Challenges in the application of CRISPR-Cas9 for genetic engineering of nile tilapia (*oreochromis niloticus)*: *systematic review*

Lesta Irmanda*, Septianto Wikan Nurhidayat, Arif Bimantara

Deparment of Biotechnology, Fakulty of Science and Technology, Universitas 'Aisyiyah Yogyakarta, Indonesia
*Email: lestairmanda@gmail.com

Abstract

Nile tilapia (*Oreochromis niloticus*) is an important aquaculture commodity in Indonesia, but productivity and disease resistance remain major challenges. CRISPR-Cas9 technology offers the potential to precisely improve growth, disease resistance, and other superior traits in a single generation. This study aims to identify technical, biological, and applied challenges in the application of CRISPR-Cas9 in tilapia and strategies that can be used to optimize gene editing results. The research method was conducted using a systematic literature review (SLR) approach to identify the challenges of applying CRISPR-Cas9 in tilapia genetic engineering. The application of CRISPR-Cas9 faces technical challenges such as mosaicism, off-target effects, and low editing efficiency, which can be overcome by optimizing sgRNA, Cas9 quality, injection methods, and multi-omics approaches. Biological challenges include infertility due to reproductive gene mutations, hormonal imbalances, and genetic compensation phenomena, which can be minimized through conditional knockout, rescue experiments, and dual targets. At the application level, mutants are often difficult to utilize due to reduced viability, so marker-based selection strategies, selection of appropriate target genes, and public regulation and socialization are necessary to support successful implementation. CRISPR-Cas9 has great potential to increase the productivity and resilience of tilapia. Its successful implementation requires an integrated approach that combines technical optimization, attention to biological effects, and application strategies that consider regulations and public acceptance.

Keywords: CRISPR-Cas9; disease resistance; gene editing; infertility; Nile Tilapia (*Oreochromis niloticus*)

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important aquaculture commodities in Indonesia, both for the domestic and international markets, due to its popular taste, affordable price, and high protein content. Based on data from the Badan Pusat Statistik (2020), Indonesia's tilapia exports reached 12.29 thousand tons, demonstrating its significant role in the national economy. However, tilapia farming in Indonesia still faces various obstacles that affect productivity. Infectious diseases are one of the main threats. Cases of streptococcosis caused by *Streptococcus agalactiae* have been reported to cause high mortality in tilapia. Research at the Cirata Reservoir in West Java found a prevalence of *S. agalactiae* infection with a significant mortality rate (Suhermanto *et al*., 2020).

Another study in Magelang Regency showed mass mortality of tilapia due to pathogenic bacterial infection with mortality rates reaching 40–96% (Sarjito *et al*., 2021). Prevention efforts through broodstock vaccination have shown positive results, although their effectiveness is still limited (Queiróz *et al*., 2024). In addition to bacteria, *Tilapia Lake Virus* (TiLV) is also a serious threat. This virus was first detected in Indonesia in 2017 and remains a problem to this day. Recent research shows that several local Indonesian tilapia strains remain susceptible to TiLV infection with high mortality rates, exceeding 70% under certain conditions (Taukhid *et al*., 2024). This condition confirms that disease resistance remains a major issue in tilapia farming. On the other hand, tilapia productivity is also affected by relatively slow growth and suboptimal feed efficiency. The Feed Conversion Ratio (FCR) in various farming systems is still quite high, ranging from 1.6 to 2.5, thereby increasing production costs (Hadiroseyani, 2023; Omasaki *et al*., 2017).

Various approaches have been used to address this problem, including masculinization with synthetic hormones such as 17α-methyltestosterone, which was chosen because male fish grow faster than females. However, the use of synthetic hormones raises concerns about food safety and environmental impacts, and is beginning to be rejected in several export destination countries (Sarjito *et al*., 2021). Other breeding approaches include conventional selection and *Marker-Assisted Selection* (MAS), which uses genetic markers such as microsatellites and SNPs to produce fish with faster growth or better feed efficiency. The drawback is that these methods take a long time because tangible results

can only be obtained after several generations, making them less efficient for addressing short-term productivity issues (Hadiroseyani, 2023; Omasaki *et al*., 2017). Transgenesis has also been applied by introducing genes from other species to improve fish performance, such as growth hormone genes, but this raises ethical, regulatory, and international market rejection issues. In terms of disease resistance, broodstock vaccination has been shown to increase fry resistance to Streptococcus agalactiae, although its effectiveness is not always permanent, and production costs are relatively high (Queiróz *et al*., 2024). The use of probiotics and environmental quality management also play a role in suppressing pathogen growth, although their success is highly dependent on cultivation conditions (Basri *et al*., 2020; Sarjito *et al*., 2021).

The various limitations of previous technologies indicate that more precise, rapid, and safe innovations are urgently needed in tilapia farming. This is where CRISPR-Cas9 becomes important. CRISPR was first discovered in 1987 when a group of Japanese researchers observed short repetitive DNA sequences in *Escherichia coli*, although the function of these sequences was not yet known (Isino, 1987 in Hsu *et al*., 2014). It was not until the early 2000s that research showed that CRISPR was part of the bacteria's defense system against viruses. A major discovery occurred in 2012 when Jennifer Doudna and Emmanuelle Charpentier successfully engineered this system into a gene editing tool that could be programmed to target specific DNA sequences (Jinek *et al*., 2012). This technology is capable of performing specific genetic modifications without adding foreign DNA, and can produce tangible results in just one generation (Doudna & Charpentier, 2014). CRISPR stands for *Clustered Regularly Interspaced Short Palindromic Repeats*, which are short, regularly spaced DNA sequences found in bacterial genomes. These sequences function as "immunological memory" to recognize viruses that have previously attacked. Meanwhile, Cas9 stands for CRISPR-associated protein 9, which is a nuclease enzyme that acts as "genetic scissors" to cut DNA at the target location (Hsu *et al*., 2014).

In general, the CRISPR Cas9 mechanism (Figure 1) involves three main components, namely target DNA, guide RNA (gRNA), and Cas9 enzyme. CRISPR-Cas9 is a gene editing technology that works by cutting DNA at specific locations determined by guide RNA (gRNA). In tilapia, the Cas9-gRNA complex is usually inserted into the zygote through microinjection. After Cas9 performs the cut, the cell repairs the DNA through natural mechanisms, either by Non-Homologous End Joining (NHEJ), which causes small mutations, or by Homology Directed Repair (HDR), which allows the insertion of new genes. In this way, important traits such as growth, sex determination, and disease resistance can be modified with precision (Li *et al*., 2021).

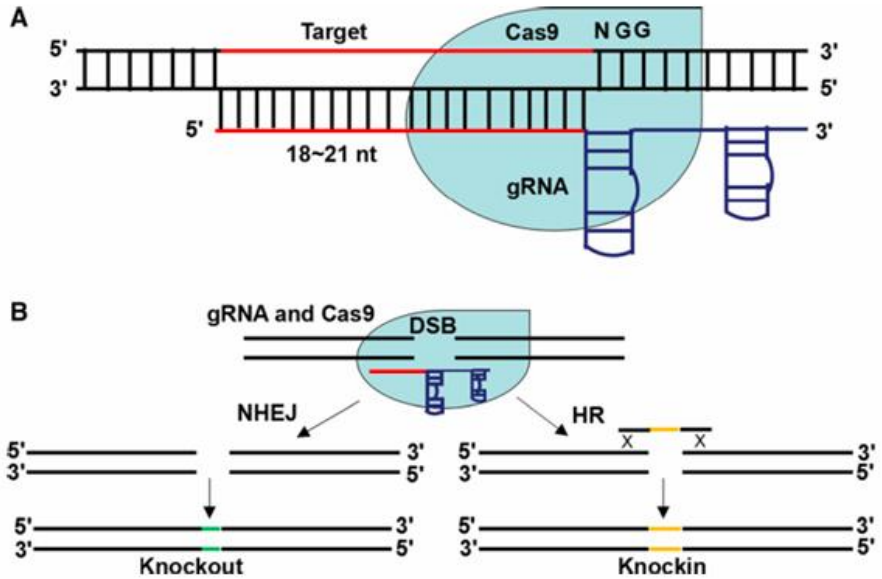

Figure 1. CRISPR Cas9 Mechanism. A) The CRISPR Cas9 system consists of Cas9 nuclease and gRNA synthesis that directs the nuclease to the target DNA precisely upstream of the 5'-NGG motif adjacent to the Watson-Crick base pairing rule. B) Double-strand break (DSB) repair promotes gene editing. (Source: Li *et al*., 2020)

Several studies have proven the effectiveness of CRISPR-Cas9 in genetic engineering of tilapia, for example in sex regulation through modification of the amh and dmrt1 genes (Li *et al*., 2020; Li *et al*., 2022), improving fertility control through the aldh1a2 gene (Zhang *et al*., 2020), and developing superior strains that are more efficient in feed utilization (Hadiroseyani, 2023). In addition, CRISPR-Cas9 is also highly relevant for improving disease resistance, given the major threat of *Streptococcus agalactiae* and *Tilapia Lake Virus* (TiLV) infections, which often cause mass mortality and economic losses in Indonesia (Taukhid *et al*., 2024). With its advantages of high precision, short breeding time, and the potential to produce non-transgenic fish that are more acceptable to the public, CRISPR-Cas9 is a strategic technology to support aquaculture sustainability, increase productivity, and strengthen the competitiveness of tilapia in the global market. However, the application of this technology still faces various challenges and obstacles that need to be further researched. Given these conditions, this study aims to identify the technical, biological, and practical challenges in the application of CRISPR-Cas9 in tilapia and the strategies that can be used to optimize gene editing results. This will serve as the basis for developing more effective and sustainable technology utilization strategies.

2. Method

This study used a systematic literature review (SLR) approach to identify studies related to the challenges of CRISPR-Cas9 application in tilapia genetic engineering. The first stage was to identify the problems and research questions. Then, an SLR protocol was created containing inclusion-exclusion criteria (Table 1), literature search strategies, databases used, and data extraction formats. The literature search strategy involved determining keywords and Boolean operators. The main keywords were derived from the research focus, namely CRISPR-Cas9 technology and tilapia (*Oreochromis niloticus*). Boolean operators were used to broaden and narrow the search; AND to combine main terms, OR to include synonyms or alternative terms, and NOT to exclude irrelevant topics. Determining the literature search database and conducting a systematic literature search. The databases used were Google Scholar, ScienceDirect, and Pubmed. Then, screening or selection of studies was carried out based on the title and abstract, as well as analyzing the full text. Entering data into the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram. Then, articles that meet the inclusion criteria are extracted based on author, year, organism, country, method, and challenges or issues that arise. The data is analyzed descriptively and compiled into a scientific article.

Table 1. Inclusion and exclusion criteria for the study

Inclusion	Exclusion
Experimental studies related to Nile tilapia genetic engineering	Studies not directly related to Nile tilapia.
Using CRISPR-Cas9	Technologies other than CRISPR-Cas9
Published in peer-reviewed journals	Studies that are not peer-reviewed
Written in English and the author's native language (Indonesian)	Written in languages other than English and the author's native language (Indonesian)
Published within the last 10 years (2014-2024)	Irrelevant literature.
Studies available in full text and/or preprint	Not available in full text

3. Results and Discussion

The results of the literature search based on keywords in the Google Scholar, ScienceDirect, and Pubmed databases are shown in the PRISMA diagram (Figure 2). Initial identification yielded 2,270 articles, which were reduced to 1,089 after duplicate removal. Then, after screening based on abstracts and titles, 939 articles remained, and 150 journals were deemed irrelevant because they were not available in English or Indonesian. After further screening, 48 articles remained that met the inclusion criteria and were available in full text, of which 27 were review articles and were therefore excluded. The final result was 21 articles from which data was extracted as shown in Table 2. The data was then analyzed descriptively and compiled into a scientific article.

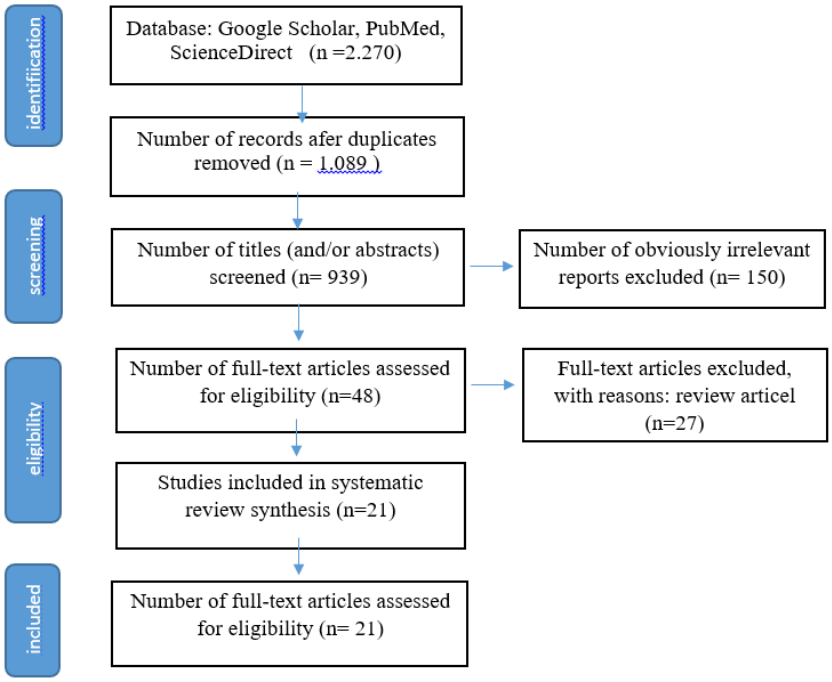

Figure 2. PRISMA Diagram Flow

An analysis of 21 research articles shows that the majority of studies on the application of CRISPR-Cas9 in tilapia were conducted in China (19 studies), while the rest came from collaborations between the United Kingdom, Korea, and China (1 study), and Israel (1 study). China's dominance in these publications reflects its strong biotechnology research capacity and government policy support to accelerate the development of modern aquaculture technology. In contrast, publications from Southeast Asia, including Indonesia, are still very limited, even though these countries are the world's leading tilapia producers. This confirms the existence of a research gap that needs to be filled by Indonesian researchers, especially in addressing local challenges such as infectious diseases and feed efficiency.

Table 2. Results of CRISPR/Cas9 application research on tilapia

No	Author and Year	Country	Organism	Target Gen	CRISPR-Cas9 Method	Challenges
1.	Li *et al*. 2014	China	Nile tilapia (*Oreochromis niloticus*)	*nanos2, nanos3, dmrt1, foxl2*	gRNA +Cas9 mRNA microinjection	Low transfer efficiency, mosaicism in the F0 generation
2.	Zhang *et al*. 2016	China	Nile tilapia (*Oreochromis niloticus*)	*dmrt6*	gRNA +Cas9 mRNA microinjection	Complexity of the spermatogenesis pathway
3.	Feng *et al*. 2015	China	Nile tilapia (*Oreochromis niloticus*)	*aldh1a2, cyp26a1*	gRNA +Cas9 mRNA microinjection	Complex regulation of retinoic acid homeostasis
4.	Li *et al*. 2015	China	Nile tilapia (*Oreochromis niloticus*)	*amhy/amhII*	gRNA +Cas9 mRNA microinjection	SNPs on the Y chromosome, off-target risk
5.	Xie *et al*. 2016	China	Nile tilapia (*Oreochromis niloticus*)	*SF-1 (nr5a1)*	gRNA +Cas9 mRNA microinjection	Risk of double and off-target phenotypes
6.	Jiang *et al*. 2016	China	Nile tilapia (*Oreochromis niloticus*)	*gsdf, dmrt1*	gRNA +Cas9 mRNA microinjection	Complex transcription regulation

No	Author and Year	Country	Organism	Target Gen	CRISPR-Cas9 Method	Challenges
7.	Jiang *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*wt1a, wt1b*	gRNA +Cas9 mRNA microinjection	Phenotypic differences in paralog genes
8.	Chen et al. 2017	China	Nile tilapia (*Oreochromis niloticus*)	*eEF1A1b*	gRNA +Cas9 mRNA microinjection	Risk of total infertility
9.	Yan *et al*. 2019	China	Nile tilapia (*Oreochromis niloticus*)	*esr1, esr2a, esr2b*	gRNA +Cas9 mRNA microinjection	Receptor function compensation complex
10.	Li *et al*. 2019	China	Nile tilapia (*Oreochromis niloticus*)	*miRNA, Vasa-3 'UTR)*	gRNA +Cas9 mRNA microinjection	Difficult validation of non-coding function
11.	Jin *et al*. 2020	UK	Nile tilapia (*Oreochromis niloticus*)	*piwil2*	gRNA +Cas9 mRNA microinjection	Mutation screening complexity
12.	Tao *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*foxh1*	gRNA +Cas9 mRNA microinjection	Risk of total infertility
13.	Jie *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*tsp1a*	gRNA +Cas9 mRNA microinjection	Complex regulation of folliculogenesis
14.	Li *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*igf3*	gRNA +Cas9 mRNA microinjection	Reproductive side effects
15.	Yang *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*rln3a*	gRNA +Cas9 mRNA microinjection	Risk of severe infertility
16.	Zheng *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*cyp11c1*	gRNA +Cas9 mRNA microinjection	Widespread hormonal impact
17.	Liu *et al*. 2020	China	Nile tilapia (*Oreochromis niloticus*)	*amh, amhr2*	gRNA +Cas9 mRNA microinjection	Genetic dose effects
18.	Li *et al*. 2021	China	Nile tilapia (*Oreochromis niloticus*)	*star2*	gRNA +Cas9 mRNA microinjection	Steroid pathway complexity
19.	Segev-Hadar *et al*. 2021	Israel	Nile tilapia (*Oreochromis niloticus*)	*Slc45a2)*	gRNA +Cas9 mRNA microinjection	Potential cultivation applications
20.	Yang *et al*. 2022	China	Nile tilapia (*Oreochromis niloticus*)	*cyp17a2*	gRNA +Cas9 mRNA microinjection	Risk of infertility
21.	Wei *et al*. 2022	China	Nile tilapia (*Oreochromis niloticus*)	*sox30*	gRNA +Cas9 mRNA microinjection	Specific gene function challenges

The main focus of CRISPR-Cas9 applications in tilapia is more directed at sex determination, fertility regulation, organogenesis, and phenotypic characteristics, while research related to disease resistance and feed efficiency is still limited. This situation needs to be understood in the context of the main problems in tilapia farming in Indonesia, namely high mortality due to *Streptococcus agalactiae* and *Tilapia Lake Virus* (TiLV) infections, as well as high production costs due to slow growth and suboptimal feed efficiency (Sarjito *et al*., 2021; Taukhid *et al*., 2024). Of all studies (Figure 3), approximately 33.3% discussed the mechanisms of spermatogenesis and male fertility, while 28.6% focused on sex determination and gonadal differentiation. Meanwhile, research on oogenesis and female fertility accounted for 19.0%, followed by studies related to receptor knockout or common reproductive

hormones at 9.5%, and non-reproductive applications such as body color characteristics and organ function, which also only reached 9.5%. These findings indicate that CRISPR-Cas9 research on tilapia is still narrow and dominated by reproductive issues, while its application to other important traits such as disease resistance, feed efficiency, and environmental adaptation is still very limited. Therefore, further diverse and applied research is needed so that CRISPR-Cas9 technology not only contributes to understanding reproductive mechanisms but also addresses real challenges in enhancing productivity and sustainability in tilapia aquaculture.

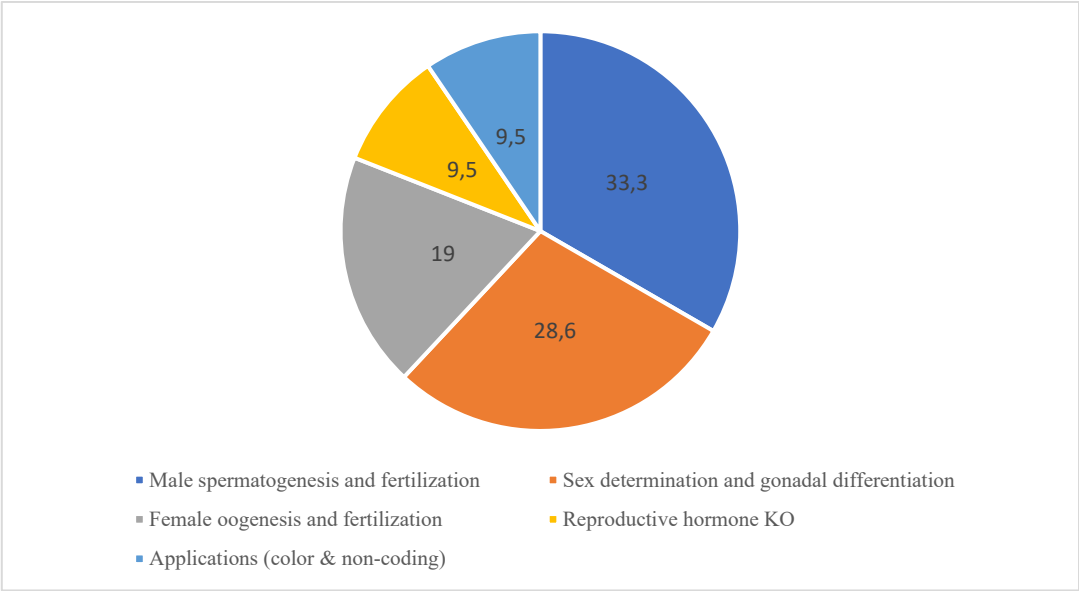

Figure 3. Research focus percentage

The application of CRISPR-Cas9 in Nile tilapia (*Oreochromis niloticus*) has opened up great opportunities in understanding genetic mechanisms, especially in terms of sex determination, spermatogenesis, folliculogenesis, and the development of new strains for aquaculture. However, the challenges faced are still quite significant. The challenges of applying CRISPR-Cas9 in Nile tilapia (*Oreochromis niloticus*) can be broadly divided into three categories: technical and methodological challenges, biological challenges, and application challenges (Figure 4).

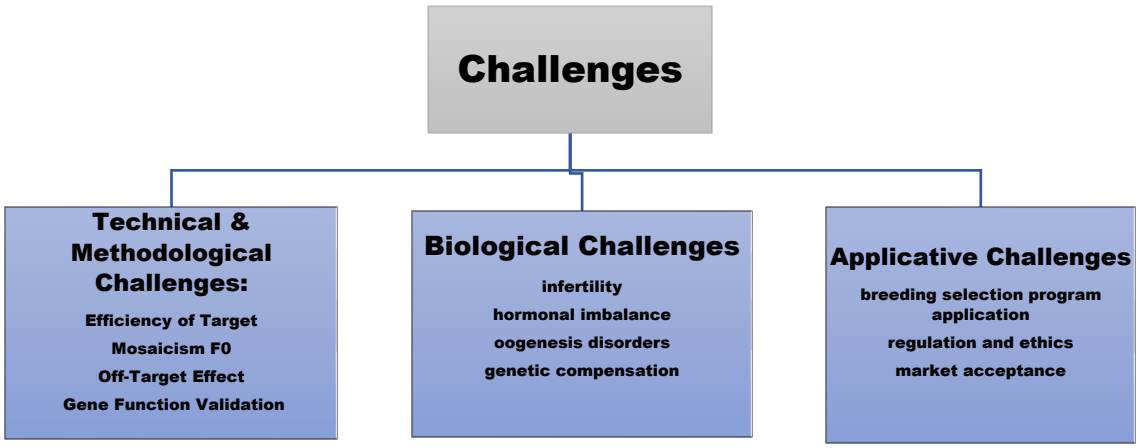

Figure 4. Challenges of CRISPR-Cas9 application in Nile tilapia (*Oreochromis niloticus*)

3.1 Technical and Methodological Challenges

The application of CRISPR-Cas9 in tilapia still faces significant technical challenges, particularly regarding gene editing efficiency. One of the main problems is mosaicism in the F0 generation, a

condition in which the results of mutation are not uniform throughout the body tissues, causing mutant instability and requiring breeding to the next generation to obtain a consistent lineage (Li *et al*., 2014). This problem can be overcome by breeding fish to the F1 or F2 generation to obtain consistent mutations, as well as using several sgRNAs simultaneously to increase editing efficiency (Feng *et al*., 2015). In addition, the potential for off-target effects, namely mutations in genes other than the desired target, is a challenge because it can produce inappropriate phenotypes and even cause new physiological disorders. To overcome this, more precise sgRNA design based on bioinformatics algorithms and validation through genome sequencing are needed to ensure more accurate editing results (Li *et al*., 2014; Xie *et al*., 2016).

Another difficulty arises in validating gene function, especially for non-coding and transcription genes such as esr and sox30, where functional effects are difficult to confirm consistently (Yan *et al*., 2019; Wei *et al*., 2022). The problem of phenotypic differences between individuals and the limitations of mutant screening methods also add to the technical complexity, as reported in studies related to wt1 and piwil2 (Jiang *et al*., 2016; Li *et al*., 2020). Another technical challenge is the still low editing efficiency, which can be improved by optimizing injection methods, enhancing Cas9 quality, and selecting target genes with higher chromatin accessibility. Meanwhile, difficulties in gene function validation can be overcome by multi-omics approaches such as transcriptomics and proteomics to ensure the comprehensive impact of mutations (Wei *et al*., 2022). This highlights the need for method optimization, from sgRNA design and injection efficiency to mutant screening techniques, as technical challenges that must be addressed before CRISPR-Cas9 can be more widely applied in tilapia cultivation.

3.2 Biological Challenges

Biological challenges are mainly related to total or partial infertility due to the deletion of genes that play an important role in reproduction. Several studies have shown that mutations in genes that play an important role in gonadal development and sexual differentiation can cause infertility in both males and females. For example, mutations in the *foxh1* gene result in arrested oogenesis, causing female fish to become infertile (Yang *et al*., 2020), while mutations in *eEF1A1b* and *cyp11c1* cause disrupted spermatogenesis, leading to sterility in males (Zheng *et al*., 2020; Jiang *et al*., 2016). These challenges can be overcome with a conditional knockout (cKO) approach that is only active in certain tissues, so that reproductive function is not completely disrupted, or by conducting rescue experiments through the addition of normal gene copies to restore the phenotype. In addition to infertility, hormonal imbalance is also a serious challenge. Mutations in genes that regulate steroidogenesis pathways such as *amh, aldh1a2, cyp17a2,* and *star2* can cause changes in hormone levels that lead to delayed gonadal development or reproductive abnormalities (Feng *et al*., 2015). Monitoring hormone profiles and administering additional hormone therapy can be used to normalize reproductive function (Li *et al*., 2021). Another complexity arises from the phenomenon of genetic compensation, where the loss of a gene's function can be partially replaced by the expression of other genes, complicating the interpretation of experimental results. The proposed solution is the use of a double knockout or dual target strategy to ensure clearer biological effects (Wei *et al*., 2022). All these factors indicate that biological aspects pose a real obstacle to achieving stable gene editing results in tilapia.

3.3 Applied Challenges

At the applied level, difficulties arise when the results of engineering are difficult to utilize in breeding programs because the mutants produced often experience infertility or reduced viability. The solution is to select target genes that are not directly related to fertility, such as genes that control growth, disease resistance, or body color, so that superior traits can be produced without disrupting the reproductive system (Chen *et al*., 2018). The stability of engineered populations can also be strengthened by combining gene editing techniques with marker-based selection so that the quality of the population is maintained. In addition, regulatory and ethical aspects pose challenges because there are still public doubts about the safety of genetically engineered products. To overcome this, a clear regulatory framework is needed, as well as public awareness campaigns about the differences between transgenic engineering and non-transgenic precision gene editing, which is relatively safer (Wei *et al*.,

2022). With these solutions, it is hoped that the application of CRISPR-Cas9 in tilapia can be more optimal and contribute significantly to the sustainability of aquaculture. Therefore, in addition to technical and biological aspects, a clear regulatory approach and public awareness are also needed so that CRISPR-Cas9-based research results can be applied in the fishing industry without causing social resistance.

4. Conclusion

Based on the results of a research analysis of 21 articles, it can be concluded that the application of CRISPR-Cas9 in tilapia (Oreochromis niloticus) has shown high effectiveness in revealing the functions of genes that play a role in sex determination, fertility regulation, organogenesis, and certain phenotypic characteristics. However, the application of CRISPR-Cas9 in tilapia faces challenges that include technical, biological, and applicative aspects. Technically, gene editing efficiency is still low, mosaicism, off-target effects, and difficulties in gene function validation arise, requiring optimization of sgRNA design, Cas9 quality, injection, and multi-omics approaches. Biologically, mutations in important reproductive genes can cause infertility, hormonal imbalances, and genetic compensation phenomena, requiring strategies such as conditional knockout (cKO), rescue experiments, and dual targets to stabilize phenotypes. At the application level, mutants are often difficult to utilize due to reduced viability or infertility; selecting target genes that focus on growth, disease resistance, or other superior traits, combined with marker-based selection, as well as clear regulation and public outreach, are key to success. Overall, the success of CRISPR-Cas9 in tilapia requires an integrated approach to ensure technical efficiency, biological stability, and optimal application acceptance. With methodological refinement and the use of these alternative strategies, CRISPR-Cas9 has great potential to become a key technology in accelerating the tilapia breeding process, both to increase productivity and disease resistance and to adapt to environmental changes. This also positions CRISPR-Cas9 as a strategic innovation in supporting the sustainability and competitiveness of the modern aquaculture sector. Research in Indonesia needs to be directed towards utilizing CRISPR-Cas9 to improve resistance to endemic diseases and feed efficiency, in line with the needs of the national aquaculture industry. Strengthening laboratory capacity, international collaboration, and targeted research policies are essential for the sustainable application of this technology and to enhance the competitiveness of tilapia in the global market.

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