The Journal of Basic and Applied Zoology

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# Biologically engineered probiotic supplement production containing phytase enzyme for livestock, poultry, and aquaculture consumption

Narjes Mohammadi Bandari[1,2], Mohammad Abootaleb[1,2*], Iraj Nikokar[3] and Mohammad Karimli[4]

## Abstract

**Background** Livestock and aquaculture feed rely heavily on cereals, fish meal, and plant proteins, but these ingredients are not fully utilized by animals, and alternative protein sources are needed due to rising demand, unstable resources, and high prices. However, plant-based materials contain phytic acid or phytate, making phosphorus less available to monogastric animals. Bacterial phytases can effectively release phosphorus from phytate in the digestive system, making them cost-effective and a potential alternative to traditional sources of phosphorus. Probiotics are helpful bacteria that have long been employed in food production and health-related products. Bioengineered probiotics are utilized to express and transmit native or recombinant molecules to the digestive tract's mucosal surface, thereby improving feed efficiency and health. Therefore, this study aimed to use a biologically engineered probiotic supplement containing phytase enzyme-producing lactic acid bacteria as a feed additive for livestock, poultry, and fish to address this issue.

**Results** The study involved multiple steps to engineer *Lactobacillus lactis* to produce the PHY protein for animal feed. These steps include identifying and designing primers for the *phy* gene, and *phy* gene was extracted from the *pMNA1* plasmid by colony PCR and cloned in *L. lactis*, confirming the presence of the PHY protein through SDS-PAGE, and harvesting the product in granular form. The *phy* gene identified and isolated using PCR and inserted it into *L. lactis*, confirming the presence of the PHY protein through SDS-PAGE. The resulting product was harvested and used as animal feed for livestock, poultry, and fish.

**Conclusions** The development of biologically engineered probiotic supplements containing phytase enzyme can enhance the nutritional value and sustainability of animal production. More research and development in this field can lead to more effective and sustainable animal production practices, benefiting both producers and consumers of animal products.

**Keywords** Phytase, Probiotic, Livestock, Poultry, Aquaculture, *Phy* gene

*Correspondence:
Mohammad Abootaleb
mohammad_abootaleb@yahoo.com
Full list of author information is available at the end of the article

## Background

Cereals, fish meal, and plant proteins are the main components of livestock, poultry, and aquaculture feed, with the latter being especially vital for carnivorous fish; however, these elements are not fully utilized by the intended audience (Gatlin et al., 2007). Due to increased demand, unpredictable resources, and high prices for grains and fish meal, as well as the expansion of the animal husbandry and aquaculture industries, research is required to explore alternate protein sources (Shepherd et al., 2017). One of the most important themes in aquaculture is using plant proteins as a substitute for fish meal in fish diets, such as soybean powder and soy protein concentrate. Plant-based products used for animal nutrition, such as cereals, other plant materials, and plant proteins, contain phosphorus in the form of phytic acid or phytate, which monogastric animals cannot consume (Arago et al., 2022).

Bacterial phytases have important biological properties, including the capacity to effectively release phosphorus from phytate in the digestive tract, resistance to inactivation during feed processing, and storage stability, which makes them cost-effective. Phytases are found naturally in microorganisms, plants, and some mammals, then bacteria being one of the microorganisms that may produce this enzyme (Rizwanuddin et al., 2023). According to research, industrial production of bacterial phytase has several applications and is economically beneficial.

On the other hand, obtaining phytase from genetically engineered plants and animals is both time-consuming and costly (Handa et al., 2020).

*Lactic acid bacteria* (LAB) can be effectively employed to express recombinant phytase. They are safe, cost-effective, and produce enzymes with excellent purity and stability. On the contrary, great species variety, ambiguity in the route of administration, and demanding monitoring due to a lack of clinical trial data provide significant hurdles in the use of these recombinant probiotic phytases (Priyodip et al., 2017).

*Lactobacillus lactis*, a probiotic microorganism, can boost the body's resilience to infections by activating the microflora and immune system. Gram-positive lactic acid bacteria (LAB) are commonly employed in the food industry to produce and preserve fermented products. Genetically modified *L. lactis* strains have potential industrial applications, including as dairy products, functional foods, probiotics, and live vaccinations. Probiotic enzyme synthesis is a topic of interest among researchers.

Phosphorus is typically deposited in cereals as phytate, which is inaccessible to chickens. As a result, substantial amounts of phosphorus are expelled in feces, causing environmental and economic implications (Lott et al., 2000).

Microbial phytases make up a significant portion of the enzymes used in the feed industry. *Escherichia coli* phytase offers several advantages over other microbial phytases, such as optimal biological activity at low pH and within the stomach's natural pH range. It is resistant to pepsin and surpasses other phytases in catalyzing phytic acid (Rizwanuddin et al., 2023).

The presence of phytate in the digestive tract inhibits the function of digestive enzymes such as alpha-amylase, lipase, and proteases such as pepsin and trypsin. According to research, including phytase enzyme in poultry nutrition improves phosphorus bioavailability, nitrogen and amino acid digestibility, apparent metabolizable energy, and ultimately leads to better poultry performance (Jain & Singh, 2016; Song et al., 2019).

## Methods

This research aimed to use a biologically designed probiotic supplement containing phytase enzyme-producing lactic acid bacteria as a feed additive for livestock, poultry, and fish.

### Bacterial strains and plasmids

*Escherichia coli* strain Top10, *Bacillus subtilis* and *L. lactis* were obtained from a gene bank in the Pasteur Institute of Iran. Plasmids used in this study, plasmid *TA* Cloning Vector as a *T. vector* and expressive plasmid *PUC19* as a carrier (Fig. 1).

### Media

Strains were maintained in LB medium bottle, LB agar plate, MRS bottle, and MRS plate (SRL, India) containing ampicillin 100 µg/ at 37 °C.

### Plasmid isolation and PCR

In this work, *B. subtilis* strain *wB600* was employed, which had the *pMNA1* plasmid carrying the *phy* purification gene. Using colony PCR, the genetic steps were taken to identify and engineer the *phy* gene from *B. subtilis*.

### Cloning and expression of the *phy* gene in *E. coli* and *B. subtilis*

The ligation was established between 500 ng of PCR product and 200 ng of *T. vector* to provide *E. coli* harboring *phy*, and the ligation mixture was subsequently transformed into *E. coli* using the conventional technique (CaCl2 method) (Abootaleb & Bandari, 2020). A total of 1000 mL of transform solutions was transferred to an LB agar plate containing ampicillin antibiotic (100 µg/mL), X-gal (30 µg/mL), and IPTG (2 mM). It was then incubated at 37 °C. After white colonies were cultivated, sorted, and used for colony PCR, and plasmid DNA containing the *phy* gene was identified by

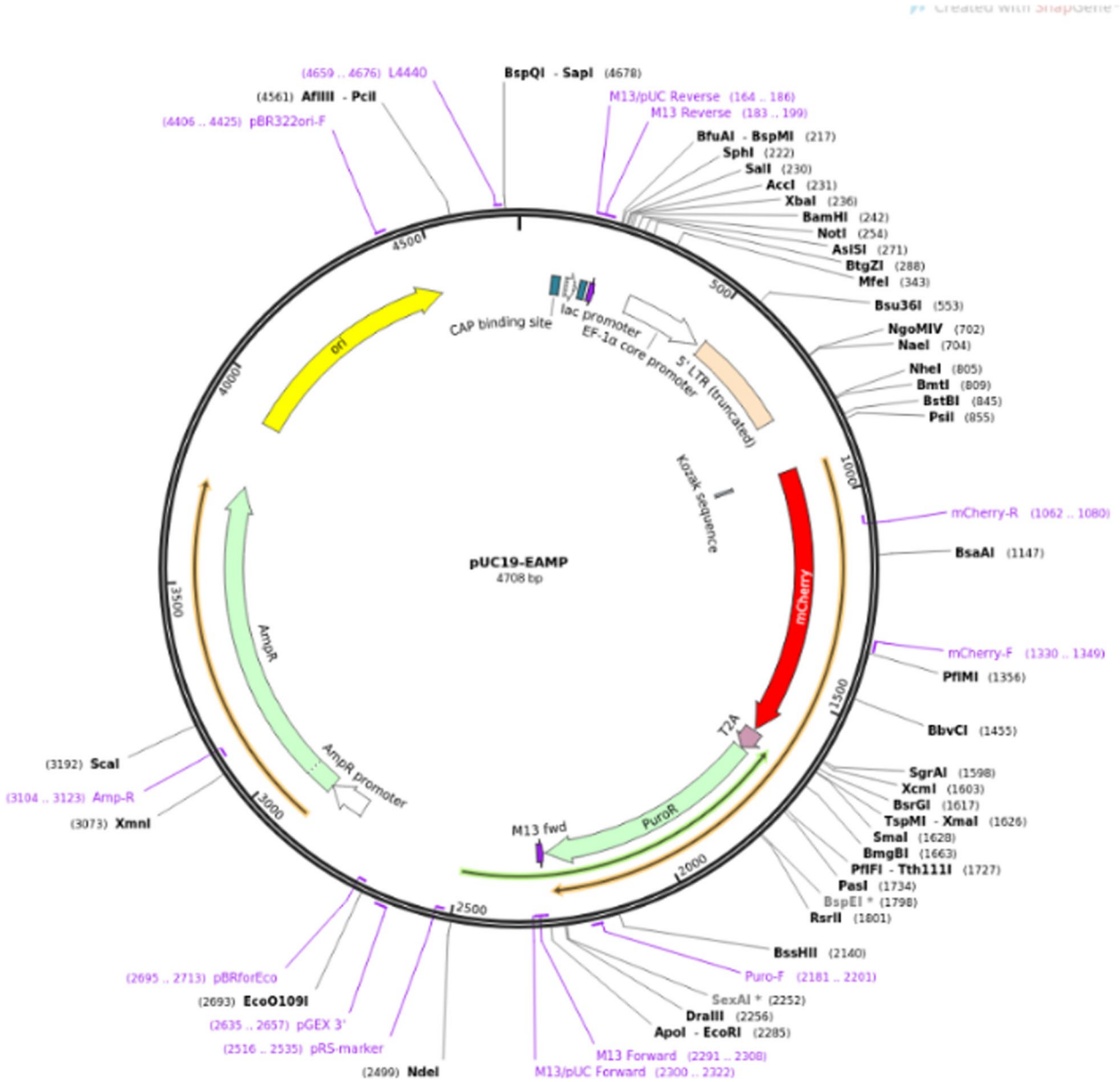


**Fig. 1** Schematic illustration of PUC19 vector

enzymatic digestion, finally, the *phy* gene was discovered and isolated. To get *L. lactis*-containing *phy*, the expression vector *PUC19* was digested with restriction enzymes sal I and BamH I, and the DNA was purified using a DNA purification Kit (Viogen, Korea). The ligation was achieved between plasmid 1 µg and gene 3 µg fragment. Following that, electroporation was carried out at 1000 v/cm for 8.5 ms. 200 µL of transform solutions was transferred to an LB agar plate containing the antibiotic ampicillin (10 µg/mL). It was then incubated at 37 °C for 48 h (Abootaleb & Bandari, 2020). The primers and PCR conditions used are listed in Table 1.

### Expression of the *Phy* gene

The next step was the expression of the target gene and confirmation of the existence of the PHY protein using SDS-PAGE. Once the protein was confirmed to be present, the engineered *Lactobacillus* culture was harvested, and the product was produced in granular form. Finally, the safety and efficiency of phytate phosphorus granules

**Table 1** Primers used and PCR condition

| Gene | Primer sequences 5′_3′ | Size of products(bp) | PCR Program | PCR volume (25 µL) |
|---|---|---|---|---|
| *phy* | F:<br>GGT CCG TTT CGT ATT CGA<br>R:<br>TCC GCT TCT GTC GGT CGA | 1065 | 1 cycle 94ċ…..13 min 30 cycle: 94ċ…..30 s 59ċ…..30 s 72ċ…..30 s 1cycle: 72ċ…..8 min | 10X PCR Buffer: 2.5 µL<br>10 mM dNTPs: 0.5 µL<br>10 mM $MgCl_2$: 0.75 µL<br>10 pmol F + R Primer: 1.25 µL<br>Taq DNA polymerase (5u $µL^{-}$): 0.2 µL<br>Template DNA: 1 µL<br>$H_2O$ up to 25 µL |

were feces determined through a digestibility experiment of samples harvested over 7-day periods.

### Statistical analysis

To analyze the obtained data, SPSS version 23.0 (SPSS, Chicago, IL) was used. T-test and Pearson's Chi-square test were employed to interpret the correlation between parameters. The level of significance in the current study was $< 0.05$.

## Results

Initially, the *B. subtilis phy* gene was extracted by colony PCR (Fig. 2). Then, the *phy* gene was inserted successfully in *E. coli* T-vector and a 1065-bp band was observed. T-vector cloning products grew on LB agar plates containing ampicillin antibiotic, X-gal, and IPTG (Fig. 3), and colonies were screened for the presence of *phy* using colony PCR.

So, the *phy* gene was separated from *TA Vector* with *Sal I and BamH I* enzymes and it was inserted to *PUC19.* The result was verified with single digestion by *BamH I* and PCR.

The GenBank accession number OR237980 corresponds to the nucleotide sequence of the *phy* gene that has been documented in the NCBI nucleotide database.

The presence of the PHY protein was confirmed using SDS-PAGE (Fig. 4). Next, the engineered *Lactobacillus* culture, which has been producing the desired phytase enzyme, was collected from the growth medium. Then, the harvested *Lactobacillus* culture was then subjected to a freeze-drying process. After the freeze-drying process was complete, the dehydrated *Lactobacillus* culture was recovered in the form of granules. These granules were used as feed complexes for animals and led to a reduction in phosphorus levels in feces samples.


**Fig. 2** Gel electrophoresis shows the PCR product of *phy*: Lane 1. Gene ruler 1-kb DNA ladder, Lane 2. Negative control, Lane 3. PCR product of phy

## Discussion

The use of probiotics in animal feed has gained significant attention in recent years as a means to improve animal health and performance (Bhogoju & Nahashon, 2022).

In this study, we successfully engineered a probiotic supplement containing the phytase enzyme for livestock, poultry, and aquaculture consumption. Our experimental design involved the bioengineering of phytase and its expression on *L. lactis*, which is a novel approach.

The cloning and expression of the *phy* gene in *E. coli* and *B. subtilis* were successful, and the presence of the PHY protein was confirmed by SDS-PAGE analysis. The engineered *Lactobacillus* culture was then harvested, and the product was produced in granular form. The resulting product was found to be safe and effective in fish, livestock, and poultry.

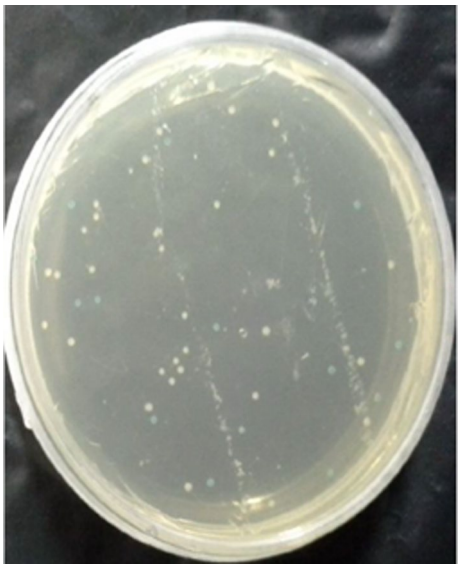

**Fig. 3** Blue/white screening of transformants

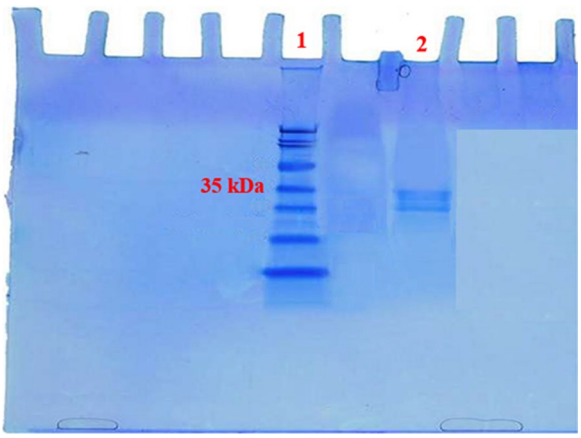


**Fig. 4** SDS–polyacrylamide gel electrophoresis (SDS-PAGE) patterns of PHY protein

Several studies have shown the feasibility and efficacy of using biologically engineered probiotics containing phytase enzymes in livestock, poultry, and aquaculture feed (Abid et al., 2018; Bhagat et al., 2020).

In one study, a recombinant *L. lactis* was developed to produce phytase with the help of a usp45 signal peptide. The phytase activity of the resulting bacteria was confirmed through zymogram analysis, and the recombinant *L. lactis* was found to increase the apparent digestibility of phytate phosphorus in broiler chicken feed. The study concludes that the engineered *L. lactis* supplement is a novel approach that shows promise for livestock, poultry, and aquaculture consumption, and focuses on the specific development of a recombinant *L. lactis* with the potential to produce phytase (Pakbaten et al., 2019), while this study provides a broader context of the need for alternative protein sources and the potential of bacterial phytases in livestock and aquaculture feed. It highlights the potential of bacterial phytases to effectively release phosphorus from phytate in the digestive system, making them a cost-effective and potential alternative to traditional sources of phosphorus. Mohammadi Ziarat et al. evaluated the effectiveness of a recombinant probiotic, *L. lactis*, in improving phosphorus availability in broiler chickens by producing phytase. The chickens were fed different diets, including a positive control diet with adequate phosphorus, a negative control diet with reduced available phosphorus, and negative control diets supplemented with different treatments, including the recombinant *L. lactis* probiotic, Hostazym®, and non-recombinant *L. lactis*. The results showed that supplementation with Hostazym® or the recombinant *L. lactis* probiotic improved growth performance, phosphorus retention, bone strength, and villi height, and reduced excretion of phytate and total phosphorus. The phosphorus content of the tibia in chickens fed the P-deficient diet containing the recombinant *L. lactis* probiotic was similar to that of the control group, suggesting that it could be a viable alternative to commercial phytase (Mohammadi Ziarat et al., 2020).

Another study investigated phytate degradation as a novel mechanism of probiotic functionality using recombinant *Lactobacillus* cultures expressing *B. subtilis* phytase. The study showed that the expression of *B. subtilis* phytase increased phytate degradation and that administration of phytate-degrading probiotic cultures improved the weight gain of broiler chickens on a phosphorus-deficient diet (Askelson et al., 2014), while our study considered the need for alternative protein sources in livestock and aquaculture feed due to rising demand, unstable resources, and high prices. It mentions the use of biologically engineered probiotic supplements containing phytase enzyme-producing lactic acid bacteria as a potential solution to address this issue.

Another study looked at how phytase isolated from *Aspergillus niger* affected growth performance, bone mineralization, phosphorous excretion, and meat quality indicators in broilers given an available phosphorous (aP)-deficient diet. In conclusion, broilers fed an aP-deficient diet supplemented with *Aspergillus* phytase excreted less phosphorus, which improved growth performance and tibia development from hatching to day 35 (Srikanthithasan et al., 2020).

The use of recombinant probiotics resulted in a similar phytate digestibility to commercial *E. coli* phytase under in vivo conditions. The effectiveness of the

enzyme in the gastrointestinal tract led to an improvement in phosphorus digestibility. Several studies have shown that commercial *E. coli* enzymes can increase phosphorus digestibility in the body by 7–13% on ordinary diets, although the results may vary depending on the phytate content and the source of the enzyme in the feed (Ptak et al., 2013; Ravindran et al., 2006).

The use of a biologically designed probiotic supplement containing phytase enzyme-producing lactic acid bacteria as a feed additive can have significant implications for the preservation and functionality of granules in fish, livestock, and poultry diets. The method involves isolating and expressing the phy gene in various bacterial strains, which leads to the production of phytase enzymes and the subsequent formation of phytate phosphorus granules. The potential impacts of this technique in each dietary scenario are remarkable (Sharma et al., 2020).

When fish consume phytase enzyme-producing lactic acid bacteria, the resulting phytate phosphorus granules can help to increase nutrient utilization and reduce environmental effect. The phytase enzyme aids in the breakdown of phytate, a typical type of phosphorus in plant-based fish diets, into more easily accessible forms of phosphorus. This can improve the digestibility of phosphorus and other nutrients, resulting in better growth performance and less phosphorus excretion in fish farming systems. The granular form of phytate phosphorus makes it easier to include into fish feed formulations, ensuring efficient transport and use within the fish digestive system (Al Gifari et al., 2022).

In the context of livestock nutrition, the addition of phytase enzyme-producing *lactic acid* bacteria and phytate phosphorus granules in feed formulations can provide various advantages. The increased availability of phosphorus owing to phytase activity can boost bone development and overall growth performance in livestock. Furthermore, the reduction in phosphorus excretion helps alleviate environmental worries about phosphorus runoff from agricultural operations. The granular nature of the phytate phosphorus allows for easy incorporation into livestock feed, ensuring uniform distribution and effective use by the animals (Derakhshan et al., 2018).

The use of phytase enzyme-producing lactic acid bacteria and phytate phosphorus granules in chicken feeds can improve phosphorus use, resulting in increased bone strength and eggshell quality in laying hens. Furthermore, reducing phosphorus excretion can help to improve environmental sustainability in chicken production systems. The granular form of phytate phosphorus allows for its incorporation in pelleted or mashed poultry feeds, ensuring uniform distribution and utilization by the birds (El Enshasy et al., 2018).

Biotechnology tools such as genetic engineering and synthetic biology offer opportunities to develop innovative and effective probiotic formulations. However, careful consideration of safety, regulatory, and economic aspects is necessary for the widespread adoption of this technology. The method described for producing phytase enzyme and phytate phosphorus granules as a feed additive for fish, livestock, and poultry has the potential to improve nutrient utilization, reduce environmental impact, and promote sustainable animal production practices across different dietary scenarios. Overall, this is a promising approach for enhancing animal feed and ensuring more sustainable food production (Chen et al., 2017; Wang et al., 2016).

## Conclusions

The new data on the recombinant *L. lactis* suggest that the production of biologically engineered probiotic supplements containing phytase enzymes has the potential to improve the nutritional value and sustainability of livestock, poultry, and aquaculture production. Further research and development in this area can lead to more efficient and sustainable animal production practices, which can ultimately benefit both the producers and consumers of animal products.

**Acknowledgements**
We would like to sincerely acknowledge all those who have contributed to this research article.

**Author contributions**
NMB, MA, IN and MK contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

**Funding**
The authors received no specific funding for this work.

**Availability of data and materials**
Data are available on request from the authors.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
We hereby grant consent for the publication of our work. We acknowledge that this consent extends to both the initial publication and any subsequent editions or reprints of the publication.

**Competing interests**
The authors declare that they have no conflict of interest.

**Author details**
[1]Burn and Regenerative Medicine Research Center, Guilan University of Medical Sciences, Rasht, Iran. [2]Agricultural Biotechnology Research Institute of Iran-North Branch (ABRII), Raika Gene Pharmed Technology Unit (Company), Rasht, Gilan Province, Iran. [3]Medical Biotechnology Research Center, Laboratory of Microbiology and Immunology of Infectious Diseases, Paramedicine

Faculty, Guilan University of Medical Sciences, Rasht, Iran. [4]Department of Biology, Faculty of Basic Science, Damghan Branch, Islamic Azad University, Damghan, Iran.

**Received: 6 August 2023 Accepted: 5 April 2024**
Published online: 13 September 2024

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## Publisher's Note