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Research Article

Anti-Influenza Activity of *Lactobacillus Johnsonii* Isolated from the Bamboo Rat (*Rhizomys Sinensis*)

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Abstract

Influenza virus infections remain a serious threat to global public health, causing considerable morbidity and mortality each year. The growing interest in probiotics as natural antiviral agents provides new opportunities for developing alternative or adjunctive strategies against influenza infection. In this study, we investigated the anti-influenza virus activity of *Lactobacillus Johnsonii* (*L. johnsonii*) isolated from the bamboo rat (Rhizomys sinensis). The isolate was identified by 16S rRNA gene sequencing and biochemically characterized using the API 50 CHL kit. Antiviral activity was evaluated through both pre- and post-treatment approaches using broth suspensions, cell-free supernatants (CFS), bacterial pellets, and heat-killed preparations. Cytopathic effect (CPE) inhibition and MTT assays were employed to assess cell viability and antiviral efficacy. The pre-treated broth suspension exhibited the highest inhibitory activity, achieving 68% inhibition, while the post-treated suspension showed 61.3%. Similarly, CFS displayed 62.6% and 52.2% inhibition, bacterial pellets 56% and 47.9%, and heat-killed bacteria 47% and 46.3% inhibition in pre- and post-treatment assays, respectively. Notably, the pre-treatment approach consistently demonstrated stronger antiviral effects than post-treatment, suggesting a preventive mechanism of action. These results indicate that *L. johnsonii* exerts significant inhibitory effects against the influenza virus, potentially through the secretion of antiviral metabolites or modulation of host cellular responses. These findings provide preliminary evidence that *L. johnsonii* isolated from the bamboo rat possesses notable anti-influenza virus activity and warrants further investigation to elucidate its underlying mechanisms and potential applications.

Keywords: Antiviral activity, bamboo rat, cytopathic effect, *influenza* virus, *L. johnsonii,* Rhizomys sinensis; MTT assay; host–microbe interaction

1. Introduction

The term lactic acid bacteria (L.A.B.) refer to a broad group of bacterial species with diverse physiological and functional characteristics, many of which exhibit strong fermentative capacity and health-promoting properties in humans. L.A.B. comprise several phylogenetically distinct species belonging to the classes *Clostridia*, *Mollicutes*, and *Bacilli* [1]. They are Gram-positive, generally non-motile, non-spore-forming, catalase-negative, and aerotolerant microorganisms that produce lactic acid as the major end product of carbohydrate metabolism [2]. Their ability to ferment sugars, lower pH through acid production, and generate bioactive secondary metabolites has made L.A.B. indispensable as starter cultures in traditional fermentation practices since ancient times [3-5]. Certain among L.A.B., *L. johnsonii* has emerged as a particularly interesting species due to its host-adapted features, and growing evidence of health benefits. Genomic and ecological data suggest that *L. johnsonii* has co-evolved with

vertebrate hosts, favouring its persistence in gut and mucosal environments [6]. In human gut settings, a strain *L. johnsonii* 456 was shown to resist gastric acidity and persist beyond initial ingestion, underscoring its probiotic potential [7]. Mechanistically, *L. johnsonii* has been shown to modulate immune cell phenotypes: for example, one study demonstrated that *L. johnsonii*-derived bacterin increased IFN-γ in splenocytes and activated dendritic cell surface markers, indicating Th1-type mounting capability [8]. In respiratory and mucosal contexts, oral supplementation of *L. johnsonii* in mice altered bone-marrow derived dendritic cell (BMDC) responses during viral infection (respiratory syncytial virus), reduced airway Th2 cytokines, and induced regulatory T-cells, indicating that *L. johnsonii* can influence systemic immune responses from a gut origin [9]. Moreover, a recent review of *L. johnsonii* outlined its potential across diverse disease models; lung, liver, gut, with immune modulation, barrier enhancement and pathogen antagonism among key mechanisms [10].

Among infectious agents, pandemic respiratory viruses are particularly concerning due to their rapid spread, especially among immunocompromised individuals, and the limited effectiveness of vaccines against novel zoonotic strains. The influenza virus, a highly contagious pathogen of global significance, belongs to the Orthomyxoviridae family and possesses an enveloped, segmented, single-stranded, negative-sense RNA genome [11]. In recent years, avian influenza viruses (A.I.V.) have emerged as major public health threats, causing severe outbreaks in humans and animals and inflicting substantial economic losses on the Chinese poultry industry [12]. The A.I.V. envelope contains 18 hemagglutinin (H.A.) and 11 neuraminidase (N.A.) subtypes [13]. In recent influenza pandemics, this virus has caused seasonal epidemics infecting an estimated 3–5 million individuals and resulting in 250,000–550,000 deaths annually [14]. More recently, reassortment events among influenza viruses in swine have given rise to novel genotypes. The genotype 4 reassortant Eurasian avian-like H1N1 virus, harboring internal genes from both triple-reassortant H3N2 and the 2009 pandemic H1N1 viruses—was identified in China during influenza A virus surveillance from 2011 to 2018 [15-18]. Swine influenza A virus remains one of the most prevalent respiratory pathogens in pigs, posing a serious threat to the global pork industry [19, 20]. The Eurasian avian-like triple SIV-H1N1 (ER-H1N1) subtype is widely circulating and has caused major epidemics among pig herds in China and Europe. Evidence from multiple studies indicates that this virus can infect both pigs and humans, emphasizing its zoonotic risk [21, 22].

The Chinese bamboo rat (Rhizomys sinensis), a rodent species of the family Spalacidae, is native to southern China, northern Vietnam, and northern Myanmar. It inhabits bamboo forests, pine woodlands, and cultivated plantations [23]. Despite the availability of vaccines, influenza remains difficult to control due to variable efficacy of immunization program [24] and the adverse side effects or resistance associated with antiviral drugsts [25]. This underscores the urgent need for safe, natural, and effective alternatives such as probiotic-based antiviral approaches. A growing body of research has demonstrated that specific L.A.B. strains exert protective effects against viral infections [9, 26], and modulate allergic and immune responses in both humans and animal models [27-30]. Studies have shown that *Lactobacillus*-fermented yogurt enhances the survival of influenza-infected mice [31] and reduces influenza incidence in healthy elderly individuals [32].

Given their Generally Recognized as Safe (G.R.A.S.) status by the U.S. FDA, probiotics represent a safe and promising avenue for developing natural antiviral therapies. Therefore, this study was designed to isolate, identify, and characterize a novel L.A.B. strain from the bamboo rat and to evaluate its in vitro antiviral activity against the influenza virus (ER-H1N1). It was hypothesized that the isolated *L. johnsonii* strain may exhibit significant antiviral potential, contributing to the growing evidence supporting probiotics as natural agents for managing viral infections.

2. Material and Methods

2.1. Isolation and identification

A healthy bamboo rat (*Rhizomys sinensis*) was ethically sacrificed, and gut samples were aseptically collected. The

bamboo rat gut was chosen due to its unique high-fiber diet and specialized gut microbiota, which may harbor distinctive *Lactobacillus* strains with potential probiotic and antiviral properties [11]. Each sample was transferred into a flask containing MRS broth as enrichment medium along with 100 mL of sterile distilled water and incubated at 37 °C for 24 h. Following enrichment, 100 µL of the culture was spread onto MRS agar plates and incubated anaerobically at 37 °C for 48 h. Individual bacterial colonies were repeatedly sub-cultured to obtain pure isolates. Final identification was carried out using both molecular and classical microbiological methods. Molecular identification involved PCR amplification, while classical characterization included Gram staining for cell morphology, oxidase and catalase tests, indole production, carbohydrate fermentation profiling, and motility assessment. A total of 178 L.A.B. strains were isolated, and for antimicrobial activity screening, all Gram-positive and catalase-negative bacilli were selected.

2.2. D.N.A isolation, PCR and 16srRna sequencing of *L. johnsonii*

L. johnsonii was selected as the most promising L.A.B. candidate and further identified using 16S rRNA gene sequencing. After 24 h of culturing on MRS medium at 37 °C, a single pure colony of *L. johnsonii* was picked for DNA extraction. Genomic DNA was isolated using the Tiangen Genomic DNA Isolation Kit (China), following the manufacturer's instructions. Species-specific primers, forward primer (LB1F) 5′-GTGATCGCAGTTGGAAAACTG-3' and reverse primer (LB1R) 3′-AAGTCTGTCCTCTGGCTGG-5', were designed for *L. johnsonii* to amplify the target 16S rRNA region. PCR was performed with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 5 min. The PCR product was analyzed on a 1% agarose gel to confirm amplification. Although the resulting fragment was 270 bp (Fig 1), this short amplicon corresponds to the V3 hypervariable region of the 16S rRNA gene, which has been widely used for genus-level and, in some cases, species-level identification due to its high discriminatory power [12]. The gel-purified PCR product was then submitted for Sanger sequencing, and the resulting sequences were analyzed to confirm species identity.

2.3. Biochemical Characterization of *L. johnsonii*

Using the API 50 C.H.L. medium system, the *L. johnsonii* strain was biochemically characterized according to manufacturer instructions. A cultured colony of *lactobacillus* was picked and re-suspended in sterile water to achieve a cell density of 10^8 CFU/ml. Briefly, a freshly grown colony of *L. johnsonii* was harvested and re-suspended in sterile water to achieve a cell density of 10^8 CFU/mL. Inversion was used to gently mix a 2 mL aliquot of the cell suspension into 10 mL of API 50 C.H.L. medium. In addition, 120µl of this suspension was inoculated into API 50 C.H.L. strips overlaid with mineral oil and incubated for 48 hours before the color change was recorded.

2.4. Heat stability examination

One-fold and ten-fold cell-free supernatant of *L. johnsonii* were exposed to various heat treatments to demonstrate stability and antiviral activity against H1N1 such as 35°C, 45°C, 60°C, 90°C and 121°C with different time intervals like 5min, 10min and 15 min respectively.

2.5. M.D.C.K. Cell Culturing

In this study, we used (M.D.C.K.) Madin- Darby canine kidney cell cultures to support viral infectivity. By using high glucose Dulbecco's modified Eagle medium (D.M.E.M.) (Sigma-Aldrich, St.louis, MO, U.S.A.) supplemented with 10% heat-inactivated standard fetal bovine serum (F.B.S.) and 1% (v/v) 100U/mol penicillin and 100µg/ml streptomycin solution at room temperature under normal atmosphere condition were used to grow M.D.C.K. cells. Every 2 -3 days, cells were subcultured at a 1:3 ratio. Shortly, tissue culture flask media were collected and centrifuged at 1500× g for five minutes. The cell monolayer was detached by adding 5 mL/flask of a trypsin-versine solution (10 mL ×10 trypsin (Sigma-Aldrich, St. Louis, MO, U.S.A.) pre-sterilized versine (EDTA) incubated for 1 minute. After the detachment, the new medium was added to the flask to neutralize the trypsin activity. Following centrifugation at 1500×g rpm for 5 minutes, the supernatant

was removed, and cells were re-suspended by adding a new growth medium. By maintaining their total media volume up to 20-22ml/flask, the cells were split 1:3 and placed back into their separate incubators.

2.6. Virus culturing

The highly pathogenic influenza virus (ER-H1N1) (Accession no: MW295758) was obtained from Guangxi University College of Medicine Nanning, China. In the allotonic cavity of 11 days old chicken embryos, the ER-H1N1 influenza virus was incubated for three days. Specifically, specified pathogen-free (SPF) chicken eggs were washed with 70% (v/v) ethanol and incubated for 11 days at 35°C in a humid environment for embrocation. A 100 µl of ER-H1N1 was inoculated in an allotonic cavity using a 1ml syringe, and the punched holes were sealed with wax. The eggs were incubated again for 2-3 days in a static humidified incubator at 35℃. After incubation, eggs were placed at 4°C overnight to collect fluid using a 3ml syringe with a 21-gauge, 1-inch hypodermic needle to collect the harvested allotonic fluid. The allotonic fluid was stored at -80℃ as an H1N1 virus stock solution. The allotonic fluid virus titer was determined as embryo infection dose $10^{5.6}EID_{50}$/0.1ml as a 50% dilution end point assay described by Reed and Munnech [33].

2.7. Cytopathogenic reduction assay

For evaluating the cytopathogenic reduction effect of *L. johnsonii* firstly, the M.D.C.K. cells were cultured in a 96-well microplate using D.M.E.M. medium with 10%(v/v) fetal bovine serum (F.B.S.), 1%(v/v) 100U/mol penicillin and 100µg/ml streptomycin solution for 24-36 hours at 37°C in a 5% CO_2 cell culture incubator. At a concentration of 1×10^5 M.D.C.K., cells per well were seeded onto a 96 wells culture plate. Treated *L. johnsonii* cell-free supernatant (C.F.S.), Bacterial pellet, bacterial suspension, and heat-killed bacteria were serially diluted two folds with 2% FBS DMEM solution. H1N1 treated with two-fold dilutions and all four types of treated *L. johnsonii* were incubated at 37°C in a 5% CO2 cell culture incubator for 1 hour. These mixtures were inoculated into M.D.C.K. cells and incubated in D.M.E.M. solution with 2% F.B.S. at 37°C in a humidified chamber with 5% CO2 for 72 hours. The plates were observed for C.P.E. after 24, 48 and 72h, respectively. The reduction of the cytopathic effect is regarded as the presence of the cytopathic effect [14].

2.8. Bacterial culture

Bacterial culture was used in four different ways (1) As cell-free supernatant (C.F.S.), (2) Bacterial pellet, (3) Bacterial suspension, and (4) Heat killed. For obtaining C.F.S., The L.A.B. was grown in M.R.S. broth for 24h at 37°C and then centrifuged and filtered using a 0.22µm syringe filter while the bacterial suspension was obtained by culturing *L. johnsonii* in M.R.S. broth overnight at 37°C anaerobically. Bacterial pellets were produced by cultivating L.A.B. in M.R.S. broth overnight at 37 °C. To eliminate any residual M.R.S. broth, the growing bacteria were centrifuged, thoroughly rinsed with PBS, and serially diluted in D.M.E.M. supplemented with 2% (v/v) heat-inactivated F.B.S. [47]. Another cultured L.A.B. was sonicated for 30 minutes, heat-killed at 121℃ for 15 minutes, and used to check antiviral activity.

2.9. Mitochondrial dependent reduction Assay (M.T.T. Assay)

The Mitochondrial dependent reduction assay (M.T.T. Assay) By demonstrating the possible positive effects of antiviral agent, the M.T.T. assay (3-4,(5 dimethyl thiazol-2 yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich U.S.A.) is employed to quantify the live cells [48]. A total of 25ul M.T.T. dye was applied to incubation reaction plates where the medium was detached. Following a 3–4-hour incubation period at 37℃, 100 l of dimethyl sulfoxide (DMSO) was added, and the mixture was shocked for a short period. A microplate reader (Bio-Tek Instruments) set to 490 nm was used to measure the optical density of each well.

3. Results

3.1. Swelling

3.1. Molecular identification of *L. johnsonii*

Finally, 58 isolates were identified as *L. johnsonii* by gel electrophoresis. The amplified fragment length was 270bp, as shown in Figure 1. The ladder or first well was 100 bp marker, and the next was *L. johnsonii* positive control, and the

succeeding wells detected the *L. johnsonii*. The isolates were selected to evaluate the antiviral activity and the high accuracy in molecular identification of *L. johnsonii*.

3.2. Biochemical characterization of *L. johnsonii*

The API 50 C.H.L. kit biochemical characterization revealed that the isolate was most closely related to *Lactobacillus acidophilus* (Table 1). However, the strain displayed a distinctive carbohydrate utilization profile that differentiates it from *L. acidophilus*. Specifically, the *L. johnsonii* isolate fermented D-fructose, D-galactose, D-mannitol, D-xylose, D-tagatose, D-arabitol, D-maltose, D-sorbitol, esculin, arbutin, D-cellobiose, salicin, D-raffinose, amygdalin, D-mannose, L-rhamnose, gentiobiose, and N-acetylglucosamine, as indicated by the color change from violet to yellow in the strip capsules. In contrast, *L. acidophilus* typically does not ferment D-raffinose, D-arabitol, or D-tagatose but ferments sucrose and trehalose, providing clear biochemical criteria to distinguish these two closely related species [13]. These carbohydrate utilization patterns, together with the catalase-negative, oxidase-negative, and Gram-positive characteristics, confirm the isolate's identity as *L. johnsonii* and rule out misidentification with *L. acidophilus* or other *Lactobacillus* species.

3.3. Heat resilience test

The heat stability of *L. johnsonii* was evaluated using a cytopathogenic assay. The 10-fold concentrated cell-free supernatant (C.F.S.) was subjected to heat treatment across a temperature range of 30–121 °C for 5, 10, and 15 min, and antiviral activity against influenza virus (H1N1) was assessed on MDCK cells at 24, 48, and 72 h post-treatment (Table 2). The C.F.S. retained strong antiviral activity (+++) at 30–60 °C for all time points, moderate activity (++) at 90 °C, and detectable activity (+) even after treatment at 121 °C for 15 min.

Table 1. Biochemical Characterization of L. johnsonii by Carbohydrate interpretation Assay (API 50 K.I.T.).

Carbohydrate	Result	Carbohydrate	Result
Control	-	Methyl-β-d-xylopyranoside	+
D-galactose	+	D-ribose	+
D-mannose-	-	D-melezitose	-
D-glucose	+	L- rhamnose	-
D-xylose	-	D-lyxose	+
Inulin	+	Gentiobiose	-
Glycogen	-	Amidon	-
Dulcitol	-	D-saccharose	+
L-sorbose	-	Potassium Gluconate	-
D-adonitol	-	N-acetylglucosamine	+
D-fructose	+	Methyl-a-d-mannopytanoside	+
L-xylose	-	Amygdalin	+
D-maltose	+	Inositol	+
D-turanose	-	D-sorbitol	-
Potassium-2-ketogluconate	-	D-mannitol	+
D-fucose	-	L-fucose	-
Potassium-5-ketogluconate	-	L-arabitol	+
Esculin	+	Arbutin	-
Erythritol	-	Methyl-a-d-glycopyranoside	+
D-cellobiose	+	Potassium-2-ketogluconate	-
D-arabitol	-	D-ribose	+
D-tagatose	-	L-arabinose	+
D-fucose	-	Glycerol	-
Xylitol	+	D-lactose	+
D-raffinose	-	Salicin	+

Note: A positive sign (+) denotes a color change to yellow, while a negative sign (-) denotes no change in color.

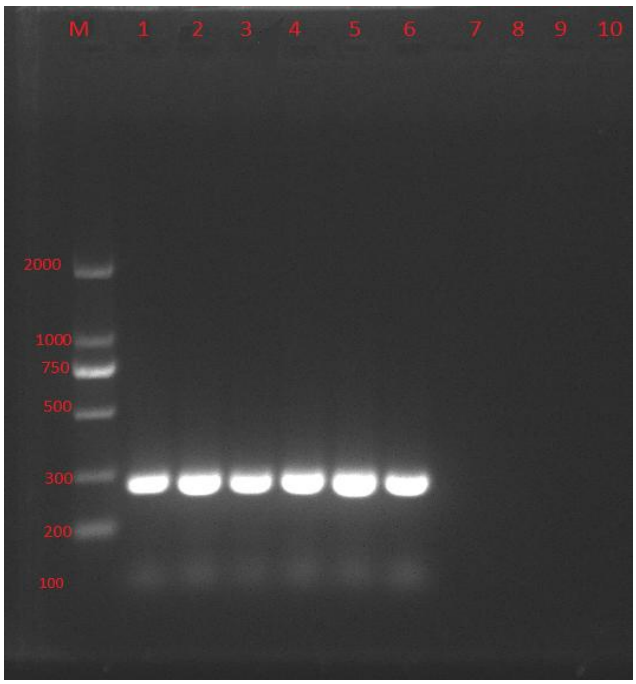

Figure 1. Gel electrophoresis of PCR-amplified 16S rRNA gene from L. johnsonii. Lane M: DNA ladder (size markers in bp); Lane 1: PCR product of *L. johnsonii* showing a single specific band at ~270 bp, confirming successful amplification. The primer pair LB1F/LB1R was designed specifically for *L. johnsonii.*

Table 2. Heat stability test of *L. johnsonii*

Temperature	Time(min)	24h	48h	72h
30℃	5	+++	+++	+++
	10	+++	+++	+++
	15	+++	+++	+++
45℃	5	+++	+++	+++
	10	+++	+++	+++
	15	+++	+++	+++
60℃	5	+++	+++	+++
	10	+++	+++	+++
	15	+++	+++	+++
90℃	5	++	++	++
	10	++	++	++
	15	++	++	++
121℃	5	+	+	+
	10	+	+	+
	15	+	+	+

Note: Symbols indicate the level of antiviral activity against influenza virus (H1N1) as determined by a cytopathogenic assay: +++, strong activity; ++, moderate activity; +, detectable activity.

These results demonstrate that the antiviral components of *L. johnsonii* are highly heat-stable, maintaining efficacy over 72 h, and suggest that they may be suitable for practical applications, including incorporation into heat-processed foods, storage, and probiotic formulations.

3.4. Cytopathogenic reduction assay

The cytopathic reduction assay was used to evaluate the in vitro antiviral activity of *L. johnsonii* in MDCK cells. In this assay, MDCK cells cultured in 96-well plates were infected with the influenza virus ER-H1N1, and untreated virus-infected cells served as the negative control, exhibiting a clear cytopathic effect (CPE) (Fig. 2D). In contrast, cells treated with the cell-free supernatant (C.F.S.) of *L. johnsonii* showed a significant reduction or complete absence of CPE at various time points, including 36 h and 72 h post-infection, as shown in Figures 2B and 2C

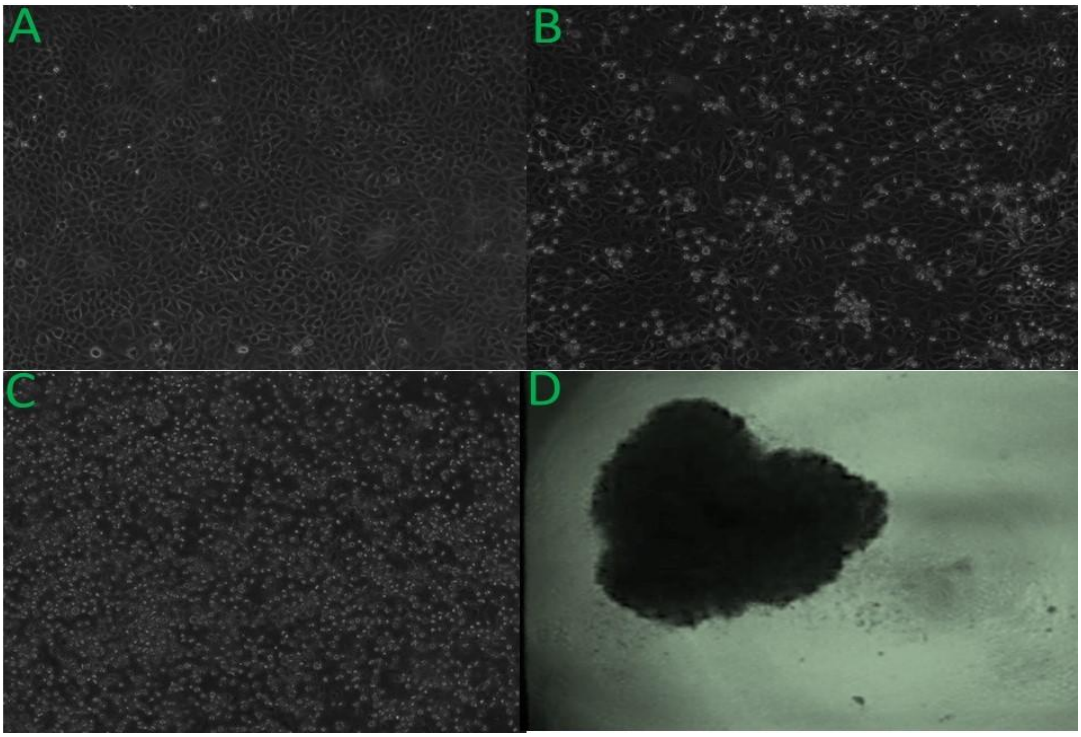

Figure 2. Cytopathic effect (CPE) of ER-H1N1 virus in MDCK cells. (A) Untreated MDCK cells (control); (B) MDCK cells pre-treated with L. johnsonii and then infected with ER-H1N1 for 36 h; (C) MDCK cells pre-treated with ER-H1N1 and then treated with L. johnsonii for 72 h; (D) MDCK cells infected with ER-H1N1 only (negative control). Scale bar = 100 µm (Applies to all panels).

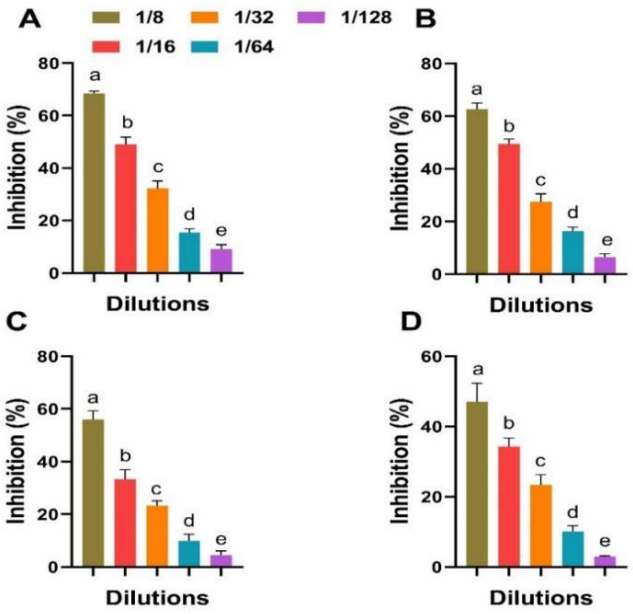

Figure 3. Percentage inhibition of H1N1 virus by pre-treatment with L. johnsonii at different dilutions. (A) Broth suspension; (B) Cell-free supernatant (C.F.S.); (C) Bacterial pellet; (D) Heat-killed bacteria. Serial dilutions (1:8, 1:16, 1:32, 1:64, 1:128) were used. Data represent mean ± SD of three independent experiments (n = 3). Different letters indicate statistically significant differences between dilutions using LSD test ($p < 0.05$).

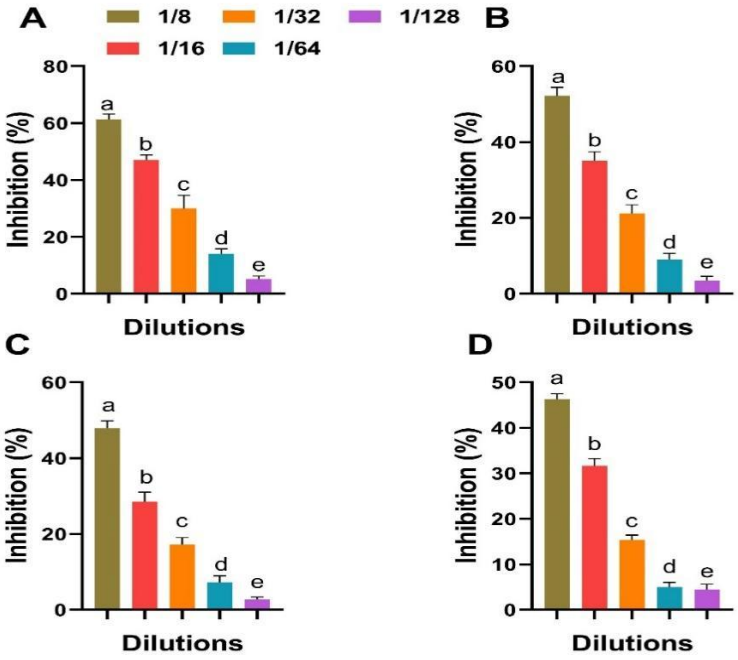

Figure 4. Percentage inhibition of H1N1 virus by post-treatment with *L. johnsonii* at different dilutions. (A) Broth suspension; (B) Cell-free supernatant (C.F.S.); (C) Bacterial pellet; (D) Heat-killed bacteria. Serial dilutions (1:8, 1:16, 1:32, 1:64, 1:128) were used. Data represent mean ± SD of three independent experiments (n = 3). Different letters indicate statistically significant differences between dilutions using LSD test ($p < 0.05$).

These results indicate that *L. johnsonii* can effectively mitigate virus-induced cytopathic effects in MDCK cells, demonstrating its potential as an antiviral probiotic against influenza virus.

3.5. Pre and post-treatment antiviral effect

The antiviral activity of *L. johnsonii* was evaluated using virus-induced cytopathic effect (CPE) in acutely infected MDCK cells. Four bacterial preparations—broth suspension, bacterial pellet, cell-free supernatant (C.F.S.), and heat-killed bacteria—were tested. For pre-treatment, 50 µL of each preparation was inoculated onto MDCK cell monolayers in 96-well plates in triplicate (n = 3) and allowed to adsorb for 90 min, followed by infection with 50 µL of H1N1 influenza virus (100 TCID50) and incubation at 37 °C for 48 h. For post-treatment, cells were first infected with 50 µL of virus for 40 min and then treated with the bacterial preparations for 48 h. Cell viability was assessed using the MTT assay, and CPE was observed microscopically.

Pre-treatment with the broth suspension exhibited the highest antiviral effect, with 68%, 49%, 32%, 15%, and 9.1% inhibition across serial dilutions (Fig. 3A). C.F.S. in pre-treatment showed 62.6%, 49.4%, 27.4%, 16.4%, and 6.4% inhibition (Fig. 3B). The bacterial pellet exhibited 56%, 33.3%, 23.3%, 9.9%, and 4.6% inhibition (Fig. 3C), while heat-killed bacteria showed 47%, 34.3%, 23.3%, 10.1%, and 2.9% inhibition (Fig. 3D). Post-treatment showed consistently lower inhibition across all preparations.

Statistical analysis using one-way ANOVA followed by the least significant difference (LSD) test confirmed that pre-treatment with broth suspension and C.F.S. had significantly higher antiviral activity compared to post-treatment and other bacterial preparations at corresponding dilutions. For example, at the highest concentration, the inhibition by pre-treated broth suspension (68%) was significantly higher than post-treated broth suspension (61.3%) ($p = 0.021$), bacterial pellet (56%, $p = 0.034$), and heat-killed bacteria (47%, $p = 0.012$). Similar significant differences ($p < 0.05$) were observed across all other dilutions, demonstrating the superior efficacy of pre-treatment.

Similarly, post-treatment with the broth suspension of *L. johnsonii* exhibited percentage inhibition of 61.3%, 47.1%,

30%, 15.2%, and 5.2% across serial dilutions (Fig. 4A). Post-treated C.F.S. showed 52.2%, 35.1%, 21.2%, 9.0%, and 3.5% inhibition (Fig. 4B). The bacterial pellet exhibited 47.97%, 28.57%, 17.23%, 7.20%, and 2.77% inhibition (Fig. 4C), whereas heat-killed bacteria showed 46.3%, 31.7%, 15.4%, 5.0%, and 4.5% inhibition (Fig. 4D). Statistical analysis using one-way ANOVA followed by LSD test indicated that the antiviral activity of post-treated preparations was significantly lower than their respective pre-treatment groups ($p < 0.05$), though all preparations still retained measurable inhibitory effects.

4. Discussion

Due to its high immunostimulatory potential, lactic acid bacteria (LAB, probiotics) are in high demand [34]. In this study, 178 LAB strains were isolated from the gut of bamboo rats, and one strain, designated *L. johnsonii*, was selected for antiviral activity. Molecular identification was confirmed by 16S rRNA sequencing, and biochemical characterization using the API 50CHL kit revealed utilization of 23 carbohydrates, classifying the strain as a Gram-positive *lactobacillus* (Table 1). Heat stability analysis demonstrated that the cell-free supernatant (C.F.S.) of *L. johnsonii* retained antiviral activity even after exposure to 121 °C for 15 min over 72 h (Table 2), indicating the presence of thermostable bioactive components.

The cytopathic effect (CPE) caused by viral infection leads to structural changes or damage to host cells, including lysis or impaired cell division. Consistent with previous reports [35, 36], probiotic *Lactobacillus* strains can reduce viral CPE in vitro. Here, the C.F.S. of *L. johnsonii* effectively inhibited ER-H1N1-induced CPE in MDCK cell monolayers compared to the negative control (Fig. 2), demonstrating its antiviral potential.

The antiviral effect of *L. johnsonii* was evaluated using bacterial broth suspension, C.F.S., bacterial pellet, and heat-killed preparations. The strain showed strong activity against H1N1, with the highest inhibition observed in pre-treated broth suspension (68%), followed by C.F.S. (62.6%), whereas post-treatment inhibition was lower (61.3% and 52.2%, respectively). While the antiviral activity of LAB is often attributed to organic acids, hydrogen peroxide, and competitive exclusion [37,38], these mechanisms can vary among strains. For example, the bacterial pellet demonstrated 56% inhibition in pre-treatment, likely due to strain-specific adhesion properties that prevent viral attachment to host cells [37]. Heat-killed bacteria retained moderate antiviral activity (47% pre-treatment), consistent with immunomodulatory effects reported for inactivated LAB [39,45-47].

Importantly, *L. johnsonii* may possess unique strain-specific factors contributing to its antiviral efficacy. Several studies have shown that *L. johnsonii* strains can produce distinctive metabolites, such as bacteriocins, short-chain fatty acids, and exopolysaccharides, which enhance adhesion to epithelial cells and modulate host immune responses [40]. These metabolites not only exert direct antiviral effects but also strengthen the mucosal barrier and stimulate antiviral cytokine production, including IFN-β and IL-10, thereby enhancing innate immunity. Furthermore, *L. johnsonii* has demonstrated superior adhesion capacity compared with other *Lactobacillus* species, mediated by surface-layer proteins, lipoteichoic acids, and mucus-binding pili (41). Such adhesion facilitates colonization of epithelial surfaces, allowing prolonged metabolite action and competitive exclusion of viral particles. In our study, pre-incubation of MDCK cells with *L. johnsonii* preparations was more effective than co-incubation, supporting previous findings that timely colonization and metabolite production are crucial for antiviral protection [42, 43]. This observation aligns with reports that *L. johnsonii* strains can condition host cells to upregulate antiviral pathways and improve epithelial barrier function prior to infection (44).

Overall, the antiviral activity of *L. johnsonii* is a result of both general LAB mechanisms (organic acids, competitive exclusion) and strain-specific factors, including metabolite secretion and cell adhesion properties, which likely enhance its ability to inhibit influenza virus replication and reduce cytopathic effects. These findings underscore the importance of evaluating probiotic effects at the strain level rather than assuming uniform activity across species.

5. Conclusion

The current study demonstrated the in vitro antiviral activity of *L. johnsonii* isolated from bamboo rats. Across all preparations, pre-treatment showed higher inhibition of H1N1 influenza virus compared to post-treatment, with the strongest activity observed in broth suspension, followed by C.F.S., bacterial pellet, and heat-killed cells. While these results highlight the potential of *L. johnsonii* as a probiotic with antiviral properties, claims regarding safety cannot be made without in vivo or clinical toxicity studies. Therefore, further research using animal models, such as mice, is necessary to validate both the efficacy and safety of this strain, as recommended in previous studies on probiotic interventions. Such investigations will provide critical insights into its therapeutic potential against influenza infections and inform future clinical applications.

Author contributions

J.Y., Z.Q., and M.J. designed the study. M.J. wrote the manuscript and performed the data analysis and curation. Y.X., S.L., X.L., Ali, S., Karim, S., Fazal, F., and J.Y revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethical approval

The animal experiments complied with the regulation of the ethics committee of Guangxi University and were approved under the specific agreement number Gxu2019-010.

Conflicts of Interest

The authors report no conflicts of interest.

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Data availability statement

The data presented in this study are available on request from the corresponding author.

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