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Characterization of Lactobacilli Isolated from Fish Gut

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Authors' Contribution
MZA designed the study and supervised the research work. NK performed the experiment and SR compiles the data. RM and AR helped in experimental work. MI and MQK analyzed the data and guided the manuscript writing. NK processed the data and wrote manuscript.

Key words
Serpata seenghala, Labeo rohita, **Lactobacillus, Biochemical characterization, Antibiotics sensitivity, Antagonistic activity**

ABSTRACT

The purpose of this study was to investigate the presence of *Lactobacillus* spp. in the guts of two freshwater fishes (*Serpata seenghala* and *Labeo rohita*) with different feeding habits. Three gut samples from the adult fish of each species were studied and a total of eight isolates were screened out. Among these eight isolates, five were obtained from *Serpata seenghala* and three from *Labeo rohita* for the isolation of lactobacilli using selective Lactobacillus De Man Rogosa Sharpe Agar. Based on the morphological, physiological and biochemical characterization, three isolates were identified as *Lactobacillus plantarum* obtained from the gut of both species, two isolates identified as *Lactobacillus casei* obtained from *Labeo rohita*, while two isolates of *Lactobacillus brevis*, and one isolate of *Lactobacillus acidophilus* were found in *Serpata seenghala*. *In vitro* findings revealed that all isolates showed antagonistic activity against clinical pathogens including *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* All isolates of *Lactobacillus*, when tested with five different types of antibiotics (erythromycin, gentamicin, doxycyclins, chloramphenicol, polymixinB) showed little resistance and appeared susceptible to most of the antibiotics. All isolates of lactobacillus were able to survive at different pH values (2, 3 and 5) and low bile salt concentration (0.5%). This study indicates that the *Lactobacillus* isolates from gut of *Serpata seenghala* and *Labeo rohita* have good probiotic potential and they can also tolerate a wide range of acidic condition.

INTRODUCTION

The production of aquatic animals rose from 179 million tonnes in 2018 to 178 million tonnes globally in 2020. Asia accounted for 70% of the world's production of aquatic animals through fisheries and aquaculture in 2020, making it the primary producer region (FAO, 2022). Because of the growing need for high-quality protein, declining wild fish harvests, and advancements in fish farming technology, it is anticipated that global aquaculture production will triple by 2050 (Ibrahim *et al.*, 2020).

Fish has traditionally comprised a sizable portion of the human diet and, in many regions of the world, is the primary source of animal protein (Sheng and Wang, 2021). Additionally, fish as a food is protective, particularly in lowering blood pressure and preventing obesity, heart attacks, strokes, and high blood pressure (Forsythe, 2020). Fish serve as only inexpensive source of a nourishment in majority of the developing nations, driving increasing demand which is predicted to increase by up to 7 kg per capita by early 2030 (FAO, 2020).

In the field of carp polyculture, *Labeo rohita* (Rohu) is a well-known Indian major carp species. *Labeo rohita* is a plankton-eating column feeder. As a juvenile or adult, *Labeo rohita* mostly eats algae and submerged vegetation, making it a herbivorous column feeder (Majumder *et al.*, 2018). It provides a significant nutritional source of n-3 PUFA fatty acids and protein (Memon *et al.*, 2010). Rohu provides the highest amount of protein out of all the IMCs (Ahmed *et al.*, 2012). It also serves as a minor supply of vitamin A and calcium (Roos *et al.*, 2003). *Serpata seenghala* (Singhara) is a catfish that is carnivorous in the

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diet. Its barbels are comparatively short, its mouth is only one-third the width of the head, and its spatulate, blunt nose sets it apart from other sperata species (Bhattacharjee *et al*., 2012). Catfish aquaculture has great potential in Asia and Africa due to its high output, quick growth rate, delicious flavor, high market demand, and immense consumer popularity (House *et al*., 2003; Shourbela *et al*., 2016).

Water quality, influenced by seasonal fluctuations, leads to an increase in bacterial infections among aquatic animals. Fish immune responses are affected by changes in water quality, which also encourage the growth of bacteria in the aquatic environment (Sunitha and Krishna, 2016; Verma and Gupta, 2015). There are numerous strategies available to safeguard farmed aquatic animals from the effects of pathogens, as intensive fish farming deals with the issue of significant loss brought on by infections. Probiotics have grown in popularity as one of these methods for treating sickness (Hai, 2015). Probiotics are the mono or mixed cultures of live microorganisms that are an excellent technique used as supplementation. The Lactobacillus genus-related Gram-positive bacteria also referred to as probiotics. Many bacteria have either been mentioned or have been proposed as biological control agents against pathogens in aquaculture (Kumar *et al*., 2013). *Aeromonas hydrophila, Escherichia coli,* and *Staphylococcus aureus* can all be effectively repelled by lactic acid bacteria (Sahoo *et al*., 2015). Some bacteria have been found to have antimicrobial properties, for instance, *Lactobacillus* can stop the growth of *Vibrio cholera*, *Aeromonas* and lower the risk of aquatic infections (Allameh *et al*., 2017).

Numerous fish-isolated lactic acid bacteria (LAB) strains are capable of producing antibacterial compounds that are effective against a variety of harmful fish bacteria as well as human pathogens (Ringo *et al*., 2018). It is increasingly popular to use LAB, particularly *Lactobacillus*, to inhibit pathogenic microorganisms (Hu *et al*., 2017). Probiotic bacteria known as LAB which are found in fish intestines, have been shown to benefit fish health by enabling them to utilize nutrients more effectively and by boosting their immune system's ability to fight off pathogenic infections (Agustina *et al*., 2022). Several investigations have demonstrated that some LAB groups have antibacterial activity *in vitro* against the common carp (*Cyprinus carpio*) (Kaktcham *et al*., 2017), as well as against a number of other freshwater fish species (Bektas, 2020) and marine fish (Alonso *et al*., 2019). Among the LAB, *Lactobacillus* is the most renowned probiotic and is employed extensively in animal husbandry, healthcare and food sector (Ghanbari *et al*., 2013). *Lactobacillus* is a very varied group of LAB that has a wide metabolic adaptability which directly contributes to their capacity to colonize a variety of environments (Wei *et al*., 2019). The present work was done to isolate and characterize lactobacillus species from the gut of carnivore and herbivore fishes of Pakistan culture system. Isolated bacteria from fish gut can be further cultured and used as probiotic in different animal feed after ruling out its probable pathogenicity and antibiotic susceptibility pattern.

MATERIALS AND METHODS

Collections of fish gut samples

A total of six freshwater fishes; three individuals of carnivore fish (*Serpata seenghala*) and three individuals of herbivore fish (*Labeo rohita*) were collected from the local fish market in Rawalpindi. The isolation and characterization of bacteria from fish gut were conducted in the Aquaculture and Fisheries Laboratory, Department of Zoology, Wildlife, and Fisheries, PMAS - Arid Agriculture University Rawalpindi. After dissecting fish, one gram gut content of each fish was homogenized in nine ml of normal saline. Homogenized fish gut was serially diluted up to 10^{-7} dilutions, pour-plated on MRS agar plates and incubated for 24 h at 37°C. The pure cultures were obtained through streak plate method.

Identification of Lactobacillus *species*

The *Lactobacillus* spp. were identified through cultural, physiological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology (Vos *et al*., 2011). The isolates were Gram stained, tested for catalase and oxidase production, observed for motility. Sugar fermentation test (glucose, galactose, lactose, mannitol and sucrose), the VP test, growth test at 15°C and 45°C, the creation of citrate, and the production of indole were all tested.

Screening of the isolated Lactobacillus *spp. for probiotic properties*

Antibiotic sensitivity test

The agar disc diffusion method (Bauer *et al*., 1966) was used to measure bacterial antibiotic resistance against five distinct types of antimicrobial drugs (erythromycin: 15µg, gentamicin: 10µg, doxycyclins: 30mcg, chloramphenicol: 30µg, and polymixinB: 300µg). The 50µl of each *Lactobacillus* isolates was plated on Muller-Hinton agar and covered with antibiotic discs, which were then incubated for 24 h at 37°C. Based on their action, the antibiotic sensitivity was noted and the zone of inhibition was determined in millimeters.

Antagonistic activity

Antagonistic activity of *Lactobacillus* spp. against the clinical pathogens (*Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa)* was determined by well diffusion method. The pathogenic bacteria were cultured in tryptic soy broth (TSB) and incubated at 37ºC for 24h. At the same time, *Lactobacillus* cultures were incubated for 24h at 37ºC in MRS broth and cell free supernatants were collected by centrifugation at 8000 rpm and at 4ºC for 5 min. To create a bacterial lawn, pathogens were spread in tryptic soy agar (TSA) using sterile cotton swabs. Each plate had its wells (6 mm in diameter) pierced, and the supernatant was then poured into the appropriate well to measure the inhibitory activity. Following a 24-hincubation period at 37 °C, diameter of clear zone surrounding each well was measured.

pH tolerance test

A change in pH was used to assess acidification. The inoculum amount for the standardized assay remained at 10^6 CFU/ml. The produced inoculum was added to MRS broth that had been previously adjusted (pH values 2, 3, and 5) using 0.5M NaOH and 1M HCl. The tubes were incubated at 37ºC and the viable bacterial counts were ascertained after 60, 90 and 120 min by using repeated dilution and spread plate on MRS agar media. The survival rate of isolates was shown as a percentage.

Bile salt tolerance

Using MRS broth tubes supplemented with 0.5%, 1% and 2% (w/v) of oxgall, standard inoculum (10^6 CFU/ml) was cultured. After incubation at 37ºC, viable cell counts were assessed after 60, 90 and 120 min using the plate count method. The survival rate of isolates was shown as a percentage.

Statistical analysis

The data were analyzed using one-way ANOVA, with $p < 0.05$ considered to be statistically significant. The results are presented as mean ± standard deviation. OriginPro software was used for data analysis.

RESULTS

Characteristics of isolated Lactobacillus *species*

A total of eight *Lactobacillus* isolates were identified based on morphological, cultural, and biochemical traits: Isolate-1, 2, 8 (*Lactobacillus plantarum*); isolate-3, 5 (*Lactobacillus brevis*); isolate-6, 7 (*Lactobacillus casei*) and isolate-4 (*Lactobacillus acidophilus*). All five isolates from carnivores and herbivores fishes were Gram-positive non-motile rods ranged in size between 0.6-0.9 µm and made white colonies. Differentiating characteristics of the *Lactobacillus* species are given in Table I. All isolates were observed to be Gram-positive and catalase-negative. The pattern of sugar fermentation varied amongst strains. All isolates were able to grow at 15ºC except isolate-4. Among the biochemical tests, motility, citrate production, indole, and VP were found to be negative.

Antibiotic sensitivity

The sensitivity pattern obtained with isolated species of *Lactobacillus* against five antibiotics is shown in Table II. All of the isolates showed a resistance to PolymixinB antibiotic except isolate-7. However, the majority of Lactobacillus species (65%) were found to be susceptible to most of the antibiotics, and limited resistance was seen. This can be viewed as a favorable characteristic for bacteria used in probiotics.

Antagonistic activity

Certain probiotics may be considered to have an antibacterial effect against pathogenic microorganisms *in vitro*. All isolates showed significant antibacterial activity against clinical pathogens as shown in Figure 1. Among 8 isolates, isolate-6 and isolate-7 showed a strong antagonistic effect against *E. coli*. Statistical evaluation ($p < 0.05$) revealed significant all results.

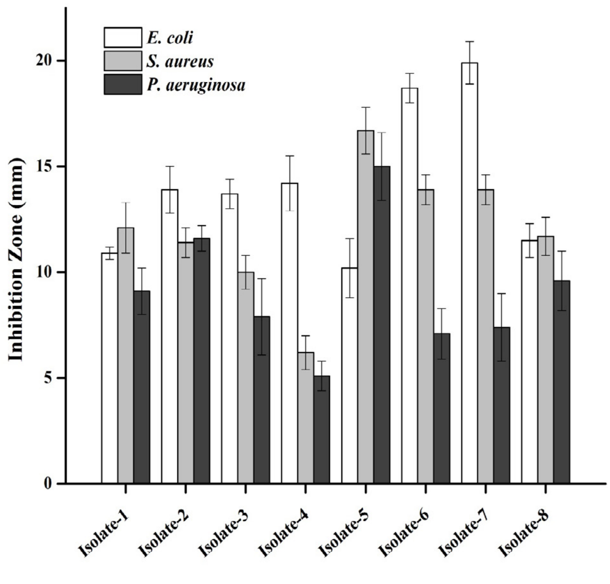

Fig. 1. Antagonistic activity of lactobacillus isolates against clinical pathogens.
Values are means of three replicates, with standard deviations represented by vertical bars. P<0.05 is statistically significant.

Table I. Biochemical characteristics of isolated lactobacillus strains.

Characteristics	Carnivore fish (*Serpata seenghala*)					Herebivore fish (*Labeo rohita*)		
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**	**Isolate 6**	**Isolate 7**	**Isolate 8**
Colony appearance	White	White	White	White	White	Cream white	Cream white	Cream white
Size	0.9µm	0.9µm	0.7µm	0.6µm	0.7µm	0.7µm	0.7µm	0.9µm
Gram's test	+	+	+	+	+	+	+	+
Morphology	Rod-shape	Rod-shape	Rod-shape	Rod-shape	Rod-shape	Rod-shape	Rod-shape	Rod-shape
Motility test	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Catalase test	-	-	-	-	-	-	-	-
Oxidase test	+	+	-	-		-	-	+
Growth 15/45 (ºC)	+/-	+/-	+/-	-/+	+/-	+/-	+/-	+/-
SCA	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-
Sugar fermentation								
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	-	+
Mannitol	+	+	-	-	-	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Ribose	+	+	+	-	+	+	+	+
Presumptive *Lactobacillus* species	*L. plantarum*	*L. plantarum*	*L. brevis*	*L. acido-phillus*	*L. brevis*	*L. casei*	*L. casei*	*L. plantarum*

+, positive reaction; -, negative reaction.

Table II. Antibiotic Sensitivity profiles of isolated lactobacillus strains.

S. No	Antibiotic	µg/disc	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
1.	Doxycycline	30	+++	+++	-	+++	+++	+++	+++	+++
2.	Erythromycin	15	-	-	+++	+++	+++	-	+++	+++
3.	Chloramphenicol	30	+++	+++	+++	+++	+++	+++	+++	+++
4.	Gentamicin	10	+++	-	-	+++	+++	+++	+++	-
5.	PolymixinB	300	-	-	-	-	-	-	+++	-

Sensitive (+++), Resistant (-).

pH tolerance

As shown in Figure 2A, test strains were grown in a modified MRS medium with different pH values (2, 3 and 5). The survival rate was determined by counting colonies on plate. Out of the eight strains, three had a survival rate of more than 70% at pH 5.0, whereas two strains demonstrated a survival rate exceeding 50% at pH 3.0. At pH 2.0, only four strains exhibited a survival rate of greater than 20%.

Bile salt tolerance

As shown in the Figure 2B, the test isolates were grown in MRS medium with different bile salt concentrations (0.5%, 1% and 2%). The survival rate was determined by counting colonies on plate. As bile salt concentrations rise, results indicate that survival rate falls. At 0.5% bile salt concentration, all isolates were able to survive. As the concentration of bile salt increases, growth of Isolate-3, Isolate-4 and Isolate-5 were inhibited, but all other isolates showed a minimum survival rate (< 30%), they tolerated even 2% bile concentration.

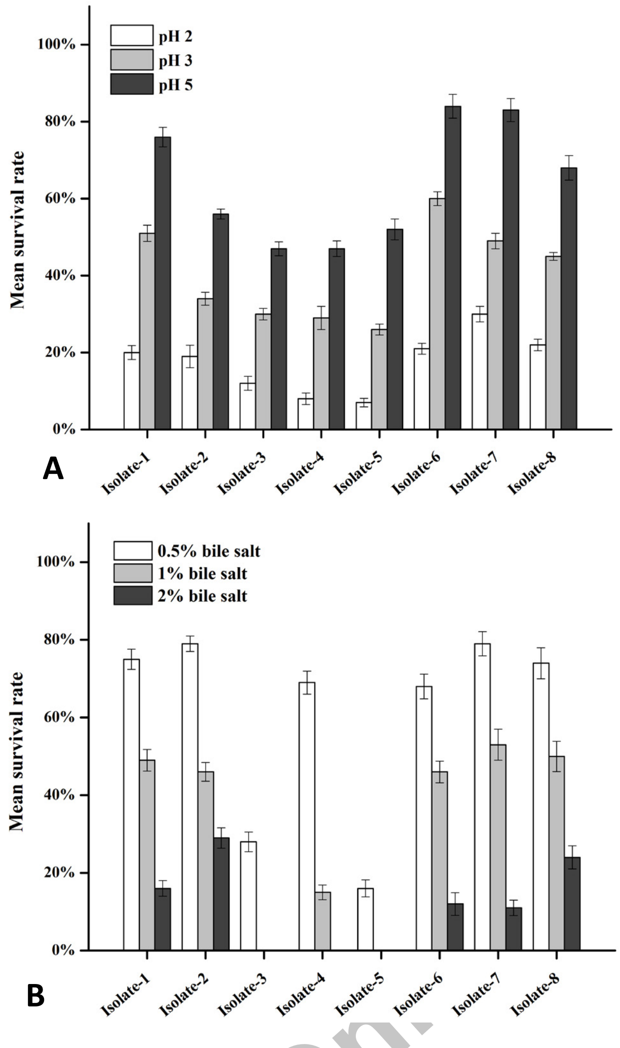

Fig. 2. Effect of different pH levels (A) and bile concentration (B) on the growth of lactobacillus isolates. Values are presented as mean ± SD and in terms of percentage. $P<0.05$ is statistically significant.

DISCUSSION

Six freshwater fishes, including *Serpata seenghala* (a carnivorous fish) and *Labeo rohita* (an herbivorous fish), were collected and tested for *Lactobacillus* species in the current study. The studies presumed lactobacilli species (Table I) were compared favorably to those reported by Bucio *et al.* (2006). According to these authors, the intestinal contents of the fish under study contained lactobacilli that were identified *as Lactobacillus coryneformis, Lactobacillus alimentarius, Lactobacillus sakei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus oris*, *Lactobacillus brevis* and *Lactobacillus casei.* The existence of Lactobacillus in singharaa (*Serpata seenghala*) and rohu (*Labeo rohita*) among six freshwater fish is consistent with the results of another researcher, who founded a maximum population of Lactobacillus in rohu (*Labeo rohita*) and catfish (*Clarias orientalis*) (Dhanasekaran *et al*., 2008). The current result is further supported by the identification of 44 strains of Lactobacillus from intestinal tract of African catfish (*Clarias gariepinus*) (Ogunshe and Olabode, 2009).

The Lactobacillus isolates were culturally, physiologically, morphologically and biochemically characterized in four groups; *Lactobacillus plantarum* (3 isolates), *Lactobacillus casei* (2 isolates), *Lactobacillus brevis* (2 isolates) and *Lactobacillus acidophillus* (1 isolate). Majority of the *Lactobacillus* species that have been recovered from an adult fish are ones that are frequently found on meat, animals, and humans, which is an interesting observation (Vos *et al*., 2011). The *Lactobacillus* species that were discovered in this investigation were distinct from those that had previously been reported. The author discovered that the isolates of *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus leichmannii, Lactobacillus fermenti, Lactobacillus Buchneri* and *Lactobacillus cellobiosus* were the most commonly occurring ones (Kvasnikov, 1977). However, only the species *Lactobacillus plantarum, Lactobacillus acidophillus* and *Lactobacillus casei* were similar in the present study. This could be the result of entirely different identification techniques, distinct environments, or different hosts.

Understanding the pattern of antibiotic sensitivity of isolated Lactobacillus strains is one of the most important factors from a safety perspective. The possibility exists that microorganisms utilized as prospective probiotics could harbor genes related to antibiotic resistance, which could then be passed on to harmful bacteria. The current understanding of LAB sensitivity to antimicrobial drugs is somewhat restricted because of the abundance of genera and species found within this bacterial group, in addition to variations in resistance spectra (Ocana *et al*., 2006). Because antibiotics may be used to replenish the intestinal microflora, the ability of LAB to tolerate them is of interest.

However, transfer of antibiotic resistance genes to an unrelated pathogens or possibly the pathogenic bacteria in gut is a serious health issue that has implications for probiotic strain safety and selection (Kumar *et al*., 2013). Out of the five antimicrobial agents that were tested against different species of lactobacillus, all of the isolates were susceptible to chloramphenicol, 87% isolates were susceptible to doxycyclins, 62% isolates were susceptible to erythromycin and gentamicin, 12% isolates were susceptible to polymixin B. These findings validated a study conducted to determine the sensitivity of *Lactobacillus* species to different antimicrobial agents (Danielsen and Wind, 2003).

In the present study, all isolates showed significant antibacterial activity against clinical pathogens. Among 8 isolates, isolate-6 and isolate-7 showed a strong antagonistic effect against *E. coli.* The ability of a lactobacilli to suppress pathogenic bacteria is due to production of organic acids and other antimicrobial compounds like diacetyl, hydrogen peroxide and bacteriocins, which increase the inhibitory activity of lactic acid bacteria (Hoque *et al*., 2010; Victor *et al*., 2011). To create a framework for use of the *Lactobacillus* to treat GI disorders in humans and animals, more research is needed to identify which compounds selectively limit the growth of harmful bacteria (Fernández *et al*., 2003; Li *et al*., 2015).

Tolerance to bile-salts and low pH are most important criteria when accepting the microorganism as probiotic. Among eight isolates of *Lactobacillus* species, maximum survival rate (>20% viability) was achieved by the four isolates at pH 2.0, suggesting that lowering the pH below 5.0 reduces the viability of a lactobacilli strains. Our findings support the results of Wang *et al*. (2010). Bile salt tolerance has also been proposed as a requirement for choosing lactobacillus strains, in addition to pH. All of the lactobacillus isolates survived at 0.5% bile salt concentration, whereas five isolates showed survival rate (< 50%) at 2% bile salt concentration, and all other three isolates were resistant to bile. These findings were consistent with Fang *et al*. (2015).

Fish may be exposed to environmental microorganisms at any point in their lives. While some of them are advantageous, others are not. A better understanding of the beneficial bacteria could help enhance the current approaches used to control diseases on fish farms. Given that Lactobacillus is naturally found in the gastrointestinal tracts of humans and other animals (Fuller, 1989) and has a variety of health benefits that have been documented (Ouwehand *et al*., 1999). Understanding that *Lactobacillus* is naturally occurring bacteria in fish could result in more uses for enhancing fish health.

CONCLUSION

The results of this study indicate that eight lactobacillus isolates were obtained from the guts of *Serpata seenghala* and *Labeo rohita*. These isolates were identified through biochemical analysis as *Lactobacillus plantarum* (Isolates 1, 2, and 8), *Lactobacillus brevis* (Isolates 3 and 5), *Lactobacillus acidophilus* (Isolate 4), and *Lactobacillus casei* (Isolates 6 and 7). The lactobacillus isolates from the gut of *Serpata seenghala* and *Labeo rohita* have a strong ability to suppress pathogenic microorganisms, including *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. They can also withstand a wide range of pH and bile salt concentrations. These traits indicate that the lactobacillus isolates from gut of *Serpata seenghala* and *Labeo rohita* have good probiotic potential.

DECLARATIONS

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IRB approval

The study was approved by institutional review board of PMAS Arid Agriculture University.

Ethical statement

In our research work, we have adopted all the guidelines authorized by the ethics committee of PMAS Arid Agriculture University Rawalpindi.

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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