \times 10^2$ CFU/g were detectable by both lateral flow detection and fluorescence detection in artificially contaminated samples of romaine lettuce.

The *stx2*-targeted thermophilic helicase-dependent amplification (tHDA)-based CRISPR/Cas12a system combined with the filter concentration was used by Kim et al. (2023) to detect low (10^3 CFU/g) levels of *E. coli* O157:H7 in fresh salad mix and employed a visual detection of amplicons under the UV light. Fang et al. (2022) developed a recombinase aided amplification (RAA)-assisted CRISPR/Cas12a system for the fluorescence detection of *E. coli* O157:H7 *rfbE* gene in beef samples. The presence of the microorganism in ground beef samples could be detected after 4 h of enrichment with the initial inoculum of 9.0 CFU/25 g. The

same target gene was used by Wang et al. (2021) in the CRISPR/Cas12a-based assay combined with the metal organic framework immunomagnetic beads enrichment for the detection of *E. coli* O157:H7 (the limit of 14 CFU/mL within 4 h) in ground beef. Zhu et al. (2023) employed the *rfbE* targeted RAA-CRISPR/Cas12a system and achieved signal visualization by both fluorescence (~ 1 CFU/mL) and the lateral flow assay (1×10^2 CFU/mL), depending on the type of ssDNA reporter used. Skimmed milk and drinking water spiked with different concentrations of *E. coli* were used in this study to evaluate the potential of the method for its practical application *in situ* and yielded satisfactory results in terms of sensitivity. Moreover, optimization of the reaction process allowed fast (55 min) signal visualization. Jiang et al. (2023) proposed a colorimetric detection of *rfbE* amplicons in *E. coli* O157:H7 by the combination of RAA-CRISPR/Cas12a and a cationic-conjugated polythiophene derivative (PMNT) mixed with the ssDNA. This technique has an advantage of signal observation with the naked eye.

A target DNA amplification-free detection of *E. coli* O157:H7 integrating a hybridization chain reaction (HCR) and CRISPR/

Cas12a was also proposed. The novel method developed by Song et al. (2023) employs the target *rfbE* gene. Its presence stimulates the *trans*-cleaving activity of Cas12a, which leads to cleavage of the ssDNA HCR initiation chain and a subsequent decrease in the fluorescence signal. If the target is absent, the *trans*-cleaving activity of Cas12a cannot be triggered and the intact HCR initiation chain is involved in the HCR reaction that leads to the generation of a fluorescent signal. Applicability of this assay for *E. coli* O157 detection was validated in the study using environmental water samples spiked with different bacterial concentrations.

Future strategies and challenges associated with CRISPR/Cas-based assays for EHEC detection include optimization and lowering of the target gene detection limits directly in food products, improvement of effective bacterial concentration in investigated samples, development of closed detection systems for prevention of sample contamination and simplification of the operation system, as well as portability of devices used for this purpose.

9.2. CRISPR/Cas-based methodology in studies of EHEC epidemiology

Analysis of CRISPR has the potential to be implemented for epidemiological studies and genotyping of EHEC. CRISPR represents one of the most rapidly evolving components of the genome comprising almost identical repeats and highly specific spacers (Bai et al. 2022). Since a given cell lineage can experience diverse genetic encounters, the spacer content (including their number and particular sequences) within the CRISPR locus may demonstrate significant divergence and polymorphism among different bacterial strains (García-Gutiérrez et al. 2015; Bai et al. 2022). Therefore, the CRISPR sequences are phylogenetically informative (Jiang et al. 2015) and this system can be used as a biomarker for genotyping (Bai et al. 2022).

In the study of Jiang et al. (2015), CRISPR alleles from STEC strains isolated from beef processing plants and belonging to the same serogroup demonstrated a similar spacer content and order, regardless of the isolation source, supporting the hypothesis that CRISPR loci are conserved among phylogenetically related strains.

Delannoy et al. (2012b) explored the genetic diversity of the CRISPR regions of EHEC using simplex real-time PCR assays for each of the seven most important EHEC serotypes and revealed that CRISPR sequences can be used as suitable genetic markers for selection of EHEC from sample materials. The study demonstrated greater specificity of the identification of EHEC serotype-specific CRISPR sequences compared with the O-antigen gene-based PCR protocols. Their finding bears an important practical implementation as the sequences derived from the O-antigen genes can be detected in Stx-negative *E. coli* strains as well. A

high-throughput real-time PCR investigation of CRISPR loci in a large ($n = 958$) collection of *E. coli* strains revealed that CRISPR polymorphisms showed a strong correlation with both O:H serotypes and EHEC virulence factors encoded by *stx* and *eae* genes. The CRISPR sequences chosen for simplex real-time PCR enabled differentiation between EHEC and non-EHEC strains. Specificity and sensitivity of the CRISPR PCR ranged between 97.5%–100% and 95.7%–100%, respectively, with the highest sensitivity recorded for the assays targeting serotypes O145:H28, O103:H2, and O45:H2. Of note, the combined usage of two simplex PCR assays targeting different sequences of the CRISPR loci for EHEC O26:H11 and O157:H7 serogroups was characterized by the sensitivity of 100% and 99.5%, respectively.

The same group of authors developed a single PCR targeting the CRISPR locus of enteroaggregative *Escherichia coli* (EAEC) O104:H4 (CRISPR_{O104:H4}). This diagnostic approach was particularly desirable due to the fact that the hybrid EAEC STEC O104:H4 strains lack unique biochemical traits which makes their detection with cultural and phenotypic tests challenging (Delannoy et al. 2012a).

Another study revealed that genotyping based on the diversity of CRISPR loci in diarrheagenic *E. coli* strains including STEC showed better discriminatory power compared with serotyping. Strains sharing the same serotype could be classified into different CRISPR types (CTs), with an example of O157:H7 serotype, which was divided into eight CTs. Moreover, the CRISPR spacer polymorphism provided clues for inferring the pathogenic potential of strains representing different CTs as a significant correlation between CRISPR typing and the number of observed virulence genes was found. Additionally, STEC strains representing different CTs demonstrated different adhesion abilities in the Caco-2 cell assay (Bai et al. 2022).

García-Gutiérrez et al. (2015) inferred the pathotype and a pathogenic potential of 126 strains of *E. coli* (including 17 EHEC strains) and other closely related bacterial species, and by comparing the number of their CRISPR-Cas repeat units with the presence of specific virulence genes. The study suggested an evolutionary connection between CRISPR and *E. coli* pathogenicity and supported a defensive role of CRISPR as a driving force that contributes to the emergence of pathogenic strains. The median number of repeats was significantly higher for commensal isolates than for the pathogenic ones, indicating a negative correlation between the number of the CRISPR units in the system and the presence of pathogenicity traits.

Pacheco et al. (2018) developed a genome-wide CRISPR/Cas9 screen for host factors contributing to EHEC infections. Their experimental design employed the library of HT-29 colonic epithelial cell line mutagenized with sgRNA. Each of the annotated human-encoding genes was targeted with four different sgRNAs and a Δ espZ derivative of EHEC strain

was used for infection (deletion of the *espZ* gene increases the T3SS secretion and the host cell death due to EHEC infection). Many of the gRNAs identified targeted loci associated with sphingolipid biosynthesis, particularly involved in production of the Stx receptor (Gb3). Two loci with unknown functions were also identified. Mutations in these loci protected against the Stx-mediated cell death and prevented cytotoxicity associated with the T3SS of EHEC. The authors speculated that the convergence of Stx and T3SS onto overlapping host targets could guide the design of host-directed therapeutic agents against EHEC.

9.3. CRISPR/Cas-based assays as a potential tool for the selective elimination/attenuation of EHEC

Intimin, encoded by the *eae* gene and an essential virulence factor for EHEC, has been the major subject of studies using the RNA-guided nucleases (RGNs) against this *E. coli* pathotype. Interestingly, their delivery to the target population has been accomplished by different vehicles.

Citorik et al. (2014) designed a phagemid vector to target intimin and showed that treatment of EHEC with Φ RGNeae resulted in a sequence-specific lethality against this *E. coli* pathotype both under *in vivo* and *in vitro* conditions. It was evidenced by a 20-fold reduction in viable cell counts and an improved survival in *Galleria mellonella* larvae infected with EHEC. The authors speculated that improvement in delivery efficiency with Φ RGNeae would have a positive effect on the treatment efficacy and outcome.

In one of the most recent studies, a lambda phage carrying the CRISPR-Cas3 system which is efficient in genome-scale deletions was engineered. The CRISPR array contained a spacer targeting the EHEC *eae* gene. Under *in vitro* conditions, the phage selectively killed EHEC without adverse effects on the growth of other laboratory- and human- intestinal commensal *E. coli* strains. It is noteworthy that EHEC did not develop resistance to the phage in this study. Mouse experiments confirmed the enhanced and strain-specific killing of this engineered phage, while the overall mouse intestinal microbiota was not disturbed (Jin et al. 2022). A potential limitation of this treatment could be the acquisition of phage or plasmid resistance due to resident CRISPR-bearing targets or CRISPR-containing members of the microbiome.

Sheng et al. (2023) engineered a self-transmissible broad host-range conjugative plasmid by introducing Cas9 and gRNA targeting a highly conserved signature sequence of intimin (*eae*) gene. The authors reported an efficient rate of plasmid transfer. The *in vitro* mating assays revealed that the conjugative transfer of the plasmid carrying the gRNA_{eae} selectively killed the EHEC O157 *eae*⁺ but not the mutant deleted for intimin, indicating that the system was specific (Sheng et al. 2023).

Since RGNs can selectively reduce bacteria that contain undesirable DNA sequences, it can be expected that the population equipped with defined genes will be reduced/removed and that the remaining non-targeted microorganisms would have a chance to dominate the microbial community (Citorik et al. 2014). It holds promise for further studies focused on controlling EHEC in cattle reservoirs. Bacterial donor cells such as the bovine commensal *E. coli* carrying CRISPR-engineered phages or conjugative plasmids with RGNs directed against EHEC could be used as probiotics. The probiotic strains could also amplify the CRISPR/Cas9 conjugative plasmids by lateral transfer broadly to other Gram-negative bacteria in the intestinal microbiota, increasing the potential for eliminating EHEC. Finally, libraries of multiplexed RGNs could also be used to simultaneously target a plethora of specific microbial DNA sequences as well as to modulate the composition of microbial communities (Citorik et al. 2014; Sheng et al. 2023).

In summary, development of the CRISPR/Cas system into a multifaceted molecular tool has paved the way for the introduction of novel approaches against EHEC. Recent advances in CRISPR methodology discussed in this review hold promise to improve laboratory detection and identification of EHEC. CRISPR-engineered phages or conjugative plasmids designed to target and inactivate genes whose products are critical for EHEC virulence and pathogenicity have the potential to be implicated as important future strategies against EHEC. There are many avirulent STEC or virulence gene mutant STEC carried by cattle and there are no known side effects. There is no need to eliminate the engineered bacteria. In fact, their persistence and amplification are the goals of the CRISPR engineered conjugative plasmid approach for both bovine treatment and maintenance of EHEC-free cattle.

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Authors Contribution

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Statements and Declarations

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