CellPress REVIEWS

Opinion

CRISPR-Directed Microbiome Manipulation across the Food Supply Chain

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The advent of CRISPR-based technologies has revolutionized genetics over the past decade, and genome editing is now widely implemented for diverse medical and agricultural applications, such as correcting genetic disorders and improving crop and livestock breeding. CRISPR-based technologies are also of great potential to alter the genetic content of food bacteria in order to control the composition and activity of microbial populations across the food supply chain, from the farm to consumer products. Advancing the food supply chain is of great societal importance as it involves optimizing fermentation processes to enhance taste and sensory properties of food products, as well as improving food quality and safety by controlling spoilage bacteria and pathogens. Here, we discuss the various CRISPR technologies that can alter bacterial functionalities and modulate the composition of microbial communities in foods. We illustrate how these applications can be harnessed along the food supply chain to manipulate microbiomes that encompass spoilage and pathogenic bacteria as well as desirable starter cultures and health-promoting probiotics.

Highlights

CRISPR-based technologies have revolutionized genome editing in the past decade.

Many CRISPR tools can be used to manipulate food microbiomes from farm to fork.

Various pathogenic bacteria and spoilage organisms can be targeted and removed using CRISPR-based antimicrobials.

Beneficial bacteria such as starter cultures and probiotics can be enhanced using CRISPR-based genome editing.

Implementation of CRISPR tools in foods requires consumer adoption and regulatory endorsement.

CRISPR Biology and Applications

Technologies based on CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) molecular machines have revolutionized molecular biology, genetics, and biotechnology due to their ability to readily empower genome editing [1]. The CRISPR molecular toolbox is derived from bacterial CRISPR-Cas systems that provide adaptive immunity against viruses and other invading genetic material in many bacteria and most archaea (Figure 1). In brief, the mode of action of CRISPR-based adaptive immunity hinges on DNA-encoded [2], RNA-mediated [3], nucleic acid targeting [4] of invasive elements such as phages and plasmids. During the adaptation step, bacteria acquire a piece of invasive DNA via a copy-paste mechanism which integrates viral DNA into the CRISPR array as a novel spacer [2]. Once vaccinated, bacteria transcribe and process the CRISPR array, during the expression stage, into a collection of CRISPR RNAs (crRNAs) that each contain the genetic information derived from an acquisition event [3]. Thereafter, these crRNAs guide nucleases for sequence-specific targeting and degradation of complementary nucleic acid [5] in the interference stage. Typically, this interference process relies on a signature nuclease which specifically and efficiently cleaves nucleic acid (see [6] for further details of the mechanism).

Over the past decade CRISPR-Cas has been broadly adopted and disseminated across the globe [7] to enable the genome-editing revolution we have witnessed, with the alteration of organisms across the tree of life, encompassing bacteria, archaea, and eukaryotes. Editing genomes has been implemented across a broad range of industries that rely on these workhorses for the genesis of next-generation products in biotechnology, agriculture, and medicine [1]. This has driven innovation spanning from improved yeast for industrial enzymes and biofuels, all the way to antivirals and cell therapies to combat infectious diseases and genetic diseases, respectively [8–10].

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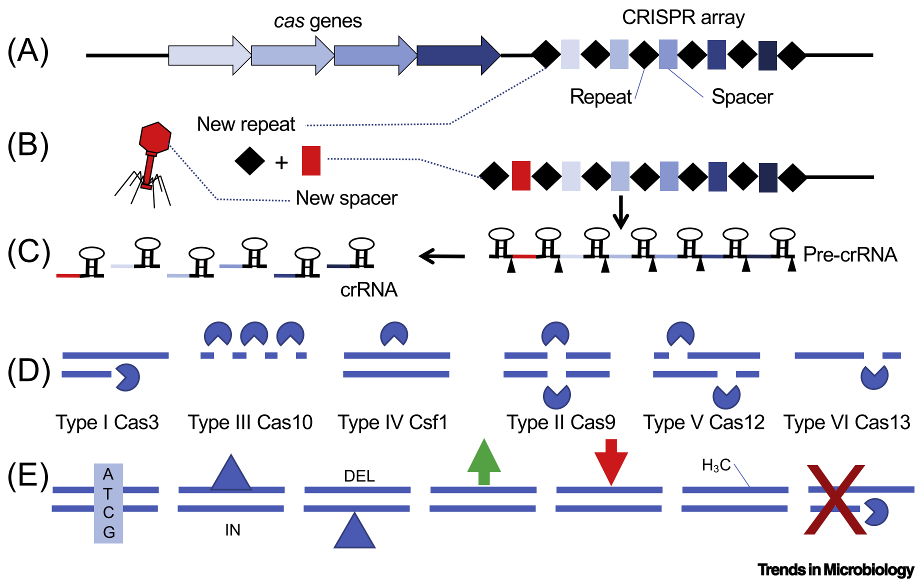

Figure 1. CRISPR-Cas Immune Systems and Applications. (A) CRISPR locus composition with a CRISPR array comprised of a series of spacers flanked by repeats, and the accompanying *cas* genes. (B) Adaptation stage during which invasive DNA is copied and pasted into the CRISPR array as a novel spacer. (C) Expression step generating pre-CRISPR RNA (crRNA) via transcription and then mature crRNAs via maturation, which each contain the genetic material derived from a vaccination event. (D) Interference step across the six major types of CRISPR-Cas system via which DNA or RNA is targeted by sequence complementarity and cleaved in various ways by signature Cas nucleases. (E) Editing applications illustrating how the genome (mutation, insertion, deletion), transcriptome (activation or repression), epigenome (methylation) can be altered, or the cell destroyed (lethal damage in bacteria).

Besides the translational medicine potential, much progress has been made in agriculture using genome editing, with advances underway to improve livestock breeding, crop traits, fruit and vegetable yields, and more [8]. There is much effort in progress to advance product development and process optimization for increased food safety and preservation along the food supply chain. Here, we discuss how CRISPR-based technologies are poised to enhance the global food supply chain, and illustrate how the CRISPR toolbox could be exploited to further advance food safety and preservation by selectively killing foodborne pathogens and spoilage bacteria, or enhancing the functional features of beneficial bacteria used as starter cultures and probiotics.

The CRISPR Toolbox and Its Many Uses

There are diverse classes, types, and subtypes of CRISPR-Cas systems in nature (Figure 1) that target DNA or RNA and rely on idiosyncratic nucleases (endo- or exonucleases) that yield various outcomes (blunt vs. sticky ends) [11]. Specifically, there are two main classes (class 1 relies on effector complexes whereas class 2 relies on single-effector nucleases); six main types (with various signature proteins, notably Cas9 for type II and Cas12 for type V) and already 34 subtypes (with varying accessory *cas* genes) have been documented to date [11]. Thus far, the Cas enzymes from class 2 systems have proven to be most popular with their reliance on single-effector nucleases that trigger precise DNA cleavage. In particular, the endonuclease Cas9 was originally used to drive genome editing in human cells using the single guide RNA (sgRNA)–Cas9 combination [12] to generate blunt DNA cleavage which is repaired by the endogenous DNA repair pathways, leading to editing precisely at the site of cleavage [13,14]. Despite Cas9 diversity in nature, most publications to date rely on the widely popular *Streptococcus pyogenes* Cas9, driven by a synthetic sgRNA. More recently, the ability of Cas12 (formerly known as Cpf1) to generate dual nicks and also generate genome editing (i.e., using the *Francisella novicida*

Cas12) illustrated the wide applicability of various Cas nucleases for genome editing. In addition to driving alteration of DNA sequences, these Cas nucleases can be catalytically inactivated by mutating nickase domains (e.g., RuvC and HNH in Cas9) to exploit deactivated Cas9 (dCas9) as a DNA-binding molecular device. This enables various editing outcomes when dCas9 is tethered to different effector domains, such as transcriptional regulators (CRISPRa or CRISPRi to activate of repress transcription, respectively), deaminases (for base editing to change one nucleotide to another), fluorophores (to tag DNA sequences), or acetylases and methyltransferases (to alter the epigenetic state of DNA). This expanding CRISPR toolbox thus enables scientists to alter the genome, transcriptome, or epigenome of organisms of interest (Figure 1) [1,6].

In addition to engineering portable Cas-based systems, native CRISPR-Cas systems that occur widely in bacteria (~46% of bacterial genomes) and archaea (~90% of archaeal genomes) can be repurposed to drive genome editing. Besides genetic engineering applications, native CRISPR-Cas systems can also be naturally exploited to build up phage or plasmid resistance, constitute genetic tags for genotyping, or be used to selectively target the bacterial genome and drive lethal damage, as programmable antimicrobials [1,6]. Indeed, the exonuclease Cas3 from type I systems is a potent DNA-targeting enzyme which can generate lethal DNA damage, and can be programmed to selectively and efficiently kill bacteria from a consortium and open new avenues for the compositional alteration of a mixed microbial population.

Modifying Bacterial Activity with Genome Editing in Food Fermentation

Application of the various CRISPR-Cas tools holds clear promise to manipulate food fermentation processes along the food supply chain. Generally defined as the microbial process through which substrates (typically carbohydrates) are converted to acids and alcohols, fermentation has widely been used for human food processing and preservation for millennia, even long before the advent of microbiology. Yeast and bacterial fermentation broadly impact desirable organoleptic properties of various foods, encompassing dairy products (yogurt and cheese), drinks (beer and wine), meat (salami and sausage), and vegetables (pickles and sauerkraut). Fermentation usually starts with a complex microbial starter culture and is further shaped by cooperation and competition within the microbial community which also encompasses the indigenous flora. The characteristics of the microbial community at the start and throughout the fermentation define the outcome, hence the final product's flavor and texture. Besides the taste benefits, acidification also prevents microbial spoilage and survival of bacterial pathogens, hereby contributing to product shelf-life and safety. Accordingly, controlling and manipulating the community structure is key to the optimization of existing food products and the development of new ones.

Controlling spoilage bacteria in food products is a high priority for food safety and extending food shelf-life to limit food waste. Here, CRISPR-Cas antibacterial technology has the potential to enhance this process by selectively removing spoilage bacteria, or by removing microorganisms that compete with spoilage-repressing bacteria such as lactic acid bacteria. As CRISPR-Cas can be delivered via engineered phage particles or extracellular vesicles into spoilage bacteria, it can specifically target essential genes within a species of interest, thereby eliminating the bacteria from a microbial population. Such a strategy would be analogous to bacteriophage therapy applications which have been shown to be successful at extending shelf life of products where spoilage bacteria, such as *Pseudomonas*, are eliminated. The specificity of this approach can be defined by the host range of the phage used for delivery, as well as the occurrence of endogenous CRISPR-Cas systems from which the endogenous machinery can be repurposed, and by the distribution pattern of the target sequence used for programming CRISPR nucleases. Furthermore, this approach can be used to specifically program the CRISPR-associated nuclease to selectively target a unique genetic feature that occurs only in *bona fide* pathogenic bacteria, such as virulence factors, toxin-encoding genes, or antibiotic-resistance cassettes. This enables specific targeting of undesirable genotypes and the maintenance of beneficial commensals and non-pathogenic bacteria that occur within the food microbiome. Yet, a broader range is in play in cases where CRISPR spacers are designed to target sequences that are conserved within a given genotype, species, of even genus, depending on the hypervariability (or not) of target sequences. Indeed, some variable sequences within broadly occurring features such as the 16S, 23S, internal transcribed spacer (ITS), or glycolysis genes can be strategically used to define the range of CRISPR specificity and targeting. This flexible programmability is a noteworthy advantage over broad-spectrum antibiotics. Future studies should determine how removing one member within a mixed population may alter the overall dynamics and composition of a whole microbiome. The emergence of spoilage bacteria might also be suppressed by increasing the robustness of the fermenting starter culture. For example, *Streptococcus thermophilus* is an essential member of a starter culture for milk fermentation, and increasing its adaptive immunity against phages via its endogenous CRISPR-Cas system would potentially contribute to a more robust starter culture. Thus, the CRISPR-Cas approach could further diversify the food preservation toolbox.

Fermented food products can also be further altered by metabolic pathway engineering of key enzymes involved in the production of chemical compounds that enhance taste and/or sensory properties of products. For example, the aroma profile of *Saccharomyces cerevisiae* used in beer fermentation has been altered to improve taste, but with reduced alcohol percentage [9]. Similarly, pathway engineering to rewire metabolic fluxes may also be applied to increase the production of secondary metabolites with probiotic properties, such as reuterin produced by *Lactobacillus reuteri* [10]. Given the rapid expansion of (i) databases with species-specific metabolic network models, (ii) computational modeling tools to predict metabolic fluxes upon genetic and environmental perturbations, and (iii) the CRISPR toolbox, it is now possible to systematically predict and experimentally verify the outcome of novel fermentation processes at a large scale [15–17]. These approaches can also be implemented in starter culture workhorses such as *Lactococcus lactis, Lactobacillus bulgaricus* subsp. *delbrueckii,* and *S. thermophilus* to enhance their functional properties such as the genesis of diacetyl flavor, uptake and catabolism of amino acids, and exopolysaccharide synthesis, respectively.

Altering Pathogenic Microbiome Composition with Targeted Killing

Although the presence and growth of spoilage bacteria in food is undesirable, it does not constitute a major health threat *per se*. This is different for pathogenic bacteria that grow and survive in food products, through which they cause health problems due to the production of toxins. Based on available data, it has been estimated that millions of annual illnesses are caused by foodborne pathogens [18]. Together with an ever-expanding global food chain, and rapid increase in demand for safe and sustainable food in developing countries and emerging markets, it is of great importance to increasingly control the growth of pathogens and spoilage organisms in foods. Traditional food processing involves the use of heat treatment (such as pasteurization), sometimes in combination with acids, salt, and various antimicrobial agents to eliminate and/or control commensal food bacteria. Even though this approach is highly effective, and there are many regulatory processes in place to control food safety and manufacturing, such as HACCP (hazard analysis and critical control points) and FSMA (the food safety modernization act), there are still foodborne disease outbreaks on a regular basis. For example, the Centers for Disease Control and Prevention (CDC) reports 839 foodborne disease outbreaks in 2016, with 14 259 illnesses encompassing 875 hospitalizations and 17 fatalities[i].

Exploiting the CRISPR toolbox along the food supply chain (Figure 2) can be used to selectively kill pathogens and thereby reduce foodborne illnesses. Such a programmable antimicrobial is very promising given its specificity and the many concerns about the rise of antimicrobial resistance.

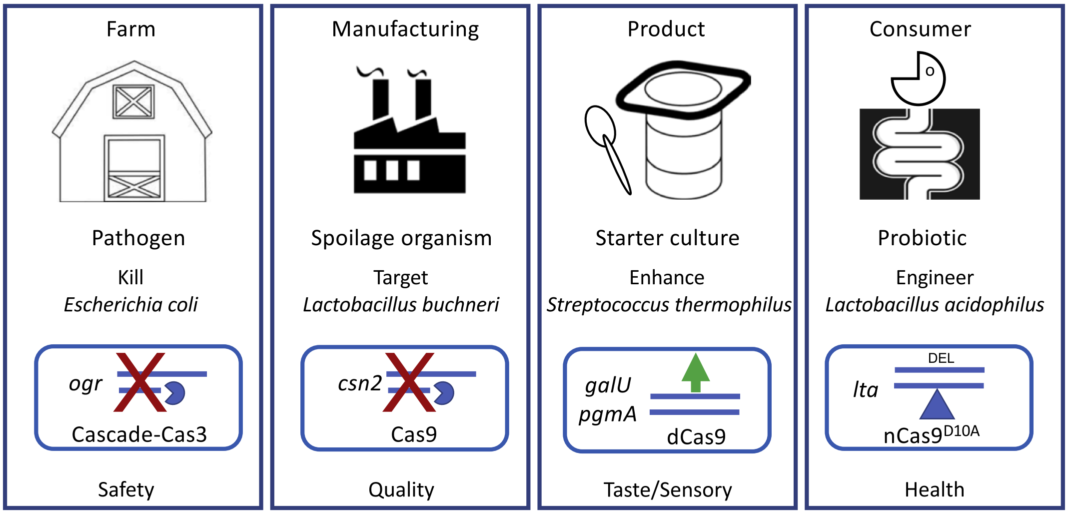

Figure 2. CRISPR Applications across the Food Supply Chain. Select examples show how various organisms, including pathogens, spoilage organisms, starter cultures, and probiotics can be targeted using various CRISPR-based technologies across the food supply chain to impact safety, quality, sensory, and health-promoting attributes, respectively. For instance, pathogenic *Escherichia coli* can be selectively killed at the farm level to promote food safety, by targeting *ogr* using the endogenous type I Cascade-Cas3 machinery. Likewise, spoilage lactic acid bacteria can be targeted during manufacturing to increase microbiological quality and shelf-life of food products, and *Lactobacillus buchneri* can be targeted by directing the endogenous Cas9 to the flanking *csn2* gene. Starter cultures, such as *Streptococcus thermophilus*, can be altered to improve sensory attributes, for instance via using dCas9 to increase the transcription level of *galU* and *pgmA,* genes involved in exopolysaccharide manufacturing to increase viscosity and impact yoghurt texture. Likewise, the surface composition of probiotic bacteria can be engineered, such as removing the lipoteichoic acid synthase *lta* gene in *Lactobacillus acidophilus* using an exogenous nickase version of Cas9, namely nCas9^{D10A}, to alter the immunomodulatory profile.

Furthermore, such a specific approach, in which virulence factors and/or antibiotic-resistant genes of pathogenic bacteria are specifically targeted, would enable the maintenance of the non-pathogenic bacterial community within the food microbiome. The potential of this approach was first demonstrated by Gomaa *et al*. with the specific targeting of *Escherichia coli* and/or *Salmonella,* as well as *S. thermophilus,* using endogenous systems [19]. This study showed how even nearly identical strains can be selectively targeted by using guide sequences uniquely present in one genome, and also illustrated how broader specificity can be achieved by targeting sequences shared between strains and even species. Shortly thereafter Bikard *et al*. and Citorik *et al.* also showed the antibacterial potential of engineered CRISPR-Cas9 systems to combat *Staphylococcus aureus* and *E. coli* [20,21]. Much effort is underway to best select effective targets, multiplex targeting using CRISPR arrays, and optimize the use of both endogenous and exogenous CRISPR-Cas systems across classes and types. Effective target design includes the selection of those genomic regions (e.g., virulence and/or antibiotic genes) which are conserved among pathogenic strains in order to kill pathogens as specifically as possible, while maintaining the rest of the microbial population untouched. Delivery vehicle engineering is another major focus at this point in time, and several approaches are based on bacteriophage- and phagemid-based deliveries as illustrated by the aforementioned studies [20,21]. With recent advances in multiplexed CRISPR array targeting, it is readily possible to concurrently target multiple sequences to enhance efficiency, or strategically target undesirable genotypes such as antibiotic-resistance or virulence genes [22].

Alternative delivery systems include staphylococcal pathogenicity islands (SaPIs), which are mobile genetic elements that encode virulence factors and produce phage-like particles via helper phages [23]. In a recent study, Ram *et al*. engineered SaPIs in order to deliver CRISPR-mediating antimicrobials in mice. Though intriguing and promising, scalability and broad implementability of these so-called antibacterial drones (ABDs) is unclear with regard to targeting a wide range of Gram-positive and Gram-negative pathogens [24]. Although these pioneering CRISPR-mediated antimicrobial studies focus on treatment of infections, they pave the way for future exploitation of the CRISPR toolbox to promote food quality and diversify our ability to prevent food poisoning and spoilage, notably for *Listeria monocytogenes*, for which the ABD-mediated CRISPR approach has been documented [24].

CRISPR Applications to Advance the Food Supply Chain

Effective measures to control pathogen contamination for food safety reasons, as well as controlling starter cultures and fermentation processes, are both important topics with respect to the food supply chain, especially given the current high and ever-increasing demand for food production globally. The food supply chain describes the various steps of food production and consumption from raw material (farming) to the final consumer product (Figure 2). CRISPR-mediated manipulation of microbiomes is anticipated to be exploited at various stages of the food supply chain, starting with the farm environment, for both plant and animal microbiomes. Many efforts are underway to unravel how microbial populations in soil and on plants are driving crop survival and yield increase, through enabling nitrogen fixation, increasing nutrient uptake and catabolism, assisting in drought tolerance, and fending off pest, insect, yeast, fungal and bacterial colonization. Likewise, much of the human probiotic success is being applied to promote host intestinal and immune health in commonly used livestock such as pigs (for pork), poultry (for chicken and turkey), and cattle (for milk and beef). Many companies are developing next-generation probiotics to promote animal growth, optimize feed formulation and conversion, and provide competitive exclusion against pathogenic bacteria *in vivo.* Moving from the farm environment into manufacturing, the fermentation and processing of raw material, such as milk, grain, and vegetables into fermented products, such as yogurt, cheese, pickles, and sauerkraut is highly relevant. Being able to detect and eliminate spoilage and pathogenic bacteria is a key priority for food processors, and being able to do so via various chemical and biological means is important at different stages of processing, through storage and distribution. Being able to use specific CRISPR techniques to suppress the growth of spoilage bacteria after processing could be very useful to extend and maximize shelf life and product consumption (Figure 2). Indeed, contamination and growth can occur at various processing stages, and being able to manage organisms such as *L. monocytogenes* that can be problematic in cold environments is important. One key area of focus is the ability to manage biofilms on surfaces in industrial settings, which are often recalcitrant to disinfectants typically used in food processing [25,26]. Other major foodborne pathogens mostly contaminate food in earlier stages of the supply chain, especially raw material, such as *Salmonella*, *Campylobacter,* and *E. coli* (including *E. coli* O157:H7). A CRISPR-based method to selectively kill and remove these pathogens when contamination is detected could be highly valuable (Figure 2). Despite the use of CRISPR-Cas after contamination, it would also be important to exploit the technique to prevent contamination of pathogens that cause food poisoning in which toxins are produced in foods, which are then consumed by consumers. While CRISPR would not address previously produced toxins, this approach could remove toxin-producing bacteria. These CRISPR-based approaches could be combined with incumbent phage therapy options currently used as sprays or rinses in the food industry [27,28][ii]. Such an integrated approach could be exploited during food processing or early in the food-packaging process as an additive. It can also be used as a spray, shower, bath, or nebulized on animals during early stages of carcass processing [28]. As the familiarity with phage therapies increases, and given the increase in the use of phage applications in the USA [28], we anticipate that interest in these approaches will increase in short order.

Concluding Remarks and Future Perspectives

Biocontrol of pathogens and spoilage organisms along the food supply chain, together with optimizing fermentation and manufacturing processes, is of key strategic importance to food safety and innovation. Given the rapid expansion of the food supply chain, the global expansion of food manufacturing and distribution, as well as the increasing demand for food in emerging and developing markets, we must increasingly provide safe and sustainable food products. Here, we propose a new strategy to enhance the fight against foodborne pathogens and spoilage bacteria, which is driven by the revolutionary CRISPR-based toolbox. The advantage of this strategy is that programmability affords flexibility with regard to both genome editing (to enhance functional traits of probiotics and starter cultures) and targeted killing (to remove pathogens and spoilage organisms), thereby allowing food scientists to optimize the composition and functionality of the various microbiomes implicated throughout the food supply chain, from farm to fork, encompassing crops, livestock, processing lines, manufacturing facilities, and the consumer. Technical enhancements are still needed to optimize specificity, efficiency, and delivery, but there is already much promise and potential for this nascent technology (see Outstanding Questions). Current efforts to enhance starter cultures encompass phage resistance for increased industrial robustness and lifespan, as well as improved exopolysaccharide production for an ideal texture and mouthfeel. Likewise, probiotics are being engineered with altered cell-surface proteins for improved host adherence and immunomodulation, as well as enhanced bile salt hydrolase transcription for bile acid pool modification, and the addition of carbohydrate transporters and hydrolases for the uptake and catabolism of prebiotics and fibers, constituting CRISPRbiotics, next-generation CRISPR-edited probiotics.

Besides the technical challenges ahead, the recent news that CRISPR-based technologies have been used for human germline editing illustrate how the success of this technology depends much on consumer acceptance and the development of regulatory frameworks that define how best to use and commercialize these tools (see Outstanding Questions). On both a local and global basis, consumers and regulators must keep up with the science and debate how to harness this powerful technology for enhanced food safety and human health. Already, lack of global harmony is illustrated by the endorsement of CRISPR-based editing in food crops by the United States Department of Agriculture (USDA), as opposed to the ruling by the European Court of Justice (ECJ) that edited crops fall under genetically modified organism (GMO) guidelines. Various tools and applications of CRISPR-based tools and endogenous CRISPR-Cas systems in bacteria should fall under different rules and guidelines, and it would be misguided to bundle all the tools in the toolbox and all the applications in agriculture, biotechnology, and medicine under one umbrella. Thus, we urge the various stakeholders, across academia, industry, governmental agencies, and consumer groups to educate themselves on the various tools and applications of CRISPR and engage in a productive and open dialogue to best ascertain how these powerful technologies should be used in agriculture to enhance our food supply chain. Indeed, harnessing CRISPR for food safety is a novel concept which opens promising avenues for the enhancement of food safety and the genesis of next-generation sustainable foods.

Outstanding Questions

Which of the available CRISPR-Cas nucleases perform best in foodborne pathogens and spoilage bacteria?

Can phages with broad host specificity be equipped with portable Cas nucleases for broad implementation?

Can antibacterial drones (ABDs) be harnessed as specific delivery systems for CRISPR-mediated antimicrobials?

How can regulatory approval of CRISPR-mediated antimicrobials in food safety and food innovation be framed?

Disclaimer Statement

R.B. is an inventor on several patents related to various uses of CRISPR, and a shareholder of DuPont, Locus Biosciences, Intellia Therapeutics, Caribou Biosciences. and Inari Ag, companies involved in CRISPR research and commercialization.

Resources

[i] www.cdc.gov/fdoss/annual-reports/2016-report-highlights.html

[ii] www.micreos.com/upload/content/file/Publications/Food%20Technology%20April%202008.pdf

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