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Review article

Exploring the synergy of CRISPR-Cas9 and IVF for precision livestock genome editing: A review of modern advancements

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Abstract

Livestock plays a crucial role in global agricultural economy. A sustainable livestock production is the key player contributing to food security. Reproduction plays a central role in ensuring a smooth Livestock production. The era of 1980s marked a significant cornerstone in Livestock reproduction by the incorporation of *in vitro* fertilization (IVF), making it easier to develop genetically superior offspring. The precision and efficiency of genetic modifications was further enhanced by the integration of IVF with CRISPR/Cas9 technology. The technological union of CRISPR-Cas9 genome editing and *in vitro* fertilization (IVF) has brought revolutionary changes to livestock biotechnology programs. The combined strategies produce accurate, fast genetic progress through robust enhancements in numerous livestock species. IVF has established itself as a tool for breeding better livestock for increased reproductive success but joining it with CRISPR-Cas9 allows breeders to manipulate embryonic genomes precisely. This study investigates the various implementation methods of this technology including manipulation in the genome through knockout (KO) or knock-in (KI) processes to generate disease-resistant variants combined with production improvements, milk-allergen reduction and creation of transgenic animal research models for pharmaceutical industries. The article examines technical developments improving embryo editing tools alongside multiplex gene modification methods and innovation in IVF protocols. This research surveys both ethical matters alongside the effects; genome-edited livestock will have on regulatory environments. The article identifies potential future trends in this dual-platform biotechnology system, highlighting its role as a critical tool for developing precision-oriented, sustainable animal husbandry.

Keywords: CRISPR-Cas9, Electroporation, Genome editing, IVF, Knock Out (KO), Livestock

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INTRODUCTION

The production of livestock plays an essential role in securing food worldwide and enabling economical expansion of this sector. The escalating demand for premium animal proteins during population growth and urbanization demands better production methods along with enhanced sustainability for livestock systems. Traditional livestock breeding techniques solely depends upon selective breeding, cross breeding and marker assisted selection (MAS), which proved effective but constrained by the limitations such as environmental variation (Hill and Mulder, 2010), lengthy breeding cycle, accuracy of selection and disease susceptibility (Berghof et al., 2019). Assisted reproductive technology has improvised the breeding efficiency by utilizing modern techniques such as fixed time artificial insemination, sex-sorted semen, in-vitro embryo production (IVEP) and embryo transfer (ET). Multiple ovulation and embryo transfer (MOET) has also revolutionized the breeding program, but embryo recovery rate is poor in this technique. Hufana-Duran et al. (2025) proposed that Somatic cell nuclear transfer (SCNT) can be helpful in producing sex-predetermined animals with superior genetics, but further studies should support the efficient processing. The current breeding methods produce small improvements but their effectiveness is limited by delayed generation times and poor calibration and external impacts on gene execution.

In vitro fertilization (IVF) aims at producing the superior livestock population selectively (Kabu and Tunç, 2024) by using laboratory techniques for oocyte maturation, fertilization as well as early embryonic development. In IVF, Somatic cell nuclear transfer (SCNT) plays a very crucial role in the rapid production of superior animals, generating embryonic stem cells as well as transgenic models. However, Kumar et al. (2024) pointed out several drawbacks of using SCNT, most important of them is the presence of abnormalities in the newborn due to the partial or deviant nuclear reprogramming. Differential gene expression (DEGs) profile in the IVF generated blastocyst at different stages of development is also identified in the SCNT embryos. Animal breeding achieved a significant breakthrough with the adoption of *in vitro* fertilization (IVF) to accelerate the selection of top-quality genetic strains due to this new advancement. IVF treatment by itself does not provide the capability to make targeted genomic changes. Biologists solved the specificity problem through CRISPR-Cas9.

'Clustered Regularly Interspaced Short Palindromic Repeats' well-known as CRISPRs are a group of small, functional DNA sequences present in bacteria and archaea providingimmunity against several extrinsic plasmids or phage virus by splicing invaders' DNA or RNA (Kaushik, 2024). The CRISPR associated protein-Cas9 is an endonuclease that moves against the foreign DNA invasion to neutralize it. CRISPR-a revolutionary genome editing technique was coinvented by Drs. Emmanuelle Charpentier and Jennifer Doudna. CRISPR works on the principle of binding the DNA at a specific location and then cutting the DNA strands. The genetic material, guided by suitable delivery system (different delivery systems are explained in Table 1 and Figure 1) can be inserted as the DNA repairs itself (Busch-Vishniac et al., 2024). DNA sequences which are intended to be edited are identified and selected as the first step during CRISPR-Cas gene edits. Then, a guide RNA is manufactured that detects special DNA arrangement patterns within this sequence, as gRNA is complementary to the desired DNA sequence. The guide RNA is incorporated into Cas9 (RNA-guided endonuclease), which is DNA-cutting enzyme. The combo is then introduced as the enzyme-complex to the target cells. This DNA-targeting complex searches for its DNA sequence target and binds to Protospacer adjacent Motif (PAM) sequence. As soon as a true match sequence is found, Cas9 proceeds towards a double-stranded break (DSB) at that identified location within DNA. DSB turns ON the cellular mechanism of DNA repair. The DNA repair mechanism may be Homology-Directed Repair (HDR) or Non-Homologous End Joining (NHEJ); both of which have their own implications (detailed comparison

is given in Table 2). CRISPR-Cas works as a genetic implementation tool that enables DNA manipulation to get desired genetic manipulations (Hille and Charpentier 2016). The other gene editing techniques like ZFN or TALEN involve lengthy and complex processes, while CRISPR/Cas9 only needs to synthesize single guide RNA (sgRNA) fragment, so that the protein corresponding to the desired DNA sequence can be produced (Y. Liu et al. 2024). Moreover, it is rapid in synthesizing and mutating the target DNA sequence, hence, the time required for gene editing is also shortened compared to other techniques. (Generalized mechanism of CRISPR is depicted in Figure 2)

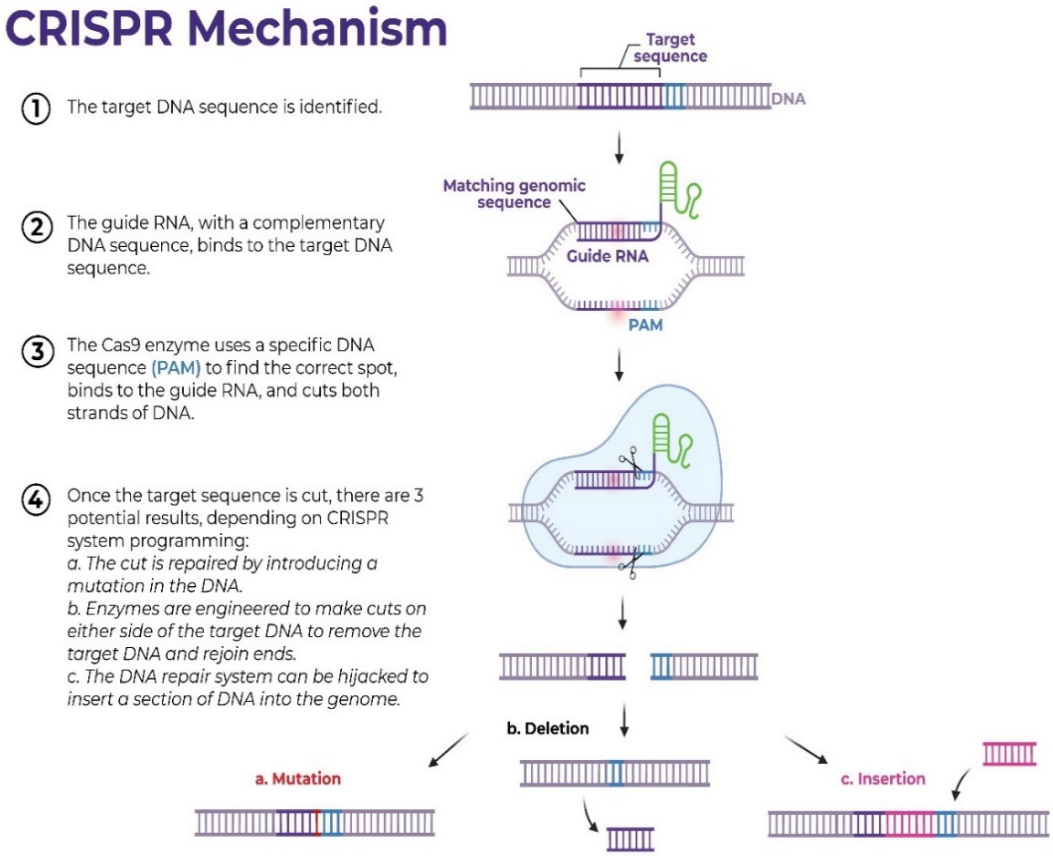

Figure 1 General mechanism of CRISPR gene editing, courtesy; UMass Chan Medical School (Jain et al. 2024).

Integration of IVF with CRISPR-Cas9 proved to be an efficient, swift and convenient method for precise genomic alteration in livestock (Wang and Doudna, 2023) improving productivity, disease resistance and adaptability to stressful environment. The application of CRISPR-Cas9 technology now allows rapid and affordable genome modifications in animals through its versatile characteristics. Integrating with IVF approaches, CRISPR-Cas9 enables scientists to directly modify embryos which produces livestock animals with advantageous characteristics including improved development rate together with disease immunity.

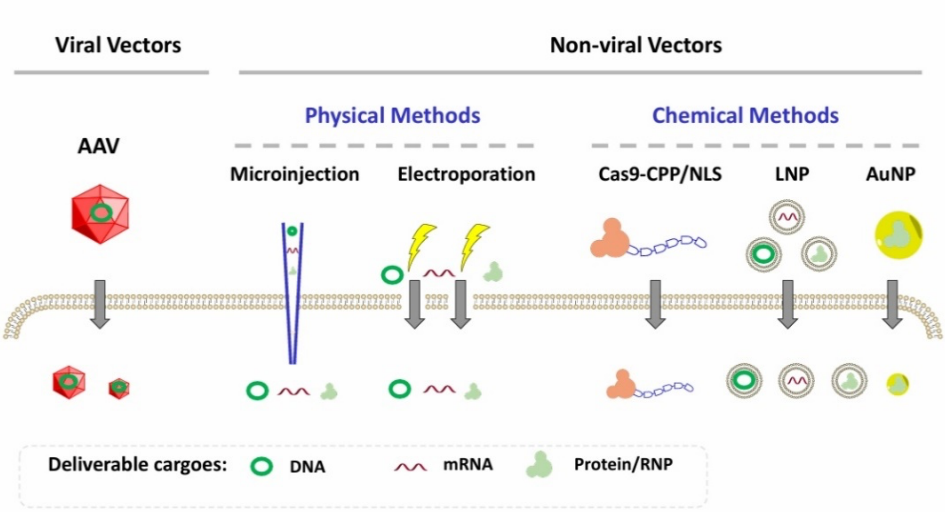

Figure 2 Various delivery methods used in CRISPR-Cas9 engineering (Glass et al., 2018).

Table 1 Various delivery methods used in CRISPR-Cas9 engineering.

Delivery Method	Type	Mechanism	Advantages	Limitations	References
Viral Vectors (e.g., AAV, Lentivirus)	Biological	Viruses engineered to deliver Cas9 and sgRNA to cells	High delivery efficiency; good for somatic cell editing	Potential immune response; size limitations (especially for AAV); integration risks	(Glass et al., 2018)
Sperm-mediated Gene Transfer (SMGT)	Biological	CRISPR cargo introduced via sperm during fertilization	Less invasive; potential for in vivo delivery	Low and variable efficiency; not standardized	(Chen et al., 2025)
Microinjection	Physical	Direct injection of Cas9 mRNA/protein and sgRNA into zygotes or embryos	Precise; commonly used in livestock gene editing	Labor-intensive; low survival and success rate; technically demanding	(Zhang and Yu, 2008)
Electroporation	Physical	Electric field makes cell membranes temporarily permeable	Simpler and faster than microinjection; high throughput	Lower editing efficiency; potential embryo damage	(Pi et al., 2024)
Gene Gun (Biolistics)	Physical	DNA-coated particles shot into cells/tissue using high pressure	Useful for plant cells and some animal tissues	Low precision; tissue damage possible	(Zafar et al., 2025)
Lipid Nanoparticles (LNPs)	Chemical	Lipid-coated particles deliver Cas9/sgRNA into cells	Non-viral; low toxicity; good for in vitro applications	Limited in vivo targeting; uptake efficiency can vary across species	(Clarissa et al., n.d.)
Ribonucleoprotein (RNP) Complexes	Chemical/Physical	Direct delivery of Cas9 protein + guide RNA into cells	Immediate action; reduced off-target effects	Short half-life; delivery to embryos still technically challenging	(Seijas et al., 2025)

Table 2 Comparison between NHEJ vs HDR (Tei, et al. 2025; Fu, et al. 2021).

Feature	NHEJ	HDR
Template Required	No	Yes (homologous DNA)
Repair Accuracy	Error-prone	High fidelity (accurate)
Cell Cycle Dependency	All phases (especially G1)	S and G2 phases only
Suitable For	Gene knockouts	Gene corrections/insertions
Editing Control/Precision	Low (random indels)	High (precise base changes or insertions)
Speed	Higher	Slower compared to NHEJ
Application in Livestock	Common for trait disruption	Challenging due to low efficiency in embryos

This review explores the advancements in genome editing by CRISPR-Cas9 followed by *in vitro* fertilization (IVF) to improve livestock genetics for different purposes, such as enhancing productivity, disease tolerance, recombinant models for human welfare. The article examines CRISPR-Cas9 technology deployment through different species for gene knockout and knock-in experiments as well as the creation of recombinant models, allergen elimination and IVF protocol optimization. The study evaluates both the ethical considerations and technical hurdles as well as the possible course of development for this revolutionary combination of biotechnologies.

GENE KNOCKOUT USING CRISPR-Cas9

Gene knockout (KO) is a genetic engineering technique, used to remove a defective/undesired gene from genome of an organism. These manipulations suppress the functioning of a specific gene to get desired results. CRISPR/Cas9 technique has been most efficient in Gene Knockout in various species. Genome editing serves as a powerful component of modern research in livestock, dealing with wide fields from breeding to disease elimination. In spite of its efficient role, very little work has been carried out in buffaloes, owing mostly due to extended gestation period, prolonged calving interval, and high maintenance costs (Punetha et al. 2024). Among all the gene editing techniques producing Knockout Genes, Ledesma and Van Eenennaam (2024) proved that CRISPR/Cas9 is the most commonly used technique. In addition, the data shows that CRISPR-KO technique is widely used for manipulating genome for enhancing Production/Yield (32%), Improving reproduction (21%) and creating Disease tolerant animals (17%). Baruselli et al. (2025) suggested that modern gene editing techniques such as CRISPR/Cas can be used along with genomic selection to generate resistant animals against harsh environment, diseases and pests. It can also help to speed up genetic gain and reduced generation interval in cattle.

Disease Resistant Embryos

CRISPR-Cas9 has been used widely to improve livestock species with disease resistant genes. A number of fatal and most prevalent disease conditions has been knocked out utilizing the gene editing tools. These advancements help boost up the global food supply in a healthy and safe way. Various mammalian species exhibit neurodegenerative disorders due to a prion disease caused by misfolding of cellular PrP (PrP^c) protein. The work of Allais-Bonnet et al. (2025) aimed at Knockout of the PRNP alleles, which encode PrP^c protein in alpine goats. Prior to the electroporation of embryos/oocytes, CRISPR-based genome editors were injected under the zona pellucida of the caprine embryo to obtain *PRNP* genome-edited alleles. The newborn kids exhibited the desired results with mutations in the PRNP alleles.

Porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease is a systemic illness, causing significant losses to pig farmers. CRISPR-Cas9 successfully modified CD163 gene in the pigs to produce PPRS-resistant animals (Hongming Yuan et al., 2022). Burger et al. (2024) further worked to generate a founder swine population that would be resistant to PPRS syndrome. Four elite lines of pigs were introduced to modify CD163 gene using CRISPR. The resistant founder population would serve as investigation models and commercial parents for next resistant generations after regulatory approval. Navarro-Serna et al. (2024) also successfully generated double knockout pig embryos by electroporation, that were resistant to different viral diseases such as PPRS and swine influenza.

Table 3 Applications of CRISPR-Cas9 in creating disease-resistant livestock.

Species	Target Gene	Disease Targeted	Outcome	References
Goats	PRNP	Prion diseases	Resistance to neurodegenerative prion disorders	(Allais-Bonnet et al., 2025)
Pigs	CD163	PRRS (Blue-ear pig disease)	PRRS-resistant pigs	(Yuan et al., 2022)
Pigs (4 lines)	CD163	PRRS	Resistant founder lines	(Burger et al., 2024
Pigs	CD163 + other	PRRS + Swine influenza	Dual resistance in edited embryos	Navarro-Serna et al., 2024)

Recombinant products and Xenotransplantation models for humans

CRISPR-Cas9 gene editing has shown that animal models for biomedical research can be produced at a low cost with ease of handling. In spite of this fact, it is a bit perplexing as several off-target effects can be encountered due to erroneous cleavage (Li et al., 2024). CRISPR/Cas9 system has improved the efficiency of genetic modification especially in pigs, as these animals serve as a perfect model for understanding human diseases as well as perfect source of xenografts for xenotransplantation. The transplantation of tissues or organs from modified pigs or non human primates into the humans in case of organ failure or other conditions, is termed as xenotransplantation. To improve the editing efficiency, Navarro-Serna et al. (2024) explored the potential of electroporation for manipulating multiple genes in a single step, which could be significant for rapid xenografts' production. Briski et al. (2024) studied various methods of gene Knockout in porcine oocytes using CRISPR/Cas9 technique to make them compatible for xenotransplantation. The genes responsible for hyperacute transplant reactions due to biosynthesis of xenoantigens were knocked out. They also worked to KO the gene responsible for Growth Hormone receptor (GHR). The motive behind was to restrict the growth of the organs to make them suitable for transplantation to humans.

A receptor known as Programmed cell death-1 (PD-1), is an immunoinhibitory receptor that suppresses the autoimmune responses in different species, including humans by modulating activity of T-cells. Nguyen et al. (2024) utilized CRISPR/Cas9 mediated electroporation into the porcine zygotes to generate PD-1 mutant animals. The main aim was to study the phenotype of offsprings with deficient PD-1 gene. The sequencing analysis revealed biallelic mutation in the piglets produced from edited zygotes, with both the PD-1 alleles successfully edited. Such animal models may be used for studying the effect of autoimmune diseases, along with therapeutic testing. Endolysosomal two-pore channels (TPCs) were discovered to be associated with the several pathophysiological roles, such as immunity, metabolic reactions, tumor production and functioning of heart and muscles. But these TPCs knock out (TPCs-KO) models were studied in mice only. To confirm the roles of these TPCs in large human-models such as pigs, Navarro-Serna et al. (2021) produced TPCs knockout (KO)

pigs by using CRISPR-Cas9. They successfully produced TPCs KO piglets by using microinjection of the *in vitro*-generated embryos.

Severe combined immunodeficiency disease (SCID) is a life-threatening condition characterized by immune system failure and prolonged ailment. To understand the biological mechanism of this disease for further therapeutic advancement, advanced gene editing techniques, particularly CRISPR has been utilized for generating model animals. Zheng et al. (2025) suggested that in addition to developing SCID models, these techniques also serve to enhance our understanding in chemotherapy against cancer, organ or stem cell transplantation as well as management of various infectious conditions. Recombinant protein, such as Human neutrophil peptide 1 (HNP1) can be produced using mammary gland bioreactors. HNP1 serves as antibacterial agent as well as modulator of immune system. Li et al. (2024) utilized CRISPR/Cas9 technology to generate transgenic goats secreting HNP1, by knocking-in (KI) the HNP1 sequence into β-casein (CSN2) gene. The milk produced by these offsprings contained significant HNP1, which presented antibacterial activity against different gram positive as well as gram negative bacteria.

Table 4 CRISPR applications in recombinant models and xenotransplantation.

Species	Target Gene(s)	Purpose	Result	References
Pigs	Xenoantigen genes, GHR	Xenotransplant compatibility	Reduced transplant rejection, organ resizing	(Briski et al., 2024)
Pigs	PD-1	Immunological modulation	Autoimmune research model	(Nguyen et al., 2024)
Pigs	TPCs	Metabolism, immunity, muscle research	Functional validation of TPC roles	(Navarro-Serna et al., 2021)
Pigs	SCID genes	SCID modeling	Therapeutic testing and cancer research	(Zheng et al., 2025)
Goats	HNP1 insertion	Biopharmaceutical protein production	Antimicrobial protein in milk	(Li et al., 2024)

Enhancing traits of economic importance

Ongoing global population explosion demands higher amount of animal proteins in the form of eggs, meat and milk. Advanced genetic engineering techniques can boost up the production in a limited period of time (Singh and Ali, 2021a). The cattle population found in some regions of America exhibit thermotolerance and have short hair, mostly owing to the natural mutations in prolactin receptor (*PRLR*) gene. Cuellar et al. (2024) demonstrated that similar mutations can be induced in the *PRLR* gene by using CRISPR/Cas9 technology to make heat stress-resistant cattle population. They developed PRLR mutation in two thermosensitive cattle breeds- Angus and Jersey. These genome-edited animals had excellent ability to regulate their body temperature, also exhibiting enhanced growth characteristics as well as increased scrotal circumference.

The development of ovarian follicle development is regulated by follicle stimulating hormone (FSH) due to expression of specific genes. Liu et al. (2024) successfully generated the first knockout library by utilizing CRISPR-Cas9 on the chromosomes 2 and 3 as well as the sheep X chromosomes. They also succeeded in generating Granulosa Cells (GCs) knockout library, as these cells secrete gonadotropins, thus stimulating development of follicles and regulating the ovulation. In sheep, *BMPRIB* gene has been recognized to be the major contributor for high fecundity. The mutation in the *FecB* allele of this gene can lead to an enhanced ovulation rate in sheep. Zhang et al. (2025) studied the effect of introducing a point mutation into the *BMPRIB* gene of ewes, by employing CRISPR/Cas9-mediated homologous-directed repair (HDR). The results were quite satisfactory with the offspring born from *BMPRIB* edited ewes exhibiting higher fecundity than normally born offsprings.

A gene named as the Suppressor of Cytokine signaling (Socs-2) hinders the growth rate in different animals. After the successful mutations in Socs-2 gene in model mice, scientists proved an improved growth rate. Keeping in view the success in mice, Mahdi et al. (2025) demonstrated Knockout of the (Socs-2) gene to improve the growth rate in sheep by using electroporation based method. The experiment successfully yielded lambs with edited genome without any off-target effects. High quality meat protein is obtained from animals with double-muscle phenotype, which is produced by mutating the Myostatin (MSTN) gene. Several mutations were introduced in sheep by the experiments of Chen et al. (2024), and generated *MSTN*^{Del73C} mutation with *FGF5* knockout sheep to obtain a heritable doble-muscle phenotype in sheep. These animals had greater number of muscle fibers at a smaller cross-sectional region, yielding more meat and protein. Similarly, Punetha et al. (2024) worked on the buffaloes to generate MSTN-edited offspring, exhibiting double-muscle phenotype by using somatic cell nuclear transfer (SCNT) and CRISPR- Electroporated zygote.

NANOS3 gene is considered to be a crucial factor in germline development by protecting apoptosis of primordial germ cells (PGCs) in different animals. However, no such data explored the role of NANOS3 gene in cattle. Mueller et al. (2023) studied this factor by producing NANOS3 knockout (KO) cattle using CRISPR-Cas9 system. They used dual gRNA approach by coinjection of guide RNA (gRNA) and Cas9 ribonucleoprotein (RNP) in the bovine zygotes produced by *in vitro* fertilization (IVF). The fetal testes in NANOS3-KO individuals were found to be devoid of PGCs on day 41 of their age, but the development of seminiferous tubules was not impaired throughout their life. Moreover, such bulls at their sexual maturity also exhibited normal libido. But in females, such NANOS3-KO heifers presented the compromised ovarian development. Hence, NANOS3 has been markedly involved with the germ cell maintenance in both sexes, especially in females. Sex determination in mammals is governed by the presence of SRY gene on the Y chromosome, which direct the development of male gonadal organs (testes). Manipulation in the SRY gene can produce changes in the phenotype of offspring. Punetha et al. (2024) worked on the buffalo embryos to clarify the effect of SRY gene, by using CRISPR-Cas9 technology. The blastocysts with mutated SRY gene enhanced the expression of Wnt4 gene which specifies the female lineage, while suppressing the expression of SOX9, which specifies the male lineage. This study also paved the way for a new insightful into the sex differentiation in buffalo.

Table 5 Trait enhancement through CRISPR in livestock.

Species	Target Gene	Trait Modified	Improvement	References
Cattle	PRLR	Thermotolerance	Improved heat stress response & growth	(Cuellar et al., 2024)
Sheep	BMPRIB (FecB)	Fecundity	Higher ovulation rate and lambing frequency	(Zhang et al., 2025)
Sheep	Socs-2	Growth Rate	Enhanced growth without off-target effects	(Mahdi et al., 2025)
Sheep/Buffalo	MSTN	Muscle Mass	Double-muscle phenotype	(Chen et al., 2024; Punetha et al., 2024)
Sheep	FSH/Granulosa	Reproduction	Knockout libraries for ovulatory gene study	(Liu et al., 2024)
Cattle	NANOS3	Germline Maintenance	Confirmed sex-specific role in fertility	(Mueller et al., 2023)
Buffalo	SRY	Sex Determination	Altered gonadal lineage in blastocysts	(Punetha et al., 2024)

Alleviating milk allergens from Dairy

Milk is an excellent source of nutrition, but certain proteins, such as β-lactoglobulin (BLG), α lactalbumin, and casein, can trigger allergies in some individuals, particularly children. The Clustered Regularly Interspaced Short

Palindromic Repeats (CRISPR)/Cas system has emerged as a powerful tool for precise genome editing, including the modification of milk allergen genes. Using CRISPR/Cas9, researchers have successfully edited BLG genes in various dairy animals, such as cows, sheep, goats, and buffaloes (Sunwasiya and Mondal 2024). In bovine mammary epithelial cells (bMECs), the BLG knockout (BLG-KO) system was achieved using three single guide RNAs (sgRNAs) and a Cas expressing system delivered via electroporation (de Souza et al., 2022; Gim et al., 2023). Western Blot analysis confirmed a significant reduction in BLG protein expression. In buffaloes, CRISPR facilitated bi-allelic editing (-/-) of the BLG gene, and somatic cell nuclear transfer (SCNT) produced BLG-edited embryos at the blastocyst stage (Tara et al. 2024). Similarly, in goats, one-cell stage embryos were co-injected with Cas9 mRNA and sgRNA to generate BLG-KO fibroblasts (Zhou et al. 2017) . These advancements demonstrate the successful application of CRISPR/Cas9 technology in producing β-lactoglobulin-free milk. Compared to traditional methods such as enzymatic hydrolysis (which is costly and may result in undesired epitopes) or Zinc Finger Nucleases (ZFNs) and TALEN-mediated editing (which are prone to off target effects), CRISPR/Cas9 offers a more efficient, precise, and cost-effective approach to eliminating milk allergens. This breakthrough holds significant promise for providing safer milk options for individuals with milk allergies.

IMPROVING DIFFERENT PARAMETERS OF IVF USING CRISPR

The CRISPR/Cas9 system has evolved to be a wonderful tool for genome editing in IVF in livestock. Different approaches were explored to identify the most effective conditions for genome editing with CRISPR, though each method has its own pros and cons. Mosaicism is a condition arising due to a genetic change in embryo after fertilization, leading to multiple cell line with varying genetic makeup. It is a significant challenge in livestock gene editing, which is characterized by distinct genome setup in different cell lines of an individual or absence of desired genotype, leading to undesired phenotype (Navarro-Serna et al., 2021). Several methods were applied by different scientists to produce nonmosaic embryos, by altering the techniques in CRISPR editing. (Salvesen et al., 2024) suggested the possible solution to the genetic mosaicism, which include bypassing the direct gene editing in zygote and utilizing the surrogate sire technique and separation of blastomeres to avoid mosaicism. Navarro-Serna et al. (2021) performed experiments on pigs to produce nonmosaic piglets by microinjecting embryos before insemination and performing embryo transfer (ET) surgically. They were successful in generating nonmosaic F0 generation animals, with biallelic mutations. Briski et al. (2024) studied different methods of gene Knockout (KO) in porcine oocytes using CRISPR/Cas9 technique such as, 1) ICSI-MGE (mediated gene editing) by co-injecting sperm and Cas9 components into the oocytes, 2) microinjection of CRISPR-Cas9 components into the oocytes before *in vitro* fertilization (IVF), 3) *in vivo* fertilized zygotes' microinjection with CRISPR-Cas9 components. Among all the techniques, ICSI-MGE stood out as the most efficient among all methods with highest biallelic mutation rate.

Different approaches named Electrofection and Lipofection serve as methods to transport genetic material including DNA and RNA into cells (Mars et al. 2015). DNA delivery through the cell membrane can be achieved either through lipofection methods by encapsulating DNA in lipid vesicles or through electrotransfection methods by applying electric pulses to produce short-lived membrane pores. Piñeiro-Silva and Gadea (2024) performed several experiments to find optimum conditions for generating IVF-edited embryos. The study indicated that genetically altered porcine embryos can be created by using lipofection with Lipofectamine 3000 or CRISPRMAX with limited equipment and little experience.

In addition, lipofection provided similar or greater efficiency than electroporation mediated genetic modification. They also demonstrated that coincubation for 8 hours resulted in optimum fertilization rate. In order to achieve high mutation rate, Pi et al. (2024) demonstrated that before electroporation, Cas9 protein must be pre-complexed with single-guide RNA (sgRNA). They successfully generated lambs that were *MSTN*-modified, achieved via electroporation by incorporating Cas9 RNPs into IVF zygotes. The work of Wang et al. (2025) on sheep oocytes demonstrated that environmental factors such as temperature, humidity and pressure greatly impact the *in vitro* maturation efficiency of oocytes. Yang et al. (2025) studied the effects of different conditions of embryo transfer to find out the more feasible method and found that the microenvironment of oviduct is most suitable for transferring frozen embryos in ewes. The study indicated that the pregnancy rate was improved when the frozen blastocysts were transferred into the oviduct, rather than the uterus.

Microinjection employs as a direct DNA or RNA gene transfer method using a thin glass micropipette to deliver genetic material into cell cytoplasm or nuclei for transgenic animal creation and gene research (Zhang and Yu, 2008). CRISPR/Cas9-mediated embryo editing by using microinjection technique is not preferrable, as it shows less efficient results due to presence of off-target mutations as well as high mosaicism rate. Park et al. (2024) demonstrated that electroporation-based embryo editing using CRISPR/Cas9 is more efficient than microinjection as it allows swift and smooth process. Electroporation also allows several embryos manipulation than single embryo in microinjection. Park et al. (2024) successfully performed electroporation of porcine embryo using CRISPR/Cas9 by targeting the NGN3 gene. The results expressed highly efficient mutation with lowest mosaicism and none off-target mutations. Torigoe et al. (2025) demonstrated the efficiency of electroporation at different events of oocyte development and stated that electroporation at different points of attachment of cumulus cells had no effects on Cas9 delivery system. They also indicted that GONAD method of genome editing may also be carried out in porcine oviduct. To find out the most feasible time for electroporation, Sardar et al. (2025) studied the gene editing by electroporation of the oocytes before and after the fertilization. The study revealed that the efficient gene editing was observed in the oocytes, which were given complete time for maturation.

Handling and editing embryos *ex vivo* is quite challenging and complicated. A technique known as “genome editing via oviductal nucleic acid delivery (GONAD) enables the manipulation of embryos *in vivo* within the oviduct.Watanabe et al. (2024) demonstrated the GONAD by injecting reagents used for genome editing into the oviduct and then subsequent electroporation of the whole oviduct *in vivo*. The study suggested that desired genome portion can be manipulated by using viral vectors containing adeno-associated particles. In routine practice, the *in vitro* fertilization (IVF) in pigs create low-quality fertilized embryos due to polysperm invasion. Oh et al. (2024) demonstrated the effects of addition of apple seed extract (ASE) in the IVF-cultured porcine embryos. In the ASE group, the apoptotic activity was reduced and increased cell survival rate was observed. They also studied the effect of embryo coculture on the endometrial cell layer and found an improved expression of insulin-like growth factors (IGF) by the genes.

Cryopreservation of semen using different cryoprotectants yield varying results in semen quality. Kamel et al. (2024) aimed at improving post thaw semen quality and fertilization rates of the cryopreserved buck semen by using L-carnitine or *M. oleifera* (Moringa) leaves extract into the semen diluent. These antioxidants provided improved post-thaw semen quality parameters, preserved sperm DNA integrity and enhanced the fertilization rates both by AI as well as IVF.

GENE KNOCK-IN USING CRISPR

The CRISPR gene knock-in process involves insertion of specified DNA sequences at precise target locations through utilization of CRISPR-Cas9 technology. Unlike CRISPR knockouts, which acts to disable genes by disrupting them, gene knock-ins (KI) enable researchers to produce targeted insertions for adding specific genetic sequences into the genome (Xue et al., 2014). Gene knock-in (KI) in early embryonic stage is very difficult due to inactive homologous recombination (HR) pathway. Hence, homology-mediated end joining (HMEJ) technique is used to knock-in the targeted gene (Leal et al. 2024). However, Yoshimi et al. (2021) carried out precise KI of plasmid DNA in rats and mice by using combination of both HDR and NHEJ pathway to edit the model embryos. Owen et al. (2021) performed experiments on the bovine embryos to knock-in sex-determining region Y (*SRY*) along with green fluorescent protein (*GFP*) template using HMEJ-based donor template and Cas9-RNP complex. The resulted offsprings successfully exhibited male phenotype with biallelic *SRY-GFP* template.

Human neutrophil peptide 1 (HNP1) serves as antibacterial agent as well as modulator of immune system. Li et al. (2024) utilized CRISPR/Cas9 technology to generate transgenic goats secreting HNP1, by knocking-in (KI) the HNP1 sequence into β-casein (CSN2) gene. sgRNA, Cas9 mRNA and a plasmid containing HNP1 sequence were mixed and injected into the cytoplasm of One-cell stage embryos. The knocked-in offsprings exhibited HNP1 in their milk. Cattle *Rosa26 (cRosa26)* is a specific locus that can support and express any exogenous gene at any stage of embryo development. Xie et al. (2022) demonstrated CRISPR knock-in (KI) of EFGP gene (Enhanced green fluorescent protein) at *cRosa26.* EFGP is known as "reporter gene" or "marker'', which exhibit green color on UV light exposure. The study proved efficiency of *cRosa26* as specific locus for this gene. One of the most crucial gene knock-in using Transcription activator-like effector nuclease (TALEN) is worth mentioning here. (Wu et al. 2015) used homologous recombination using TALEN-mediated knock-in of the SP110 gene to generate cattle resistant to *Mycobacterium bovis* (tuberculosis). Both *in vivo* and *in vitro* trials produced the desired resistant animals.

Table 6 CRISPR-Cas9-based gene knock-in applications in Livestock

Species	Target Gene (KI)	Strategy Used	Purpose/Result	References
Goat	HNP1	Cas9 mRNA + sgRNA + plasmid donor	Antibacterial protein expression in milk	(Li et al., 2024)
Cattle	SRY-GFP	HMEJ donor + Cas9-RNP complex	Male phenotype, GFP as marker for lineage tracking	(Owen et al., 2021)
Cattle	EGFP at cRosa26	Cas9 + gRNA to Rosa26 locus	Visual gene expression under UV light	(Xie et al., 2022)
Cattle	SP110	TALEN-mediated HR insertion	Tuberculosis resistance against *Mycobacterium bovis*	(Wu et al., 2015)

Multiplex gene editing using CRISPR

The Cas9 system allows high-efficient multiplex genome editing through its ability to either express or provide multiple gRNAs. Several research methods exist for delivering multiple gRNAs in living organisms through multigene cassettes (Kurata et al., 2018). Complex genome editing occurs at multiple DNA sites simultaneously through which the approach enables researchers to disable multiple genes along with eradicating paanimal scientistsrticular chromosomal areas. Off-target effects might be minimized by Cas9-dimers which run successfully only when two gRNAs are concomitantly expressed. Specific gene expression or methylation status becomes efficiently controlled using dCas9 together with activators and repressors when multiple gRNAs are present (Minkenberg et al., 2017).

Most of the experiments performed in past mostly focused on the knockout of single gene/allele. To check whether the multiple gene KO system would work efficiently in large animals, Ren et al. (2024) performed experiments on pigs and bovines. They successfully created porcine fibroblasts by using Cas12i^{Max} techniques. These fibroblasts yielded the simultaneous KO of 4 genes (*IGF2, ANPEP, CD163*, and *MSTN)* in single step to obtain stable pigs. The gene edited animals had improved muscle quality as well as better growth. In the similar way, they worked on bovine fibroblasts to knockout 3 genes (*MSTN*, *PRNP and* amino acid Q-G in *CD18*) simultaneously. This experiment also resulted in improved animals without any off-target effects. The research of Wang et al. (2016) involved sheep embryo co-injection with Cas9 mRNA and guide RNA (gRNA) against MSTN, ASIP, BCO2 genes at the one-cell stage embryo. Mutations were detected through genetic and morphological analyses along with no detected off-target effects. These results demonstrated that CRISPR/Cas9 system can function as an effective tool for multiple livestock trait improvement.

Table 7 Multiplex gene editing using CRISPR/Cas systems in livestock

Species	Genes Edited Simultaneously	Method/Tool Used	Outcome Achieved	References
Pigs	IGF2, ANPEP, CD163, MSTN	Cas12iMax	Enhanced growth, muscle traits, disease resistance	(Ren et al., 2024)
Cattle	MSTN, PRNP, CD18 (AA Q-G)	Cas12iMax	Improved muscle & resistance	(Ren et al., 2024)
Sheep	MSTN, ASIP, BCO2	Cas9 mRNA + gRNAs	Morphological trait enhancement	(Wang et al., 2016)

ETHICAL AND REGULATORY ISSUES ASSOCIATED WITH CAS-EDITING

CRISPR-Cas9 integration with IVF possesses great power but creates numerous substantial ethical questions for society to solve properly. The top ethical dilemma pertains to the wellbeing of animals. The genetic editing and reproductive procedures which are used together may produce abnormal developmental outcomes and health problems and reduced life expectancy in produced animals. The process requires frequent welfare checks coupled with extended observation to guarantee proper treatment of animals. Gene editing in livestock has been viewed in different perspectives at global level. Different ethical and welfare concerns of the animals hinder the continuous experimentation using CRISPR-Cas9 technology. According to the survey carried by Yunes et al. (2021), different public opinions were based on the type of editing. The genome editing for improving animal welfare received welcoming comments, while the trait improvements for economical purpose remained unacceptable. International guidelines should remain in a state of ongoing harmonization because this harmonization will create the regulatory foundation needed for safe global adoption of genome-edited animals. The essential elements for earning societal trust will be public dialogue combined with ethical deliberation and expressive risk-benefit information dissemination.

The misperception about CRISPR in the general public needs to be addressed to make this emerging trend acceptable, keeping in light the aspects of risk/benefit, informed consent as well as legal ethics (Lange and Kappel, 2022). Genetic diversity stands as an important ethical consideration when genetic manipulation is executed. The exclusive use of selected edited genes in livestock breeds creates potential vulnerabilities, which reduce population resilience to new diseases and environmental stressors. The implementation of security measures should protect both precision genome editing methods and natural genetic diversity collection. The FDA has approved using CRISPR for human welfare such

as cancer research and treatment of chronic ailments, while maintaining the freedoms of model animals (Wiley et al., 2024; Cetin et al., 2025). Genetic mosaicism, offtarget effects, unexpected outcomes and some legal concerns hinder the wider usage of Cas system for gene editing (Zhang, 2025). Some countries banned the genome editing at mass level due to ethical concerns, while others have been investing a lump some of their resources to optimize CRISPR/Cas9 editing for human welfare (Ahmad, 2025). The advantages provided by CRISPR-IVF technologies risk falling into the dominant control of wealthy nations and big farming corporations. The priority needs to be equalized benefit distribution along with strengthening capabilities in low- and middle-income regions.

Public concerns genome editing as 'playing God' and hence questions the moral status of genetically modified organisms (GMOs). The study of Kropf (2025) about slippery slope arguments concluded that conceivable traits in a single individual can be incorported in the entire genome, leading to ethical dilemmas. Such traits may give rise to aberrant gene expression, leading to new diseases' risks. Animal welfare advocates carry a strong stance that genome editing with CRISPR is instrumentalizing animals, leading to devaluation of their intrinsic worth (Singh and Ali, 2021b). The goal of genome alteration is to conserve and enhance the desirable traits, which may subdue biodiversity. Some of the off-target effects may lead to the irreversible detrimental ecosystem impacts, either in the form of deviation from natural capabilities endowed to the animals or unknown consequences of their wild counterparts (Li et al., 2024).

FUTURE PROSPECTS AND CHALLENGES

CRISPR-Cas9 integrated with IVF shows great potential to revolutionize livestock improvement over the coming years. Scientists focus on improving the functionality of genome editing instruments as one primary developmental pursuit. Base editing and prime editing represent current developments which allow for exact single-nucleotide modification without requiring double-strand break formation (Saber Sichani et al., 2023). The development of newer platforms provides additional capabilities to minimize embryo genetic mutation effects along with increased safety during gene modification procedures. CRISPR-Cas system has revolutionized the genomic editing with day-to-day advancements in the protocols of its utilization. Greater precision in IVF zygotes/embryos had made it possible to easily alter the desired part of the genome. Integration of different multi-omics technologies with CRISPR is enabling genome-wide association studies (GWAS) to provide more precision and accuracy towards the goal of achieving desirable traits of economic importance in livestock. Using metabolomics, transcriptomics, proteomics and epigenomics has improved feed efficiency, pattern of gene expression, biomarkers for disease surveillance and reproductive efficacy respectively (Wadood et al., 2025). New IVF methods will gain from using rapid embryo quality assessment technology which evaluates transcriptomic and proteomic data to help researchers select embryos for transfer that have the best chances of being healthy. Novelties in microfluidics and nanotechnology generate potential solutions for better delivery methods of CRISPR components without causing extensive invasiveness.

The implementation of artificial intelligence and machine learning algorithms within genome editing workflows improves both target gene selection and sgRNA optimization as well as genomic interaction modeling. All such technological advancements will boost precision and individualized editing methods for multiple different types of animal species. According to the reports of Yuan et al. (2025), the use of deep learning (DL) as well as machine learning (ML) models with guide RNA (gRNA), enables more precise tracking of CRISPR activities to get less mosaic outcomes and fewer off-target effects. The expanded use of artificial intelligence

and nanopore sequencing to proceed the CRISPR technology has made it possible to obtain reduced erroneous cleavage and more accuracy in editing with less human errors (Anyaegbunam et al., 2025). Livestock serve as bioreactors for pharmaceutical manufacturing while the scope of their use in production is expected to increase. Targeted gene modifications in animals enable production of high-value pharmaceutical molecules which can be recovered from milk or blood streams thus minimizing dependence on conventional manufacturing facilities. CRISPR-IVF technologies present significant potential to address worldwide issues involving climate change alongside food security while reducing zoonotic disease emergence. These technologies enable exact solutions to help develop livestock systems that provide increased productivity together with environmental efficiency and future capabilities in public health and nutrition needs.

Despite the extensive revolution in livestock production and genetic enhancement, CRISPR presents different technical, regulatory and ethical challenges. Animal scientists are struggling day by day to overcome or reduce the risks posed by these challenges. Off-target effects leading to undesired editing in the genome as well as mosaicism casuing complicated phenotypic expression due to editing errors in some of the target genes, are the most common challenges (Hennig et al., 2020). Exploiting the optimized delivery system for CRISPR components offers technical difficulties. Similarly, genome editing for polygenic traits, such as milk production, fertility, disease resistance also hinders the efficiency of this advanced technique, as it may require manipulations at several loci for considerable genetic improvement (Garcia, 2023). Besides polygenes, such traits are also impacted by environment, which should also be kept in consideration along with other factors (de Almeida Camargo and Pereira, 2022). Different countries have their own set of regulations for producing GMOs, some having strict checks on genetic manipulation may also restrict global scientific collaboration in this regard (Eski et al., 2025). Similarly, lower efficiency of IVF and SCNT in livestock also impacts the widespread embryo editing using CRISPR-Cas9, as all are interconnected in the genome editing (Xiong et al., 2014). All these challenges should be worked out to improve the efficiency and credibility of using CRISPR genome editing.

DISCUSSION

In vitro fertilization (IVF) combined with CRISPR-Cas9 technology delivers one of the greatest innovations to livestock biotechnology since the last few decades. The combination of CRISPR-Cas9 with IVF technology strengthens genetic improvement speed and accuracy while creating organisms for agricultural and biomedical research purposes. Biotechnology researchers from various livestock sectors including cattle, goats, pigs and sheep and buffalo have achieved exceptional results with gene-editing operations through their combined CRISPR-Cas9 and IVF system that lets experts regularly change or completely remove desired genes for qualities linked to production characteristics as well as disease prevention and reproduction abilities. The most beneficial aspect of CRISPR-IVF technology serves to produce livestock which possess resistance against diseases. Scientific studies that featured the PRNP knockout in goats (Allais-Bonnet et al., 2025) together with PD-1 knockout in pigs and other immune-related gene manipulations proved successful in disease resistance enhancement, while establishing improved biomedical model potential (Yuan et al., 2024). Dairy goat milk production receives new possibilities through knock-in strategies like HNP1 integration which creates opportunities for therapeutic protein production. The review demonstrates that multiple gene editing has become possible based on the research showing Cas12iMax simultaneously editing three genes in cattle and four genes in pigs. Through multiplex editing systems researchers can quickly achieve efficient changes in desired traits which otherwise needed hundreds of years of

selective breeding to develop. Scientists developed electroporation together with *in vivo* gene delivery (GONAD) to solve mosaicism and embryo damage issues which enhanced editing accuracy and minimized unintended side effects (Watanabe et al., 2024).

The technology faces several restrictions during use. Commercial application faces barriers from different regional ethical frameworks alongside evolving regulations for genetically modified animals. So as to address the worries regarding genetic diversity and long-term animal welfare and unintended ecological impacts, researchers must conduct complete ethical examinations with rigorous risk assessments (Aboelhassan and Abozaid, 2024). Despite these challenges, the benefits of CRISPR-Cas9 and IVF integration outweigh the limitations. This animal management system combines flexible procedures with quick implementation and economical advantages to provide both food production growth solutions and better animal health management with environmental impacts minimized in livestock farming fields.

CONCLUSION

Modern livestock genetic engineering has experienced a revolutionary transformation because of the combined strength of CRISPR-Cas9 genome editing with IVF technologies. Diverse genome editing capabilities become possible through this strong combination which lets researchers make exact genomic adjustments to animals leading to new solutions about productivity boost, disease resistance and allergen reduction alongside medical research model creation. This review illustrates how planned gene modifications and combined multiplex editing practices together with improved IVF techniques have remodeled the field of livestock biotechnology. Advancements in genome editing precision together with delivery system developments and supportive IVF environments will probably overcome existing limitations which include mosaicism and off-target effects during the upcoming years. The universal establishment of ethical and regulatory frameworks will act as a key factor for successful adoption and market expansion of genetically modified livestock. The combination of CRISPR-Cas9 with IVF positions itself to create sustainable agricultural systems combined with improved animal welfare and better human healthcare, hence establishing its central function in future livestock enhancement approaches.

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AUTHOR CONTRIBUTION

The author solely reviewed the literature and compiled the most recent advancements in Livestock genome editing.

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