

Antiviral Effects of a Probiotic Metabolic Products against Transmissible Gastroenteritis *Coronavirus*

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Abstract

The Transmissible Gastroenteritis Virus (TGEV) causes severe diarrhea and other symptoms which end up in death in young piglets. We have studied the protective effects of the probiotic *Lactobacillus plantarum* strain N4(Lp) which metabolic products were added to the swine testis (ST) cells with three different orders using MTT cell proliferation assay and CPE analysis. Metabolic products led to dose-dependent rescue of viability of infected cells in a certain order: pre-treatment, post-infection, co-incubation. Pre-treatment of cells with probiotic metabolic products reduced viral proliferation up to 78% at non-cytotoxic concentration 1/4 dilution. The viral yields in pre-treatment groups were reduced by over three log₁₀ units. Quantitative real-time PCR revealed the optimal inhibition of TGEV RNA replication was 24 h apply pretreatment way that up to 71% of N gene. Analysed the composition of metabolic products by GC-MS revealed the major component is sugars. Then Exopolysaccharides (EPS) was extracted and showed inhibition effect that co-incubation with TGEV.

Keywords: *Lactobacillus plantarum*; Metabolic products; Transmissible gastroenteritis virus; Antiviral; Exopolysaccharides

Introduction

Transmissible Gastroenteritis Virus (TGEV) are the highly contagious causative agent of the enteric and respiratory pathology and diarrhea, vomit, dehydration clinical symptoms which ends up with death of newborn piglets. Currently available vaccine including ordinary or gene engineering vaccine cannot provide solid protection to piglets [1]. Therefore, finding effective and potent anti-TGEV agents to control and prevent TGEV infection is important. Probiotics are defined as living organisms with health-promoting attribution when ingested adequate numbers [2]. Beneficial probiotic bacteria are demonstrated to promote the host defense and to modulate immune response [3-5]. *Lactobacillus* species as feed additive which widely used in gastrointestinal disorders treatment and intestinal balance adjustment [6]. Lactic Acid Bacteria (LAB) have functional properties of antibacterial activity and antiviral activity against diverse human and animal viruses. Significant inhibition of HIV, HINI, RV, TGEV *in vitro* and reduction of viral multiplication *in vivo* with markedly elevated interferon levels and other antiviral factors were monitored [7-9]. *Lactobacillus plantarum* Probio-38 are selected as candidates for application because of the capacity of inhibition of TGEV, enteric bacterial pathogens as well as the bile tolerance and the antibiotic resistance [10]. *Lactobacillus plantarum* CRL1506 are able to against viral infection and diminish inflammatory-mediated tissue damage also enhance antiviral immunity by oral administration [11-14]. A variety of metabolites, such as plantaricin, Exopolysaccharides (EPS), lactic acid, acetic acid, γ -aminobutyric acid produced by probiotic are capable of enhance body immunity, anti-tumor, antisepsis. EPSs are biological high-molecular long-chain polysaccharides that are secreted into the media of microorganisms [15]. The EPS secreted by *Lactobacillus acidophilus* were proved inhibit TGEV infection and

involved elevated levels of IFN- γ , IL-6, IL-8 [16]. However, the study on the antiviral activity of the active components of *Lactobacillus plantarum* has not been reported.

The present study aimed at investigate the antiviral potential of the *Latobacillus plantarum* metabolic products and the screen on its antiviral ingredients. Swine Testicle (ST) cell line were used to test the protective effects of *Latobacillus plantarum* metabolic products on TGEV infection in terms of cell survival and viral replication as wells as antiviral active ingredients.

Materials and Methods

Cells and virus

The epithelial swine testicle (ST) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibico) with 10% fetal bovine serum (Gibico), and 1% penicillin/streptomycin (Gibico) at 37°C in 5% CO₂ atmosphere. The TGEV strain CQ was isolated and identified by our laboratory. Virus were passages in ST cells monolayers. When 80% Cytopathic Effect (CPE) was observed, supernatant was collected by centrifugation at 4000 rpm. Virus was stored at -80°C.

Bacterial and culture conditions

Lactobacillus plantarum strain N4 (Lp) provided by our laboratory, which have been identified were diluted 10-fold from 10⁻¹ to 10⁻⁷ by doubling dilution methods. After rotary shaking, 1 mL of the dilution was spread over MRS agar medium and incubated at 37°C for 48 h.

MOI determination

Multiplicity of infection (MOI) is the average number of virus particles infecting each cell. MOI was used to determinate inoculation upon cell amount. 50% tissue culture infective dose (TCID₅₀)

endpoint is regarded as the standard dosage caused CPE in 50% of the infected cells, calculated with Reed-Muench method. $MOI = \text{Plaque Forming Units (PFU) of virus used for infection} / \text{number of cells}$. $PFU/mL = 0.7^* \text{TCID}_{50}$.

Assessment of cellular toxicity of bacterial supernatants

Bacterial culture supernatants were collected from growing bacterial cultures in MRS under anaerobic conditions for 12 h at 37°C. Bacteria were removed after centrifugation at 3000 rpm for 12 min, then filtrated through 0.22 μm membrane. Serial two-fold dilution of supernatants in DMEM without supplements were added on the monolayer of ST in a 96-well plate (Corning, USA) for 90 min at 37°C before carefully washing away. Mock-treated cells served as control. 3 repeated were set up for each group. Then Methylthiazolyl-diphenyl-Tetrazolium Bromide (MTT) method was used to assay cell viability. 10 μL of MTT was added to the cells per well and incubated for 4 h at 37°C, then the excess MTT was replaced by 150 μL DMSO each well until the formazan crystals totally dissolved. The absorbance of each well was measured at 490 nm. The cell survival rate was calculated as the equation: $\text{cell viability} = [(\text{OD experimental group} - \text{OD bank control}) / (\text{OD negative control} - \text{OD bank control})] \times 100\%$. Cell viability more than 50% could work as maximal atoxic concentration.

Supernatants antiviral assay

To investigate the best order of supernatants antiviral effect, three experimental procedures were used as follow:

Pre-treatment of cells with supernatants of bacteria: Washed monolayers of ST cells were incubated with serial two-fold dilution of supernatants for 90 min at 37°C. Then ST cells challenged with 0.1 MOI TGEV after washed off supernatants with DMEM for 60 min at 37°C. Cells were washed three times and kept in memdium containing 2% fetal bovine serum.

Supernatants of bacteria were added post-infection: 0.1 MOI TGEV was absorbed to ST cells for 60 min at 37°C, then the unabsorbed virus were removed with DMEM, and media containing serial two-fold dilution of supernatants were added for 90 min at 37°C. Cells were washed three times and kept in medium containing 2% fetal bovine serum.

Co-incubation of supernatants of bacteria and TGEV: Serial two-fold dilution of supernatants and 0.1 MOI TGEV were mixed and incubated at 4°C for 90 min. The mixture added to ST cells for 60 min at 37°C. The unabsorbed virus and supernatants were washed with DMEM, and replaced with memdium containing 2% fetal bovine serum.

Mock-treated cells and cells treated with TGEV served as controls. The antiviral effect was observed using MTT assay and CPE assay. Each experiment was perfumed in triplicate.

Virus titration

Supernatants with different concentrations were added to confluent monolayers of ST cells grown in six well tissue culture plates (8×10^5 cells/well) after removed the cell culture supernatants for 90 min at 37°C. 0.1 MOI TGEV was incubated for 60 min 37°C after exorcizing the supernatants. The plates were incubated at 37°C and 5% CO_2 . The virus suspension were collected 12 h, 18 h, 24 h, 36 h separately after infection, subjected to three freeze-thaw cycles, serially diluted from 10^{-1} to 10^{-10} , and added to ST cell in 96-well culture plates. Each

dilution was added to eight wells. The TCID_{50} was calculated by the Reed and Muench method. The mock-treated cells and cells treated with TGEV at each time point served as controls.

RNA extraction and quantitative real-time PCR

ST cells grown in six well tissue culture plates and were treated with pre-treatment method, as previously described. The total RNA was extracted using the RNAiso plus (Invitrogen, USA) reagent and subjected to reverse transcription with GoScript™ Reverse Transcription System (Promega, USA). Quantitative real-time PCR (qPCR) analysis was performed to amplify nucleocapsid (N) gene suing the cDNA as the template and the β -actin gene as the internal standard. The qPCR reaction contained 10 μL of SYBR Premix Ex Taq II (Takara Bio, China), 0.5 μL of forward primer, 0.5 μL of reverse primer, 1 μL of cDNA template, and RNase-free ddH_2O to 20 μL . The reaction protocol was as follow: 95°C for 30 s, followed by 39 cycles of 95°C for 5 s and 54°C for 30 s; the melting curve stage comprised rise 0.5°C temperature from 65°C to 95°C every second. Each samples repeated three times. The primers used as follow:

The N gene primers F: 5'-TTCAACCCCATACCCCTCCAACAA-3' and

R: 5'-GGCCCTTCACCATGCGATAGC-3'.

The β actingene primers F: 5'-CTCTTCCAGCCCTCCTTCC-3'and

R: 5'-GGTCCTTGCGGATGTGC-3'.

Data analysis was based on the measurement of the cycle threshold (Ct). The relative expression level of the N gene with pre-treatment method was calculated by using the 2-Ct method.

Analysis of the composition of metabolic products

Gas chromatography-coupled mass spectrometry (GC-MS) was used for culture supernatants composition analysis. The method as previously described [17,18]. Briefly, 0.2 ml of sample was dissolved in 0.5 ml methano and 20 μL of L-2-Chlorophenylalanine (1 mg/mL stock in dH_2O) as an internal standard to the sample. Vortex mixing for 10 s, then Centrifuge for 15 min at 13000 rpm, 4°C and evaporated to dryness. supernatants underwent a two-step derivatization procedure, firstly, added 20 μL of methoxy amination hydrochloride (20 mg/mL in pyridine), incubated for 30 min at 80°C, then added 30 μL of the BSTFA reagent (1% TMCS, v/v) to the sample aliquots, incubated for 1.5 h at 70°C. Mix well for GC-TOFMS analysis.

Isolation of the EPSs and its antiviral assay

After cells were removed by centrifugation, the crude EPS was collected from the concentrated supernatant by ethanol precipitation at 4°C overnight. The EPSs was prepared according to the previously described [19]. The curde EPS was dissolved in distilled water (50%, w/v) and treated with Sevage reagent (chloroform: n-butanol=4:1, v/v) for three times to remove protein and small molecules. The supernatant was dialyzed by using a dialysis bag (8-14 KDa) in the distilled water at 4°C for 48 h. Then the solution was used for further assay or dried by vacuum at 40°C for temporary storage. Serial two-fold dilution of solution was added on the monolayer of ST in a 96-well plate for 90 min at 37°C before carefully washing away. Mock-treated cells served as positive control. Virus treated cells served as negative control. Three repeated were set up for each group. MTT method was used to assay antiviral ability. TGEV inhibition rate= $[(\text{OD}$

experimental group-OD negative control)/(OD positive control-OD negative control)] \times 100%.

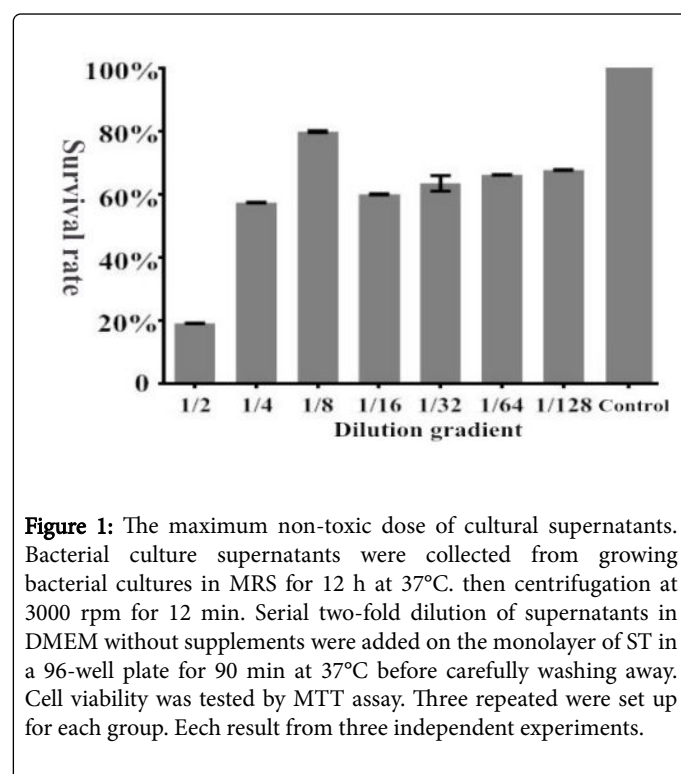
Data analysis

All calculations were performed with SPSS 20.0. All data are given as the mean \pm SD or with the standard errors of the mean. One-way ANOVA and T-test were employed to determine statistical differences among multiple groups. P-values less than 0.05 were considered statistically significant (*P-value less than 0.05, **P-value less than 0.01).

Results

Assessment of cellular toxicity of bacterial supernatants

The bacterial culture supernatants can be used for interference studies, the concentration range in which its addition to cells is non-toxic was defined, and 50% above the cell survival rate is regarded as a non-toxic of culture supernatants. The results (Figure 1) show that bacterial culture supernatants were non-toxic up to 1/4 dilution.

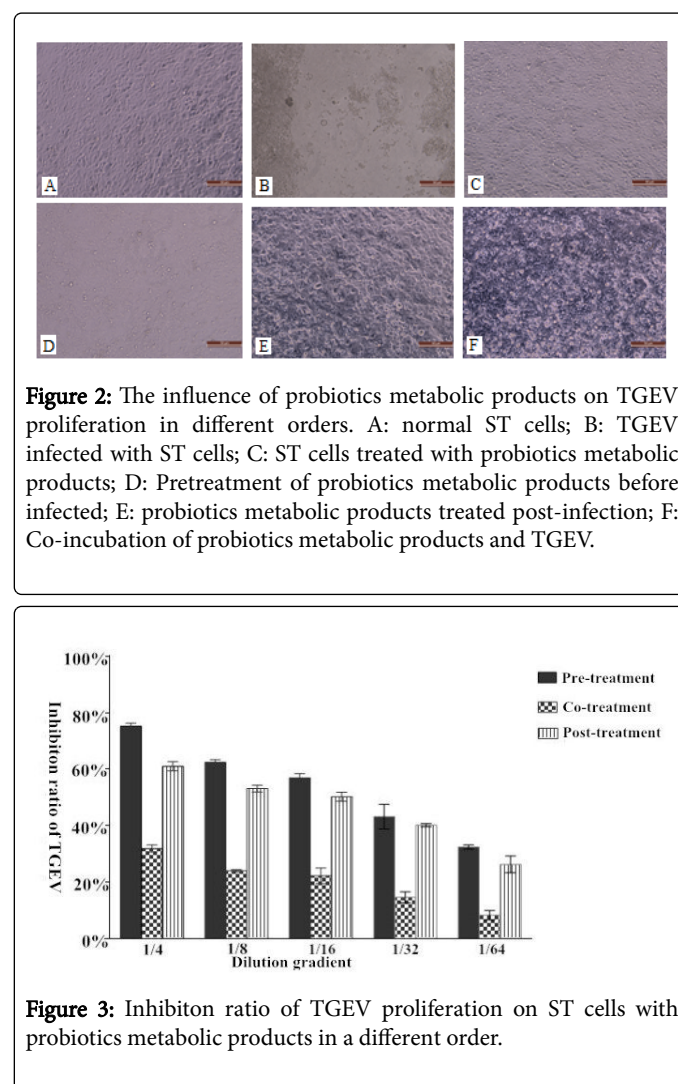


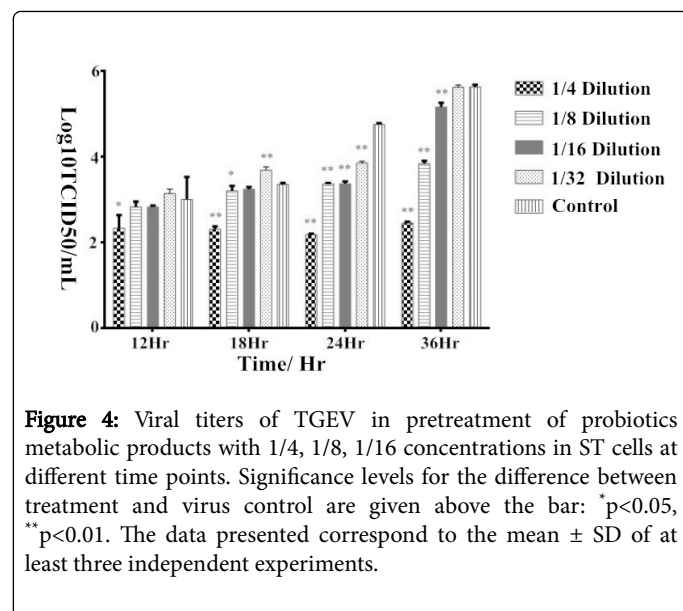
Assessment of supernatants antiviral assay during infection of ST cells with TGEV

It is known that ST cells infected with TGEV can cause a severe CPE. Whether treatment of the host cells with supernatants of Lp have a protective effect, it should decrease the characterization of virus-induced CPE. The results (Figures 2 and 3) show that it provided protection from TGEV in both pre-treatment and post-treatment of cells with supernatants of bacteria, but the pre-treatment group provided a better inhibition effect than post-treatment group. Meanwhile, Figure 3 indicate that the effect of culture supernatants on TGEV was decreased with the increase of dilution times, and it was dose-dependent.

Virus titration

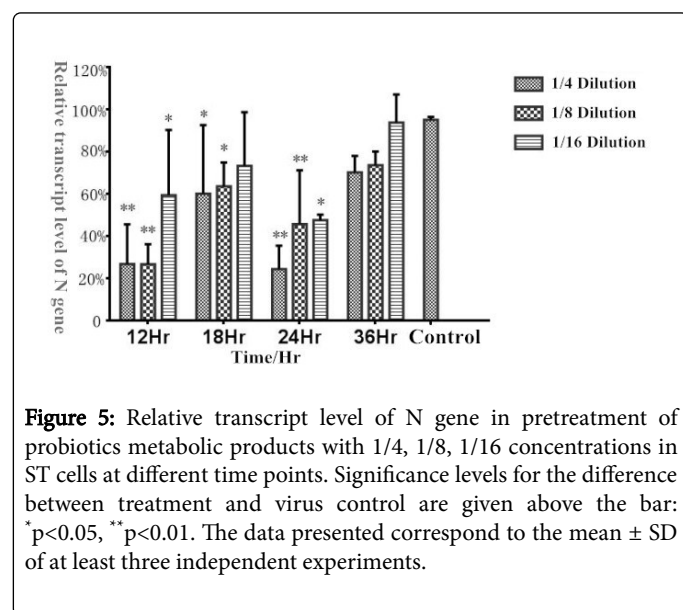
The anti-TGEV activity of supernatants of bacteria was confirmed by measuring virus yield in the culture medium using a TCID₅₀ assay. As the results (Figure 4) were consistent with those from the inhibition ratio of TGEV proliferation, since virus yield was found reduced by pre-treatment with supernatants. The viral titer was highest at 24 hours, when the cells had been exposed to the 1/4 dilution, the average value is 102.15 TCID₅₀/mL. Compared with the control group at the same time, the viral titer decreased by 398 times.





Quantitative real-time PCR of TGEV N gene

It's well known that the nucleoprotein is required for efficient coronavirus genome replication, since the relative transcript level of N gene were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in pretreatment of probiotics metabolic products with 1/4, 1/8, 1/16 concentrations in ST cells at different time points. As expected, the results (Figure 5) show that the levels of N gene mRNA were significantly downregulated in TGEV-infected cells, compared with the control group. Furthermore, the relative expression of N gene mRNA was low at 24 hours, especially with the best effect in 1/4 dilution.



Metabolic products composition

The metabolic products composition was analyzed via GC-MS. The results (Table 1) reveal there may contain around 123 compounds, and

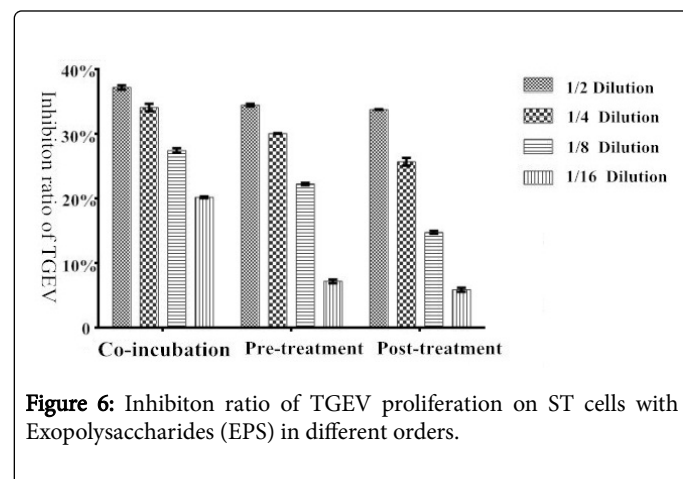
the major component of metabolic products were sugars, acids and alcohols. And sugars have the highest percentage of components, 20.33%. Thus we choose the sugars for the following antiviral study.

Possible materials	Percentage
Acids	13.82%
Alcohols	14.63%
Sugars	20.33%
Amines	8.94%
Amino acids	4.88%
Glycosides, Peptides	9.76%
Phenol, Ketone, Ester	6.50%
ATCGU and other derivatives	3.25%
Unknown substances	17.89%

Table 1: GC-MS result analysis of metabolic products composition.

Antiviral assay of the EPS

The inhibition of EPS at different dilution gradient and in different orders against TGEV were summarized in Figure 6. The inhibition effects of EPS on TGEV significantly decreased along with the increasing dilution-fold, the co-incubation of the ST cells with EPS resulted in the overall inhibitory effect was superior to the other two groups. It is clear that significantly higher inhibition ratios were observed against TGEV at 2 folds dilution (ranging from 30%-40%) than that of other dilution fold.



Discussion

Lactobacilli can produce acids, hydrogen peroxide (H₂O₂), bacteriocins and biosurfactants and thus confer protection of the host [20]. *Lactobacillus* species have been widely used in both humans and animals to prevent or treat gastrointestinal disorders, and several bacteriocins of lactic acid bacteria have been reported. However, the antiviral ability of probiotics is different from that of strain genotypes and virus types. Thus we detected the inhibition effect of the *Latobacillus plantarum* metabolic products. The cells were infected with TGEV at 0.1 MOI, as this more closely reflects natural infection.

Assessment of supernatants antiviral assay during infection of ST cells with TGEV show that the pre-treatment group provided a better inhibition effect, other groups also contributed to against TGEV, but the effect is not as good as the pre-treatment group. Several studies have demonstrated the antiviral mechanism of probiotics include colonization of the epithelium with the *Lactobacilli* sterically hindered the adhesion of TGEV to the surface and to bind to the receptor (such as pAPN) probiotics form complexes with the virus. Meanwhile, probiotics and their metabolites may also indirectly interfere with virus by altering the state of cells, stimulating innate and/or adaptive immunity. Wang have reported that *Bacillus subtilis* OKB105 and the surfactin have antiviral activity against TGEV entering IPEC-J2 cells by adsorbing virus particles and blocking viral attachment by competitive inhibition [21]. In this study, TCID₅₀ assay in which released at least of virus titers were found in 1/4 dilution and relative transcript level of N gene assay show similar results. Previous studies have shown that probiotics metabolites could have a direct inhibitory effect on the proliferation of the virus, such as entering directly the nucleus to destroy the virus nucleic acid affecting the virus particle assembly and producing toxic composition for virus [22]. However, what kind of ingredients do the work of antiviral we still do not know, and the mechanism of anti-TGEV of supernatants need to be further study.

The lactic acid bacteria could produce metabolites antagonistic to the growth of the pathogen, and these metabolites can be low-or-high-molecular-weight compounds. Several Lactic Acid Bacteria (LAB) are known to synthesize Exopolysaccharides (EPS) which may confer health benefits to people such as immunomodulatory, antitumor, antibiofilm and antioxidant activity. That nature of the *Lactobacillus plantarum* metabolic products we have studied previously found that resistant to catalase and pepsin, as well as high temperature (the results were not put on the essay), and the metabolic products composition analysis also indicated that the major component is sugars. Thus we speculated the major antiviral active substances in the metabolites may be polysaccharides. Callahan et al. have found that sulfuric acid extracellular polysaccharides can interfere with the ability of HIV to initially adhere to target cells, leading to blocking viral entry [23]. In this study, the extracellular polysaccharide of *Lactobacillus plantarum* was successfully extracted and detected effect on TGEV inhibition based on three different treatments, the results suggests EPS has a certain inhibitory effect on TGEV, and the effect of co-incubation at 1/2 dilution is the best. Damonte have reported: in most cases, the neither pretreatment setups resulted in a significant inactivation of virion infectivity or, fixing the effective antiviral doses. Only when compounds were incubated in the simultaneous presence of virus and cell the inhibitory effects were observed [24]. Therefore, we hypothesised whether there was a forthright interaction between the EPS and TGEV to form the compounds or not, and TGEV is not released from the compounds. We are under way to substantiate this hypothesis.

Conclusion

In this study, results from the MTT, CPE, TCID₅₀ and qRT-PCR analysis indicated that the *Lactobacillus plantarum* metabolic products have antiviral effect, and the best antiviral concentration of supernatants was at 1/4 dilution by pre-treatment. GC-MS revealed supernatants may contain around 123 compounds, and the proportion of sugar is highest. And MTT analysis indicated the major antiviral substance is EPS. All of these results revealed the metabolic products of *Lactobacillus plantarum* strain N4 have anti-TGEV activity.

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