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Gene Editing in Livestock: Bridging Gut Microbiome Modulation and Antibiotic Resistance Mitigation

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Abstract: The crisis of antibiotic resistance caused by the overuse of antibiotics has become a major threat to global agriculture and public health, and traditional livestock strategies have demonstrated significant limitations in addressing this challenge. Gene editing technologies, in particular the CRISPR-Cas9 system, offer innovative solutions to the problem of antibiotic resistance by regulating the gut microbiota of livestock and directly interfering with resistance genes. This study systematically explores cross-cutting applications of gene editing technology in livestock health management, focusing on three major themes: (1) Editing host genes to optimize gut microbiota balance; (2) Directly editing resistant genes of pathogenic bacteria to restore their sensitivity to antibiotics; (3) Developing collaborative strategies (phage delivery systems and probiotic engineering). The results showed that pigs with CD163 gene knocked out by CRISPR-Cas9 had complete resistance to porcine reproductive and respiratory syndrome. Engineered probiotics can remove 99.9% of resistant bacteria; The bifunctional delivery strategy reduced the intestinal drug-resistant bacterial load to <100 CFU/g. In addition, multi-omics techniques provide a precise tool for dynamically assessing the effects of gene editing on microbiome function. Conclusion: Through the new strategy of "host-microbiome co-editing", gene editing technology has the potential to be transformative in livestock microbiome regulation and drug resistance management, but challenges such as delivery efficiency, ecological risks and ethical controversies still need to be addressed, and its widespread application depends on technological innovation, interdisciplinary cooperation and the coordinated development of an international regulatory framework. To achieve the dual goals of agricultural sustainability and public health security.

Keywords: CRISPR-Cas9, gut microbiome, antibiotic resistance, probiotics, phages

1. Introduction

Genome editing technology has revolutionized livestock research by making precise modifications to an animal's genome to improve disease resistance, productivity and welfare. CRISPR-Cas9 is the leading genome editing system due to its ease of assembly and manipulation. Traditional applications of gene editing in livestock have focused on increased muscle mass, such as MSTN (Myostatin) gene knockout, and resistance to pathogens, such as CD163-edited pigs that are resistant to Porcine Reproductive and Respiratory syndrome (PRRS) [1]. However, global agriculture is facing new challenges, in particular the overuse of antibiotics and the escalating crisis

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of antimicrobial resistance. To address these challenges, the world needs innovative strategies that go beyond traditional approaches. The gut microbiota is an important agent of host health. It plays a key role in nutrient metabolism, immune regulation and pathogen elimination. Dysbiosis of the microbiome is increasingly associated with disease susceptibility and suboptimal growth in livestock, often driving the dependence on antibiotics. Alarmingly, the overuse of antibiotics in animal husbandry has accelerated the spread of resistant genes such as mcr-1 and blaCTX-M, posing a serious threat to both veterinary and human medicine [2].

This review proposes a synergistic model of using gene editing technology to simultaneously regulate gut microbiota and combat antibiotic resistance in livestock. By targeting host genes that influence microbial colonization or directly disrupting the determinants of resistance in pathogenic bacteria, antibiotic dependence can be reduced while improving animal health. Three interrelated topics are explored: (1) Host genome editing to foster beneficial microbial communities; (2) CRISPR-Cas gene editing technology used to intervene resistance genes in intestinal pathogens; (3) Ethical and regulatory considerations for the application of host-microbiome strategies. Through these three aspects, this review aims to highlight the transformative potential of gene editing in addressing two pressing issues in modern agriculture-microbiome driven health and antimicrobial resistance mitigation-while highlighting the need for interdisciplinary collaboration to address technological and societal challenges.

2. The application of CRISPR-Cas gene editing technology in the research of livestock gut microbiome

Probiotics are defined as a class of live microorganisms that can have beneficial effects on the host organism by improving the balance of the intestinal microbiota. Numerous probiotics have been shown to produce substances such as bacteriocins and acids. These substances can inhibit the growth of pathogenic bacteria and maintain the balance of the intestinal microenvironment. This can cause a positive impact on intestinal health and even disease treatment. Therefore, they possess great potential both in disease prevention and treatment. However, there are still obstacles to their use in clinical treatment due to the unclear exploration of the specific mechanisms of probiotics actions. The CRISPR-Cas gene editing technology can more simply, conveniently, and effectively edit the genes of probiotics, thereby achieving some expected improvements. For example, it can enhance the stability of probiotics, reduce the drug resistance of specific strains, enable probiotics to produce some metabolites more specifically, stably, and controllably, or endow them with some extra functions. Besides, functional gene segments for treating certain diseases can also be encoded into the genome of probiotics [3,4]. This technology has been successfully developed for studying the functional gene segments of probiotics and enhancing some advantages. It has achieved remarkable results in various traditional probiotics, including Escherichia coli Nissle 1917 (EcN), Lactobacillus, Bacillus subtilis, Bifidobacteria, and Saccharomyces [4]. Enhancing the specific advantages of probiotics is the earliest developed gene editing effect. For example, Saccharomyces has the advantage of a low off-target rate. This well compensates for the defect of CRISPR-Cas being prone to off-target effects. It became the earliest microorganism that has been successfully gene-edited through this new technology. Researchers first used CRISPR-Cas systems to pre-breakdown the δ site on the yeast chromosome. Then they increased the copy number of the integrated gene through δ-integration, enabling the yeast to produce carboxymethyl cellulase (CMCase) with an activity as high as 559 U/L [5]. This improvement is of great significance for the production of veterinary drugs, and it has certain potential in treating indigestion in herbivorous livestock.

Additionally, there are also numerous cases use gene editing to endow probiotics with the function of secreting specific active metabolites. In the application of antibiotics in animal

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husbandry, intestinal absorption after oral administration into the body is relatively common. However, this method of drug administration also has drawbacks. One of them is the intestinal residue of antibiotics. Most of the orally administered antibiotics are absorbed into the bloodstream through the small intestinal epithelial cells in the front part of the intestine. A small part still remains in the intestine and has a bacteriostatic effect on the probiotics in the distal part of the intestine. This can affect the normal gastrointestinal function of animals and lead to the emergence and proliferation of diseases or drug-resistant pathogenic bacteria. In response to this problem, some researchers have proposed using the CRISPR-Cas gene editing technology to insert the gene encoding the antibiotic-binding peptide into probiotics. The engineered probiotics thus produced will colonize in the distal part of the intestine and secrete neutralizing peptides that specifically bind to antibiotics [6]. The advantage of this solution is that it can ensure the normal absorption of antibiotics and protect the probiotic population in the distal part of the intestine. Thus avoiding the side effects and disease occurrence caused by the destruction of intestinal probiotics by antibiotics.

3. The strategies of CRISPR-Cas gene editing technology for reducing the use of antibiotics

Porcine PRRS is a viral infectious disease caused by Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). It can seriously endanger the reproductive and respiratory systems of pigs, characterized by high infectivity and high mortality. Currently, PRRS has been widely prevalent in the major pig production regions around the world, causing immeasurable economic losses to the nursery piglets and fattening pigs. It is also one of the diseases that have the greatest impact on the global pig farming industry. In the field of disease prevention, a vaccine that can be widely applied and effectively prevent PRRS has not been developed yet. Due to the lack of an adequately effective vaccine, it is almost impossible to expect the infection situation caused by PRRSV to gradually and spontaneously improve and be eliminated in regions with a high level of infection. Pork producers can currently only control secondary infections by isolating the source of infection and administering antibiotics, but this can only alleviate the negative impacts of PRRS to a certain extent. Therefore, the pig farming industry urgently needs to seek other ways to solve the serious problems that PRRS poses to the health of pigs. In recent years, due to the research and proposal of the CRISPR-Cas gene editing technology, a new idea of using CRISPR-Cas9 to edit virus-related genes to directly reduce or even eliminate the risk of infection has become one of the effective solutions proposed for PRRS. PRRSV has specific cell tropism and mainly binds to and infects porcine alveolar macrophages (PAM). This internalization process depends on specific cell receptors to be completed [7].

Up to now, at least seven receptors related to the entry of PRRSV into cells have been identified, such as Sialoadhesin (Sn) which can mediate virus internalization, and Heparan Sulfate (HS) which acts as an attachment factor for PRRSV. However, an increasing number of studies have shown that a cysteine-rich scavenger receptor CD163 is an essential receptor for the action of PRRSV [8]. In view of this, some researchers have carried out experiments using the CRISPR-Cas9 technology to artificially breed pigs lacking CD163 and have demonstrated that these pigs are resistant to the PRRSV isolate NVSL 97-7895. After knocking out a specific fragment of CD163, even if piglets are inoculated with PRRSV and continuously exposed to an infected pig herd, the piglets did not show corresponding clinical symptoms. However, the experimental data and results are only based on three kinds of animals and one PRRSV isolate, namely NVSL 97-7895. Since there are multiple virus isolates of PRRSV, resistance tests of CD163-deficient pigs against other PRRSV strains also have been conducted in subsequent experiments. Testing results from multiple research teams have demonstrated that CD163-deficient pigs are resistant to various PRRSV isolates [1]. CRISPR-Cas technology also holds great promise in addressing various malignant infectious diseases in other animals at the genetic level. It offers a more appealing new strategy for enhancing the disease

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resistance of livestock and poultry, reducing economic losses in the livestock industry, and improving the welfare of animals. For instance, pigs resistant to Classical Swine Fever Virus (CSFV) infection have been bred by inserting short hairpin RNA (shRNA) into the pRosa26 locus followed by CRISPR-Cas9-mediated knock-in [9]. Additionally, chNHE1-KO homozygous mutant chickens with resistance to Avian Leukosis Virus (ALV) have been obtained by knocking out the ALV receptor [10]. Moreover, CRISPR-Cas9 can modify the chANP32A gene to control diseases by reducing the replication of Avian Influenza Virus (AIV) in chicken cells [11].

4. The direct interventions of gene editing in antibiotic resistance

Antimicrobial resistance (AMR) is a global health problem that causes significant mortality and economic burden [12]. Limited antibiotic development and rising antibiotic resistance in livestock threaten to undermine the prevention and treatment of infectious diseases. In particular, carbapenem resistant enterobacteriaceae bacteria (CRE) and pathogens carrying multiple resistance genes (such as blaNDM-5, mcr-1, tet (X4)) pose a serious threat to clinical treatment. Therefore, there is an urgent need to develop innovative strategies for drug resistance genes. Traditional methods are often ineffective against multi-drug resistant bacteria. The CRISPR-Cas9 system, with its high efficiency, precision and programmable characteristics, has become a powerful tool for direct intervention of drug-resistant genes. It blocks the spread of resistance by cutting resistant genes or eliminating resistant plasmids and restoring the bacteria's sensitivity to antibiotics.

Guiding Cas9 nucleases to accurately identify and cut resistant genes (blaNDM-5, mcr-1) by specific single-stranded guide RNA (sgRNA) can lead to gene inactivation or plasmid loss, and then restore bacterial susceptibility to antibiotics. For example, by constructing a conjugative CRISPR plasmid (pCas9-oriT-N) carrying targeted blaNDM-5, the researchers achieved a resistance clearance efficiency greater than 94% in E. coli, significantly reducing the minimum inhibitory concentration (MIC) of meropenem from 16 μg/mL to 0.06 μg/mL. Similarly, for multi-resistant mcr-1 and tet (X4) genes, engineered conjugative systems such as pTra/tet (X4)-glnA delivered through the body successfully reduced the number of resistant bacteria to 1% and restored sensitivity to colistin and tigecycline. In terms of delivery strategies, conjugation delivery achieves efficient delivery of the CRISPR system by adding conjugation sites to modified plasmoid and utilizing natural conjugation mechanisms of bacteria, such as the conjugation efficiency of pCas9-oriT-N up to 95.83%, while in vivo experiments, oral delivery of donor bacteria can significantly reduce intestinal drug-resistant bacterial load (200 CFU/g) in mice. In addition, receptor bacteria preloaded with the CRISPR system (such as strains carrying pCas9-N) can block the horizontal transfer of exogenous drug-resistant plasmids (such as pNDM-5), reducing the number of zygotes by 26 times, highlighting its preventive potential [13]. Despite its significant advantages, the technology still faces challenges such as limited delivery efficiency, off-target effects, and ecological risks. How to efficiently deliver the editing tool to the target bacteria in the intestinal flora is a key problem.

In response to the antibiotic resistance crisis, phage-based delivery systems have significantly improved the delivery efficiency and targeting of CRISPR-Cas9 technology through multi-dimensional engineering. Phages are viruses that infect bacteria and are able to inject their genomes into host bacteria. High specificity, high infection efficiency, and the ability to avoid the selective pressure caused by antibiotic use are advantages of phage delivery systems [14]. However, traditional phage delivery may face host range limitations and immunogenicity. By modifying the capsid or tail protein (gpJ) of bacteriophage, it can specifically recognize the receptor of target bacteria, so as to accurately deliver CRISPR-Cas9 system or base editor [15]. By reconstructing phage tail proteins (e.g. gpJ and the side tail fibre STF of bacteriophage λ), the research team constructed a Mosaic tail filament (λ-p2 STF, λ-K5 STF) and gpJ variants (A8, IA2) to specifically

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recognize high-expression receptors in intestinal bacteria (e.g. OmpC, LamB). Successfully increased delivery efficiency to 93% and covered 21/23 OmpC variants. Another key point of phage delivery systems is to avoid persistent transmission of genetic material. The phage-induced chromosomal island (PICI) replication mechanism can be used to develop non-replicating DNA vectors, which only briefly express the editor in the target bacteria without replication, and no vector remains 42 days after single administration, effectively avoiding the risk of horizontal gene transfer [15].

To enhance host defense, the researchers further developed a dual-function delivery strategy: Phage partially targeted drug-resistant bacteria to deliver CRISPR-Cas9 to cut the drug-resistant gene (blaNDM-5), and the lipidnanoparticle (LNP) carried a base editor (ABE8e) to target host cells, and relieved the epigenetic inhibition of antimicrobial peptide genes (DEFB1, LYZ) in intestinal epithelial cells through C→T editing. The expression of antimicrobial peptide was increased by 5-10 times. In a mouse model, this synergistic strategy reduced the intestinal drug-resistant bacterial load to less than 100 CFU/g, and meropenem MIC recovered from 16 μg/mL to 0.06 μg/mL. This technology has both high precision (off-target rate <0.1%) and safety (time-space specific promoter restricted activity range). In the future, AI can be used to predict the receptor binding domain to build a broad-spectrum phage library, and combined with self-limiting Cas9 variants to optimize clinical transformation paths, providing a multi-dimensional prevention and control plan for the future antibiotic era [15].

5. Integrated management: the combination of gene editing and microbiome engineering

The combination of gene editing technology and microbiome engineering provides a new strategy for disease resistance strategies. By using engineered probiotics (e.g. E. coli Nissle 1917) equipped with optimized conjugative plasmids (e.g. TP114), the CRISPR-Cas9 system can efficiently eliminate antibiotic-resistant and pathogenic bacteria in the gut. Experiments have shown that a single dose can eliminate >99.9% of chloramphenicol resistant E. coli. In addition, pathogenic targeted bacteria were completely eliminated in the infection model within 4 days [16]. This strategy combined with laboratory accelerated evolution technology (ALE) to improve plasmid delivery efficiency. It improved the delivery efficiency of TP114 mutant eB-TP114 by 768 times and achieved precise activation of antibacterial genes through dynamic regulatory design (such as hypoxic response promoter). At the same time, host gene editing can cooperatively enhance the collective body defense mechanism: repair intestinal mucosal barrier genes, optimize immune cell responses or regulate metabolic signal receptors and forming a dual barrier of "in vivo antibacterial + host reinforcement"[17]. However, there are still technical challenges: 1. How to improve the delivery efficiency of conjugation plasmids through accelerated evolution in the laboratory, and improve the efficiency of host gene editing by binding non-viral vectors (such as nanoparticles). 2. Strict biological control strategies should be developed to avoid the persistence of engineered bacteria or plasmids in the environment. 3. Through the engineered conjugation system, expand the range of targeted bacteria and improve the universality and adaptability of treatment. 4. International standards for microbes and hosts of gene editing need to be developed to balance innovation and risk [18].

6. Future prospects and ethical issues

In the future, the development of efficient and safe drug delivery systems is the main research direction of gene editing technology in the regulation of intestinal microorganisms and antibiotic resistance management in livestock. The aim is to break through the existing technical bottleneck and form a comprehensive strategy of "editing - inhibition - substitution" to reduce antibiotic

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dependence. The TP114 plasmid, by combining the CRISPR-Cas9 system, cleared 99.9% of resistant E. coli from the mouse gut after a single dose [16]. At the same time, multi-omics techniques such as metagenomics and transcriptome can be used to accurately analyze the effects of gene editing on bacterial flora function. For example, the safety and effectiveness of gene editing technology can be dynamically evaluated by monitoring the change in the expression level of drug-resistant genes or the inhibition effect on virulence factors [19].

However, ethical and regulatory challenges remain. The global regulatory framework is fragmented, and each country's policies on gene-edited animals differ. The United States' classification of gene-edited animals as "animal medicines" has led to the outflow of research and development technology to countries with liberal policies such as Brazil, while the European Union's conservative attitude may limit the application of gene-editing technology [12]. In addition, the public's acceptance of gene-edited meat is biased by their own experience. Therefore, it is necessary to enhance social trust through transparent labeling system and scientific communication (such as the case of PRRS resistant pigs) [20]. In terms of ecological risks, biological containment strategies such as suicide switches need to be developed. For example, a certain range of survival should be designed to prevent the escape of gene-edited microorganisms from causing irreversible ecological disturbance when the environment is beyond the tolerance of microorganisms. In conclusion, technological breakthroughs need to go hand in hand with ethical review, international policy coordination, and public participation in order to achieve the dual goals of agricultural innovation and public health security.

7. Conclusion

Gene editing technologies, such as the CRISPR-Cas9 system that has emerged in recent years, have brought new opportunities and development ideas for the regulation of the gut microbiota in livestock and also the reduction of the livestock industry's dependence on antibiotics. For example, genetic modification of intestinal probiotics can enhance the advantages provided by probiotic communities to the host's body, strengthen the colonization ability and antibacterial capacity of probiotics, and optimize the intestinal microenvironment of livestock. Alternatively, by editing the genes of pathogenic bacteria, it is possible to target and cleave their antibiotic resistance genes, restoring the sensitivity of bacteria to commonly used antibiotics. Numerous gene editing tools, including the CRISPR-Cas system, can also act directly on the host genes. Experiments have demonstrated that knocking out certain specific gene segments can endow animals with resistance to specific pathogenic bacteria, reducing the frequency or dosage of antibiotic use during the outbreak of infectious diseases. In addition, multi-dimensional engineering modifications such as phage delivery systems and dual-functional delivery strategies have improved the delivery efficiency and targeting of gene editing tools.

Gene editing is undoubtedly an innovative tool with great research value and potential for exploration. However, it also faces numerous challenges during practical applications. At the fundamental technical level, issues such as limited delivery efficiency and off-target effects exist. In terms of ecology, the safety and risks associated with editing the genes of animals and microorganisms should also be considered. For instance, the potential risk of the spread of antibiotic resistance genes and the ecological controllability of microorganisms are issues that cannot be ignored. Moreover, at the social and legal levels, there are significant differences in the regulations for gene editing animal experiments formulated by various countries. The global regulatory framework is also fragmented. Meanwhile, from an economic perspective, the acceptance of gene-edited livestock products by consumers also needs to be improved.

Nevertheless, gene editing technology has great potential in the regulation of the gut microbiota of livestock and the application of antibiotics. By editing host genes and precisely regulating the gut

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microbiome of livestock, it is expected to construct a clinically applicable dual antibacterial and disease-resistant barrier of "in vivo antibacterial agents + host enhancement", to improve the health level of livestock. The combined use of gene editing technology and microbiome engineering is highly likely to enhance the prevention and treatment capabilities for diseases, especially severe infectious diseases, and reduce the livestock industry's dependence on antibiotics. This is of great significance for preventing the emergence of multi-drug-resistant bacteria and even pan-drug-resistant bacteria and ensuring public health safety. It also has a profound impact on improving the health index and welfare level of livestock and poultry, and promoting the development of animal husbandry and animal medical industry. To achieve this goal, governments should introduce legal and international regulatory frameworks that properly address the ethical risks of gene-editing technology. At the same time of continuous interdisciplinary research and technological innovation, the technology can be effectively and safely applied.

Authors contribution

Xinyi Chen is responsible for Abstract, Keywords, Introduction, Literature review 4, 5, 6 and References. Xinke Li is responsible for Literature review 2, 3 and Conclusion.

All the authors contributed equally and their names were listed in alphabetical order.

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