

# Low Molecular Weight Sulfated Chitosan Efficiently Reduces Infection Capacity of Porcine Circovirus Type 2 (PCV2) In PK15 Cells

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## Research Article

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# Abstract

## Background

Porcine circovirus type 2 (PCV2)-associated diseases are a major problem for the swine industry worldwide. In addition to vaccines, the availability of antiviral polymers provides an efficient and safe option for reducing the impact of these diseases. By virtue of their molecular weight and repetitious structure, polymers possess properties not found in small-molecule drugs. In this perspective, we focus on chitosan, a ubiquitous biopolymer, that adjusts the molecular weight and sulfated-mediated functionality could act as a efficient antiviral polymer by mimicking PCV2-cell receptor interactions.

## Methods

Sulfated chitosan (Chi-S) polymers of two molecular weights were synthesized and characterized by FTIR, SEM-EDS and elemental analysis. The Chi-S solutions were tested against PCV2 infection in PK15 cells *in vitro* and antiviral activity was evaluated by measuring the PCV2 copy number upon application different molecular weights, sulfate functionalization, and concentration of polymer. In addition, to explore the mode of action of the Chi-S against PCV2 infection, experiments were designed to clarify whether the antiviral activity of the Chi-S would be influenced by when it was added to the cells, relative to the time and stage of viral infection.

## Results

Chi-S significantly reduced genomic copies of PCV2, showing specific antiviral effects depending on its molecular weight, concentration, and chemical functionalization. Assays designed to explore the mode of action of Chi-S revealed that exerted antiviral activity through impeding viral attachment and penetration into cells.

## Conclusions

These findings help better understanding PCV2-porcine cells interaction and reinforce the idea that sulfated polymers, such as Chi-S, represent a promising candidate for uses in antiviral therapies against PCV2-associated diseases.

## Background

The chitosan (Chi) biopolymer is a derivative obtained from chitin, which is a natural polysaccharide, poly- $\beta$ -(1,4)-D-glucosamine-N-acetyl-D-glucosamine, present in the cell wall of fungi, yeasts, and in the exoskeleton of invertebrates [1]. The main source of chitin corresponds to crustacean shells and insect cuticles [2]. It is possible to obtain Chi from partially deacetylated chitin (50%), which corresponds to a

linear cationic biopolymer formed by glucosamine and N-acetyl D-glucosamine subunits, which are chemically linked by covalent O-glycosidic  $\beta$ -(1-4) bonds [3, 4]. The molecular weight of Chi ranges between 50 and 1000 kDa with a degree of deacetylation > 50% [2, 5, 6].

Chemical modification of Chi occurs selectively in nucleophilic amino groups (position C-2 in repeating units of glucosamine), or in hydroxyl groups (positions C-3, C-6 in repeating units of acetyl glucosamine and glucosamine) or indistinctly in amino and hydroxyl groups [7]. The chemical surface of Chi can be modified using sulfate groups and their derivatives can produce polyampholitic chains similar to those found in the structure of sulfated glycosaminoglycans (GAGs), which represent a special class of charged polysaccharides involved in the formation of extracellular matrix, such as chondroitin sulfate and heparan sulfate [8]. GAGs can interact with various proteins in the extracellular matrix, thus signaling to mediate various cellular processes, such as adhesion, migration, proliferation and differentiation [9, 10]. GAG sulfates have numerous important pharmacological properties and biological activities such as antiviral and anticoagulant among other effects [11]. Therefore, sulfated GAG derivatives, such as sulfated chitosan (Chi-S), represent prominent candidates for use in several biomedical applications [12–15]. In specific for antiviral effect, the material's degree of sulfation (DS) (i.e., number of sulfate groups per monosaccharide unit) is an especially important parameter for antiviral activity [16]. There is a positive correlation between increasing DS and antiviral potency [17]. For several human viruses, including HIV, was originally thought that sulfated polysaccharides work through dual mechanisms: blocking viral adsorption and inhibiting viral replication [18]. It is now believed that the predominant mechanism is the inhibition of viral adsorption and syncytium formation [19].

Porcine circovirus type 2 (PCV2) is a small non-enveloped virus, member of the genus *Circovirus*, family *Circoviridae* [20]. PCV2 is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS) and other diseases in swine referred to as porcine circovirus associated diseases (PCVAD) [21]. PMWS was first described in Canada in 1991, and nowadays it is described in pig populations worldwide, producing a serious economic impact on swine industry, therefore the development of antiviral options is critical [2].

This work presents the synthesis and characterization of Chi-S and its antiviral effect against PCV2 on PK15 cells (a porcine kidney-derived cell lines highly permissive to PCV2 infection), and discuss how these effects depend on its sulfate functionalization, molecular weight and concentration. In addition, to explore the mode of action of the Chi-S against PCV2 infection, experiments were designed to clarify whether the antiviral activity of the Chi-S would be influenced by when it was added to the cells, relative to the time and the stage of viral infection.

## Materials And Methods

### Material and equipment

Reagents of the highest available grade were used. All experiments and solution preparations were performed using fresh Milli-Q water (Labostar™ TWF, Evoqua Water Technologies LLC, Warrendale, PA, USA) and Ultra-pure water (18.2 MΩ) was obtained from the LaboStar™ 4-DI/UV water system. Fourier transform infrared spectra analysis (FT-IR) was performed on a ATR/FT-IR interspec 200-X spectrometer (Interspectrum OU, Toravere, Estonia). Scanning electron microscopy - energy dispersive X-ray spectroscopy (SEM-EDS) analysis of Chi-S and Chi-C samples was performed in a JEOL JSM-IT300LV microscope (JEOL USA Inc., Peabody, MA, USA), connected to an energy dispersive X-ray detector for elemental analysis with computer-controlled Aztec EDS system software from Oxford Instruments, Abingdon, UK. Real-time PCR reactions were performed using the LightCycler® Nano Instrument (Roche Molecular Systems, Inc). High resolution for PCV2 viral copy number determination and melting curve analysis was achieved with this instrument.

## Synthesis of sulfatated chitosan

To obtain Chi-S, commercial Chi (Sigma-Aldrich) of two different molecular weights were used. Low molecular weight (LMW) Chi (50-190 kDa) and high molecular weight (HMW) Chi 310-375 kDa were subjected to a sulfation reaction using the modified method described by [23]. Briefly, 5 g of each Chi was dissolved in 200 mL of cold sulfuric acid (Sigma-Aldrich) (4°C) and stirred for 2 h. Then, the mixture was precipitated dropwise in 1 L of cold ethyl ether (Sigma-Aldrich) (2°C) under constant stirring. The precipitated product was filtered, washed with abundant cold ethyl ether, and products were collected by adding them to 200 mL of ultra-pure chilled water (4°C). The water was obtained from first capsule filters with 0.2 μm flow (U.S. Filter), and furthermore, ultra-pure water (18.2 M Ω) was obtained from the LaboStar™ 4-DI/UV water system. The pH was adjusted to 7.6 with a cool 30% NaOH aqueous solution (Merck) and the mixture was dialyzed against distilled water on Spectra/Por 3 cellulose membranes with 3.5 kDa pore size for 3 days at room temperature. Distilled water was obtained from an automatic water still, model 700-Pobel. The volume of dialysis solution was changed every 24 h. Finally, the product obtained was subjected to evaporation under reduced pressure in a rotary evaporator equipment (Heidolph Laborotta 4001 Efficient), until obtaining 20 mL, which was lyophilized using the Christ Alpha 1-4 equipment, LOC-1M.

## Characterization of chitosans

The elemental analysis of Chi sample was performed on an organic elemental analyzer (INICUBE®, Elementar Americas Inc) designed for simultaneous carbon, hydrogen, nitrogen, and sulfur analysis in solid and liquid samples. This analysis allows the detection of differences in the presence of sulfur atoms between Chi-S and Chi-C, which indicates sulfation degrees of Chi-C. Furthermore, Fourier transform infrared (FTIR) spectra of Chi-S and Chi-C were obtained with an ATR/FTIR interspec 200-X spectrometer. The FTIR spectra were recorded at 4 cm<sup>-1</sup> resolutions and 64 scans. Additionally, surface morphology and micro-analysis of Chi-S and Chi-C samples were observed by scanning electron microscopy (SEM model JSM-IT300LV, JEOL USA Inc.), coupled with energy dispersive X-ray analysis (EDS Aztec, Oxford Instruments); the elemental analysis was performed using a computer-controlled software (Aztec analysis software, Oxford Instruments).

# Viruses and cells

PCV2 genotype b (strain 0233) was passed for three passages and titrated on PK-15 cells as previously described [24]. PK-15 cell line (ATCC CCL-33, passage 10) was maintained in minimal essential medium (MEM) (Corning™ Cellgro™), supplemented with 15% fetal bovine serum (Corning Cellgro), 0.5% antibiotic/antifungal (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B and 1% glutamine (Corning™ Cellgro™). Aliquots of the virus were stored at -80 °C until required

## Cell seeding and infection of cell cultures

PK-15 cells were seeded at  $2.0 \times 10^4$  cells /well in 96-well microplates (Corning™ Cellgro™) and incubated for 12 h at 37°C/5% CO<sub>2</sub> until 80% confluence. Before adding Chi samples or the virus, or when quantifying the results, the monolayers were washed twice with phosphate buffered-saline (PBS, pH 7.4 at room temperature). In all the experiments, the following controls were included: cell control (cells that were not infected with the virus or treated with Chi samples), virus control (cells that were infected only with the virus but not treated with Chi samples in the antiviral activity assays).

## Cytotoxicity assay

The toxicity of the polymers was determined using the method described by Pourianfar *et al.* [25] with modifications. Briefly, the media of the 80% confluent PK-15 cells was aspirated and replaced with 100 µl of each polymer solution diluted in MEM/15% FBS (at four concentrations, 0.5,1.0,1.5, and 2.0%). After incubation at 37 °C/5% CO<sub>2</sub> for a further 3 days, the results were quantified using CellTiter 96® AQueous One Solution MTS (Promega Corporation), with the absorbance set at 490 nm, according to the manufacturer's instructions. Cell viability percentages were measured based on the number of living cells in polymer-treated cells relative to cell controls (defined as 100% viability). The cytotoxicity curve was then generated by plotting cell viability percentages against compound concentrations.

## Antiviral activity assay

The infectivity of PCV2 on PK-15 cell cultures treated with LMW and HMW Chi-S was evaluated through a plaque infection assay. The monolayers were infected with 10 µL of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>), then the microplate was incubated at 37°C/5% CO<sub>2</sub> for 1 h. After the incubation time, the residual viral inoculum was removed by washing the cell monolayers with 1X PBS and immediately covered with 100 µL of MEM supplemented with 15% FBS, 0.5% antibiotic/antifungal and 1% glutamine, which contained four concentrations of Chi-S (0.5,1.0,1.5, and 2.0%). The incubation continued for 24 h at 37°C/5% CO<sub>2</sub>, after that the supernatant was removed and the cell cultures were treated with D-glucosamine (300 mM) and incubated for additional 48 h. After the incubation period, the monolayers were subjected to 3 freeze-thaw cycles (-80°C) and the lysates of all wells were obtained, which were centrifuged at 2,500 g to store the supernatants and interpolating the viral DNA copy number by qPCR assays, to examine the effect of Chi on PCV2 replication. The degree of inhibition of the virus was recorded by quantify the genomic copies of PCV2 in log<sub>10</sub> DNA copies/mL.

## Time of addition assay

To identify the stage in which the viral infection can be inhibited, cells infected with 10  $\mu\text{L}$  of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) were incubated with Chi-S and Chi-C at different concentrations (0.5, 1.0, 1.5, and 2.0%). The antiviral effect was evaluated at different times of infection, as previously described [25] with few modifications. For this, 100  $\mu\text{L}$  of Chi-S at the above-mentioned concentrations were added 1 and 2 h before infection of cells (Preinfection), at the time of infection (Time 0) and 1 to 2 h post-infection. The tests were performed in independent plates and incubated at 37°C/5% CO<sub>2</sub> for 24 h, then the supernatants were replaced with fresh media containing D-glucosamine (300 mM) and incubated for 48 h. After incubation, the monolayers were subjected to freeze-thaw cycles and the cells were collected. The PCV2 DNA copies were measured by qPCR assays to determine the effect of Chi-S on PCV2 titer.

## Attachment assay

The ability of Chi-S to inhibit viral attachment to PK15 cell line was evaluated as previously described [25] with modifications. The 80% confluent monolayers were pre-cooled at 4°C for 1 h followed by (i) Cellular infection with 10  $\mu\text{L}$ /well of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>), supplemented with 100  $\mu\text{L}$  of MEM containing Chi-S at the following concentrations (0.5, 1.0, 1.5, and 2.0%), (ii) infection with 10  $\mu\text{L}$ /well with virus suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) that had been mixed and pre-incubated with 100  $\mu\text{L}$ /well of each compound at 4 ° C for 30 min. All plates were kept at 4°C for another 3 h, after which the monolayers were washed twice with PBS to remove excess compounds and any unbound viruses. The plates were then filled with 100  $\mu\text{L}$ /well MEM and incubated for 24 h at 37°C/5% CO<sub>2</sub>, where the supernatant was removed and the cultures were treated with D-glucosamine (300 mM) continuing with the incubation with the above-mentioned conditions until completing 48 h. After incubation, the monolayers were subjected to 3 freeze-thaw cycles (-80°C) and the lysates of all wells were obtained, which were centrifuged at 2,500 g to store the supernatants and quantifying the genomic copies of PCV2 by qPCR assays. The degree of inhibition of the virus was recorded by quantifying the viral genome in log<sub>10</sub> DNA copies/mL.

## Viral penetration assay

The viral penetration assay was conducted using the modified penetration test as previously described [25], to evaluate the ability of the compounds to inhibit viral entry. Confluent cells (80%) were pre-cooled and infected with 10  $\mu\text{L}$  of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) and incubated at 4°C for 3 h to allow viral attachment. After this step, the cells were treated at room temperature with 100  $\mu\text{L}$ /well of each Chi-S using the same four concentrations (0.5, 1.0, 1.5, and 2.0%), followed by incubation at 37°C/5% CO<sub>2</sub> for 20, 40, and 60 min (separate plates) to allow the compound to interrupt the penetration of virus into cells. Each time point was tested separately and independently. The supernatant was aspirated followed by washing the cells with alkaline PBS (pH 11) for 1 min to inactivate viruses that did not penetrate the cell, after which, acidic PBS (pH 3) was added to neutralize the pH. Then, the PBS was aspirated and neutralized by washing the cells with PBS (pH 7.4) and the cells covered with 100  $\mu\text{L}$  of MEM. The plates

were incubated at 37°C with 5% CO<sub>2</sub> for 24 h at 37°C / 5% CO<sub>2</sub>, where the supernatant is removed and the cultures are treated with D-glucosamine (300 mM) continuing with the incubation with the above-mentioned conditions until completing 48 h. After incubation, the monolayers were subjected to 3 freeze-thaw cycles (-80°C) and the lysates of all wells obtained, centrifuged at 2,500 g. Supernatants were kept for quantifying genomic copies of PCV2 by qPCR assays. The degree of inhibition of the virus was evaluated by quantifying the viral genome in log<sub>10</sub> DNA copies/mL.

## Viral copy number measuring by quantitative real-time PCR (qPCR)

Viral DNA was extracted according to the manufacturer's instructions (Axigen, Biosciences). PCR was performed using primers that amplify a specific region of PCV2 ORF2 (PCV2qPCR-F 5'-ATGTCACGC CAGGAGG - 3 Position 1523-1541 and PCV2qPCR-R 5'-CGYTGGAG∇ GG∇ ∇ ATGGCATC - 3 Position 1603-1627). The SYBR Green system (Kapa Sybr) and a real-time thermal cycler (LightCycler Nano, Roche) were used for real-time PCR following the manufacturer's instructions. To generate the standard curve, the plasmid pGEM::PCV2 was used, in which the complete PCV2 DNA genome sequence was cloned. The plasmid DNA was extracted from the bacterial strain *E. coli* DH5α, with a commercial kit (Plasmid Midi Kit, Qiagen), according to the manufacturer's instructions. The quantification was conducted by measuring the OD<sub>260</sub> using NanoDrop spectrophotometer and DNA copies by using the online NEBio calculator (<https://nebiocalculator.neb.com/#!/ligation>). Serial dilutions (10<sup>1</sup>-10<sup>5</sup>) were prepared in duplicate of known molar concentrations of plasmid DNA. The viral DNA copies were interpolated from the standard curve obtained.

## Statistical analysis

Each Chi concentration was tested in triplicate. Data were expressed as mean ± standard deviation (SD). The reduction in viral DNA copies was determined by the difference between the average value of DNA copies of PCV2 obtained in the control (virus) and the average value of viral DNA copies obtained with all 0.5, 1.0, 1.5, and 2.0% concentrations of Chi-C or Chi-S polymers. The reduction in the genomic copies of PCV2 was expressed as a percentage, using Eq. (1).

$$\frac{10^{(LCConcentration)}}{10^{(LVControl)}} \times 100 = X$$

$$100 - X = \%DNAcopiesreduction$$

Where, LC concentration and LV control correspond to the average of log<sub>10</sub> DNA copies/mL obtained at each polymer concentration and the average value obtained in the control (virus), respectively. The results of the antiviral activity tests were previously analyzed with an ANOVA test with multivariate regression model. Tukey's honest significant difference test (HSD) was then used to compare the antiviral activity

between the type and concentration of Chi. A significance level ( $p$  value) set to  $< 0.05$  was considered to compare the group means.

## Results

### Characterization Of Chi-s And Chi-c Samples

The presence of sulfur (Tables 1 and 2) and the sulfate groups (Figure1) of Chi-S samples was confirmed by elemental analysis and FTIR spectroscopy, respectively. Table 1 presents the results obtained from the elemental analysis of LMW Chi-S, a theoretical elemental composition of Chi-C and reported raw material of Chi-C. However, similar results showing Chi-S have been reported by [26] (4.13% N, 21.62% C, 4.53% H and 12.91% S).

Table 1  
Elemental analysis of Chi and Chi-S.

Sample	%N	%C	%H	%S
LMW Chi-S	5.22 ± 0.15	26.62 ± 0.61	5.02 ± 0.27	8.5 ± 0.54
Chi-C	8.7*	44.7*	6.8*	0.0*
Chi <sup>a</sup>	7.51	40.04	7.11	0.0

*The elemental analysis represents an average of three measurements. The symbol \* represents theoretical idealized elemental composition of Chi-C. <sup>a</sup>[40]. Abbreviations: N, nitrogen; C, carbon; H, hydrogen; S, sulfur. LMW Chi-S, low molecular weight sulfated chitosan; Chi-C, commercial chitosan; Chi, chitosan.*

Table 2  
EDS measurements of LMW and HMW of Chi and S-Chi samples.

Sample	%N	%C	%O	%S	%Na	Total wt-%
LMW Chi	8.1	50.54	41.36	-	-	100
LMW Chi-S	-*	37.76	44.28	10.82	7.14	100
HMW Chi	8.08	49.46	42.46	-	-	100
HMW Chi-S	7.56	33.43	43.63	8.77	6.6	100

*C, O, N, S, and Na are the percentage amounts of carbon, oxygen, nitrogen, sulfur and sodium atoms in the Chi sample. The asterisk \* indicates that the atom is present but was not measured and - indicates the absence of the atom. Abbreviations: N, nitrogen; C, carbon; H, hydrogen; S, sulfur; wt, weight; LMW Chi, low molecular weight chitosan; LMW Chi-S, low molecular weight sulfated chitosan; HMW Chi, high molecular weight chitosan; HMW Chi-S, high molecular weight sulfate*

Comparing the FTIR spectra of LMW and HMW Chi-S and Chi-C different absorption bands appears due to the sulfation reaction. The FTIR spectrum of Chi-C exhibited the characteristic bands at  $1642 \text{ cm}^{-1}$

(amide I),  $1548\text{ cm}^{-1}$  (amide II) and  $1410\text{ cm}^{-1}$  (amide III). Additionally, the characteristic bands are observed at  $1027$  and  $1072\text{ cm}^{-1}$  corresponding to the stretching of C-O. However, for Chi-S the FTIR shows new absorption bands at  $815$  and  $1218\text{ cm}^{-1}$  corresponding to the C-O-S and O=S=O groups, respectively, confirming that the sulfation reaction was successful and these bands are characteristic of the sulfate groups in the Chi-S chains [27]. Indeed, the band around  $815\text{ cm}^{-1}$  is attributed to the vibration of the stretching of C-O-S and the band at  $999\text{ cm}^{-1}$  can be assigned to the sulfate group (Pires et al., 2013) (Figures. 1a, b).

Additionally, surface morphology and micro-analysis of Chi was observed by SEM-EDS analysis (Figure 2). SEM images of representative Chi samples show homogenous surfaces of typical raw Chi material. Indeed, EDS measurements were performed to determine the elemental composition of LMW and HMW Chi-S and Chi-C. We determine that in the absence of sulfate groups of LMW and HMW Chi samples, the chemical composition of Chi does not either show a sulfur atom nor differ from theoretical predictions according to unmodified raw Chi, therefore for both LMW and HMW Chi-C samples, the sulfur atom was absent. However, when EDS measurements were performed on functionalized LMW and HMW Chi-S consistently show sulfur atoms, in both cases, in the range of 10-8%-Wt, respectively, as observed in Table 2.

## **Molecular weight dependence of antiviral activity of sulfated chitosan**

When analyzing the antiviral activity of Chi, the viral DNA copies values obtained with LMW Chi-S and Chi-C show significant differences. For LMW Chi-S, all concentrations reduced the viral copy numbers, however, only at 2% concentration was statistically significant, compared with the control (virus), reducing it by  $2.30\text{ log}_{10}$  DNA copies/mL, which represents a reduction of 99.50% (Figure 3). The LMW Chi-C did not present statistically significant effect. Moreover, when comparing the same polymers, but with HMW Chi, no significant differences were obtained. However, for HMW Chi-S, we observed that at 2% concentration significantly reduced viral DNA by  $2.02\text{ log}_{10}$  DNA copies/mL, which is equivalent to 99.04% (Figure 3). When comparing HMW and LMW Chi-S, significant differences were observed, where the LMW Chi-S presented the lowest viral DNA copy number. None of the Chi polymers showed significant cytotoxicity at concentrations 0.5 – 2% at which they demonstrated effective antiviral activities (data not shown).

## **Antiviral activity of sulfated chitosan depends on application timing**

The application of the polymers with different timing was performed to determine the stage in which the infection by the virus could be more efficiently inhibited. Comparing application of LMW Chi-S and Chi-C revealed that there were significant differences between them. Such differences were evidenced by simultaneously infecting the cells and exposing to the polymer (time 0), presenting a greater reduction in viral copy number upon application of LMW Chi-S. Interestingly, when times of chitosan application, different from time 0, were compared (times -2, -1, +1 and +2 of the infection), there were not significant differences. Therefore, exposure of the virus to the sulfated polymer upon infection significantly reduces the viral DNA copies *in vitro* (Figure 4A).

All the sulfated polymer concentrations at time 0 showed significant differences with respect to the control, however the most effective to reduce the viral copy number was 2% concentration, reducing the viral DNA in 1.30 log<sub>10</sub> copies/mL equivalent to 94.99%. The concentration at 1.5% presented a similar drop, 1.27 log<sub>10</sub> copies/mL, corresponding to 94.63% (Figure 4A). For LMW Chi-C, no significant differences were observed among all experimental times, neither between concentrations nor between control (data not shown). Furthermore, comparative analysis of HMW Chi-S and Chi-C established that there were significant differences between both polymers and, in the case of Chi-S, it was only significant at time 0, where all concentrations showed differences with respect to the control. Here, 1.5% and 2.0% concentration reduced the viral copy number by 1.16 and 1.03 log<sub>10</sub> copies/mL, equivalent to 93.08 and 90.67%, respectively (Figure 4B). There were no significant differences between all experimental times analyzed for HMW Chi-C, nor were there differences in the viral copy number values between concentrations (data not shown).

Alternatively, when analyzing both Chi-S (HMW or LMW), it was observed that there were no significant differences in the viral copy number values obtained with each Chi, however, when applying LMW Chi-S, the viral DNA copies are further reduced. Our results reinforce that sulfated GAG derivatives, such as Chi-S, represent prominent candidates for use in antiviral therapy against PCV2-associated diseases, since the specific blocking effect of Chi-S, on the PCV2, is better evidenced at a given concentration of the Chi-S (2%). This effect was more evident in the presence of Chi with a MW <75 kDa, but was not observed with Chi of higher molecular weights (Figure 4B). Remarkably, this effect would depend on the early exposure time of the Chi-S to the PCV2 virus.

## Sulfated chitosan blocks viral cell attachment

To assess the ability of the polymers to inhibit viral binding, an attachment assay was performed. The results obtained to evaluate the ability of Chi to inhibit viral attachment reveal that there were significant differences between LMW Chi-S and Chi-C, where Chi-S reduce the viral copy number of cell cultures to a greater extent, compared with Chi-C. When the group “without incubation” (time 0) was compared with the group “with 30 min incubation” in the presence of LMW Chi-S, it showed significant differences, for which the virus-chitosan pre-incubation had a greater effect in reducing viral DNA copies. On the other hand, in both groups it can be seen that there are significant differences for all concentrations used, compared

with the control. Remarkably, when pre-incubation (virus-sulfated chitosan) was performed, all concentrations were significant, but the concentration of 1.5 and 2% were more effective, decreasing the viral copy number by 3.00 log<sub>10</sub> copies/mL (99.90%) and 2.93 log<sub>10</sub> copies/mL (99.88%), respectively (Figure 5).

Additionally, significant differences were also observed between HMW Chi-S and Chi-C, where Chi-S with pre-incubation (30 min), more efficiently reduced the viral copy number of infected cells versus without pre-incubation treatment (Figure 5).

In this way, with pre-incubation, also all concentrations significantly reduced viral DNA copies with respect to the control, although the most effective concentrations were 1.5 and 2%, which reduced the viral copy number by 2.18 log<sub>10</sub> copies / mL (99.34%) and 2.71 log<sub>10</sub> copies/mL (99.81%), respectively (Figure 5). HMW Chi-C had the same reduction behavior as LMW Chi-C, however, concentration effects did not differ significantly from each other.

## Sulfated chitosan reduces viral cell penetration

The penetration test was used to evaluate the ability of Chi-S to inhibit viral entry. The results reveal that high and low molecular weight sulfated polymers could significantly reduce the viral copy number. In experimental times, LMW Chi-S applied for 20 min significantly reduced the viral copy number when it was used at 1.0% (reduction of 1.52 log<sub>10</sub> copies/ml (96.98%), 1.5% (reduction of 1.80 log<sub>10</sub>copies/mL) and 2%, this last concentration achieved the greatest reduction in 2.64 log<sub>10</sub> copies/mL (99.77%).

Application of LMW Chi-S for 40 min produced the most significant reductions when Chi was applied at 1.5% and reduced the viral DNA by 1.87 log<sub>10</sub> copies/mL (98.65%). A similar value was obtained with 2% concentration, which reduced it by 1.54 log<sub>10</sub> copies/ml (97.12%). Finally, when the experiment lasted 60 min, only the 2% concentration was significant and reduced the viral copy number by 1.20 copies/ml log<sub>10</sub> (93.69%). The reduction of viral DNA copies did not depend on the time interval, however this reduction was less effective with 60 min treatment than with time intervals between 20 and 40 min (Figure 6). For HMW Chi-S, 20 min produced reduction of viral copy number significantly for 2% concentration, which achieved a decrease of 2.68 log<sub>10</sub> copies/mL (99.79%), with 40 min the concentration of 2% was also significant, which reduced the viral copy number by 1.86 log<sub>10</sub> copies/mL (98.62%) Finally, treatment of 60 min with concentrations 1.5% and 2%, decreased the viral copy number by 1.56 log<sub>10</sub> copies/mL (97.25%) and 1.59 log<sub>10</sub> copies/mL (97, 43%), respectively (Figure 6). The reduction in viral DNA copies did not depend on the time interval, however this reduction was less efficient at 20 min compared with 40 and 60 min time intervals. In the case of LMW and HMW of Chi-C samples, they did not generate significant reductions in viral copy number in this experiment (data not shown).

## Discussion

According to many studies, sulfated polysaccharides are a promising source of antiviral molecules. In fact, in this work, Chi-S could inhibit the *in vitro* viral infection of PCV2, indicating that the presence of

sulfate groups specifically improves the antiviral activity of Chi. Other factors influencing the antiviral activity would be: the presence of anionic groups, the degree of sulfation, charged density and molecular weight, affecting the virus penetration by altering intracellular signals and delaying the replicative cycle of viruses [28]. Other studies indicated that hypersulphated polysaccharides interfere with electrostatic interactions between the positively charged region of the viral envelope glycoproteins and the negative charges of the heparan sulfate surface receptor chains [29].

It was observed in this study that the LMW Chi-S produces the lowest viral copy number among all polymers tested (Figure 3). A possible explanation of this effect, is that the depolymerization of the polysaccharide during the sulfation process, allow it a better interaction with the virus particle, generating greater antiviral effects, thus inhibiting the spread of the virus through the cell. However, at present there is no uniform statement in the literature on the effect of molecular weight on the antimicrobial properties of Chi, and some conclusions are contradictory [30]. According to [31] the antiviral activity of Chi increases as its molecular weight decreases and as its degree of acetylation increases. Thus, data from other studies indicate that HMW Chi possesses higher antiviral properties [31, 32].

Additionally, it is possible to determine that the inhibition of the virus using Chi-S, occurs in the initial stages of infection, determining it as an effective antiviral agent. Since Chi-S appears to prevent the attachment of the virus to the host cell receptor by modifying relevant subsequent processes and delaying intracellular events that occur during post-entry and replication process. Therefore, if the virus-receptor interaction is hindered, processes such as attachment, signaling, internalization, endocytosis, and replication would be affected [28]. These ideas are inferred, since the reduction of viral copy number was observed when the infection was simultaneous upon Chi-S application (Figure 4). However, the "preventive" application in the cells before the infection does not exert a protective effect on them or a long-lasting antiviral state. As a final point, although it was not significant, to apply Chi-S, 1 and 2 h after infection (+1 and +2), reduced viral DNA copies. Therefore, the Chi-S would also affect the later stages, such as the penetration of the virus.

The results obtained are similar to those reported for other polysaccharides, where it is deduced that the most acceptable mechanism of action would be by impeding the viral attachment of PCV2 to sulfated polysaccharide receptors. Thus, when heparan sulfate and Chi-S are chemically compared, it is possible to observe that they present a structural analogy, composed of alternating linear sulfated polymers of uronic acid- (1 → 4) -D-glucosamine and consequently they can mimic their biological functions [7, 8].

It was also observed that pre-incubating the virus with Chi-S produced a greater reduction in viral copy number, but it was also reduced, although less significant, when applying the Chi-S at the time of infection (Figure 5). Therefore, it could be established that there is a blockage of viral adsorption through direct interference with the virus particle. It has been reported that 3,6-sulfated Chi directly inhibits human papillomavirus (HPV) by binding to viral capsid proteins and, therefore, blocking viral adsorption [33]. Other sulfated Chi-oligosaccharide derivatives block the interaction between HIV-1gp120 and CD4 + cell surface receptors, thereby inhibiting virus-cell fusion and subsequent virus entry. This induces a masking

effect that eventually inhibits the attachment and subsequent penetration of the virus into host cells [34]. Recently, it was demonstrated that sulfated polysaccharides possess a strong antiviral effect due to their direct interaction with SARS-CoV spike protein [28]. Therefore, our results indicate that Chi-S could interact directly with viral capsid of PCV2 to inactivate it, and thus allowing strong antiviral responses. To further support our evidence, [35], have also investigated low-molecular-weight sulfated chitosan naturally obtained from the squid *Sepia pharaonis*, demonstrating that the antiviral activity of this polymer against Newcastle disease virus is by binding to the surface glycoprotein. Therefore, its antiviral activity would be mediated by a competitive inhibition of the attachment of the viral particle to avian erythrocytes, thus functioning as a potential antiviral agent. Our results suggest that the Chi-S chemically obtained from industrial waste has great potential as an anti-PCV2 product that can be further validated *in vivo*.

According to our results, significant reductions of viral copy number were observed in the early stages of infection, therefore, Chi-S interferes with the penetration of the virus, since a reduction in viral DNA was also observed when viral adsorption in cell culture was allowed (Figure 6). These reductions were important in the first 40 min, however at 60 min a reduction in viral DNA was still observed, but not as efficient as it was for early post-infection stages (Figure 6). Recent research indicates that sulfated polysaccharides interfere with the internalization of the virus by interacting with its membrane proteins, that is, they bind with carbohydrate groups linked to the polypeptide chains of the virus to inhibit their penetration [37]. Additionally, sulfated polysaccharides bind to the allosteric site of the viral capsid, preventing the virus from initiating its cycle within the host cell. Indeed, Lee *et al.*, 2004 investigated sulfated polysaccharides from green algae and synthetic sulfated xylan exhibit potent antiviral activities against HSV-1 (herpes simplex virus) [38]. The authors showed that some sulfated polysaccharides not only inhibited the early stages of viral replication, such as viral attachment and penetration into host cells, but also interfered with the late stages of replication.

We have previously described the ability of generic Chi-C (LMW) to bind with PCV2 virus-like particles and that this conjugate would be useful and efficient for developing mucosal vaccines against PCV2 virus, since Chi, additionally, is an excellent adjuvant of the mucosa-associated immune system [36].

Here, we have shown that Chi-S have significant anti PCV2 activity. The mechanism of action would be through inhibition of viral adsorption, acting as a mimetic of the heparan sulfate receptor, binding to the viral capsid proteins and therefore blocking the attachment of the virus to the host cell, which reflects that Chi-S can be considered a “clone” coreceptor for virus binding, which would compete in the attachment with heparan sulfate or chondroitin sulfate, the virus's natural receptors on the cell surface. It has been well demonstrated that PCV2 uses these GAGs for attachment to porcine monocytic 3D4/31 and porcine kidney epithelial PK-15 cells [39].

## Conclusions

Our work suggests that Chi-S would affect early stages of the viral cycle, such as attachment or penetration into the host cell, however, further research is needed. Although, sulfated polysaccharides have been extensively studied for a long time in search of evidence of their antiviral properties, no previous research has been conducted regarding on the antiviral activity of CHI-S on PCV2 infection, therefore, we highlighted the potential of this biopolymer as a specific therapeutic compound. These warrants further studies to fully investigate the specific mechanisms that Chi-S plays in PCV2 infection.

## Abbreviations

Chi

chitosan

Chi-C

commercial chitosan

LMW Chi

low molecular weight chitosan

HMW Chi

high molecular weight chitosan

LMW Chi-S

low molecular weight sulfated chitosan

HMW Chi-S

high molecular weight sulfated chitosan

SEM-EDS

scanning electron microscopy - energy dispersive X-ray spectroscopy

FT-IR

Fourier transform infrared spectra

DS

degree of sulfation

MEM

minimal essential medium

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

### **Competing interests**

The authors declare that they have no competing interests

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### **Authors' contributions**

DJA experimental methodology, data curation. SAB conceptualization, writing- original draft preparation. ANA, SAB visualization, investigation. SAB, ANC supervision. DJA PCR software, validation. DJA, SAB, ANC, AAH, VN writing- reviewing and editing. All authors read and approved the final manuscript.

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Not applicable

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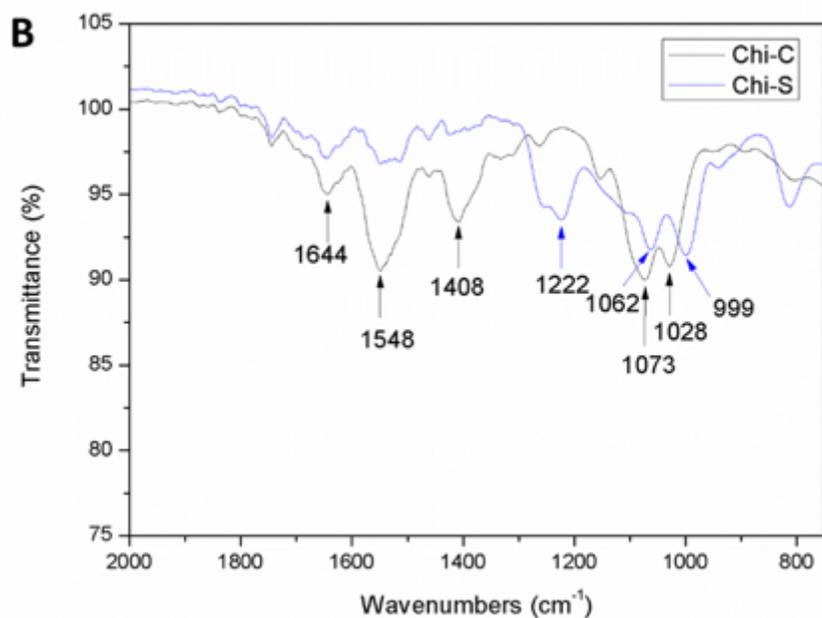
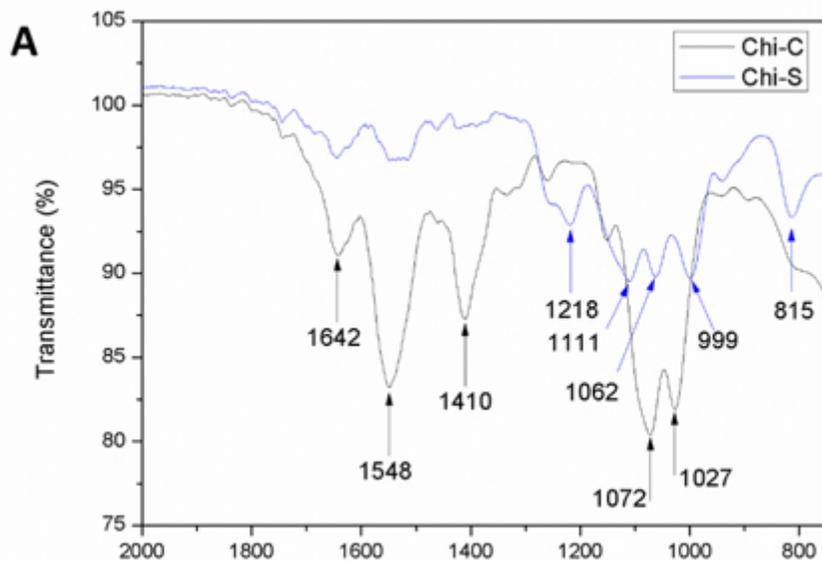
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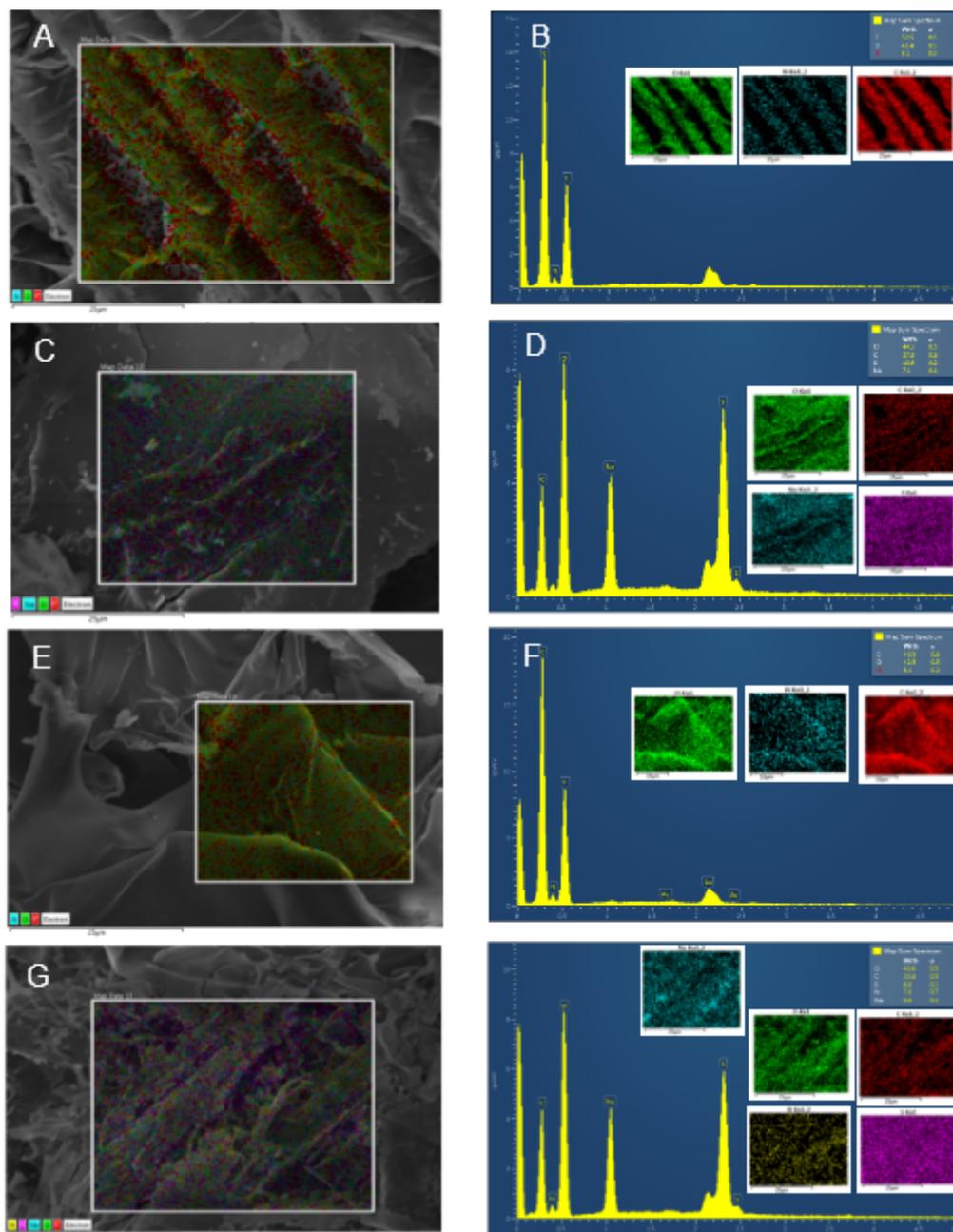
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## Figures



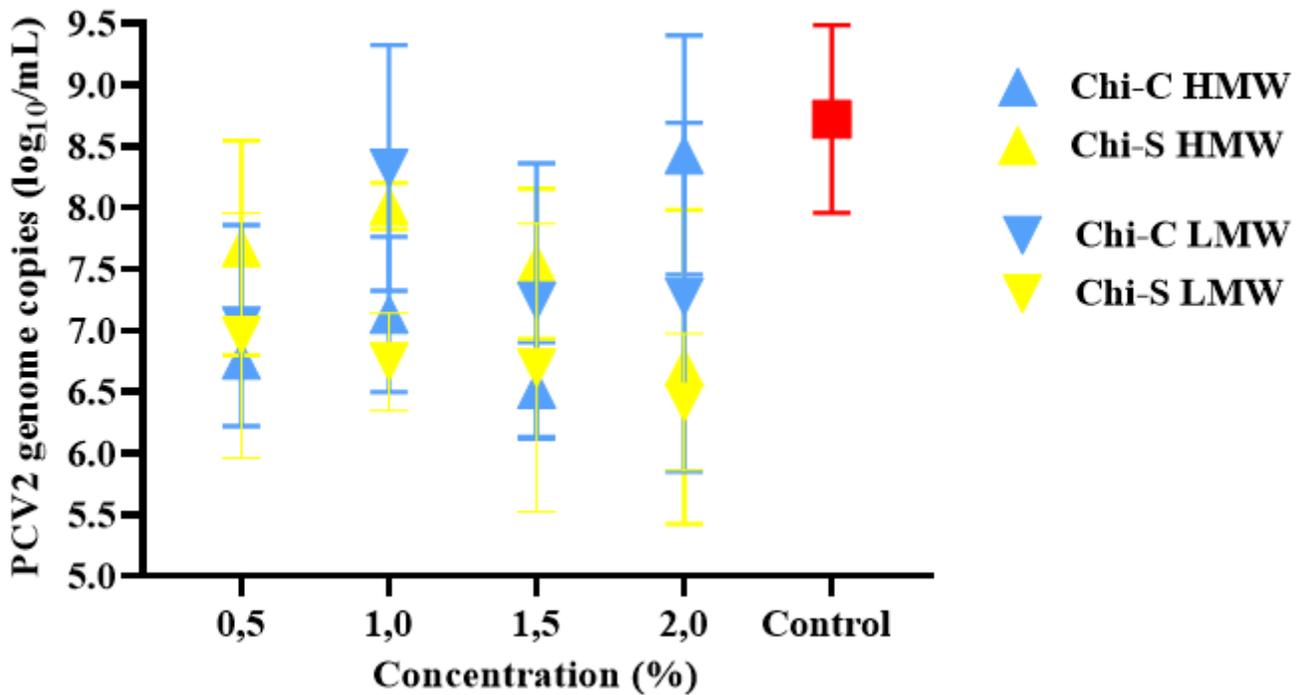
**Figure 1**

Illustrate the infrared spectra (FTIR) obtained for LMW Chi-S (blue line) versus LMW Chi-C (black line) (panel A) and HMW Chi-S (blue line) versus HMW Chi-C (black line) (panel B) using a ATR/FT-IR interspec 200-X spectrometer. Blue arrows show specific absorption bands at 815, 999 and 1218  $\text{cm}^{-1}$  corresponding to the C-O-S and O=S=O. Abbreviations: LMW Chi-C, low molecular weight commercial chitosan; LMW Chi-S, low molecular weight sulfated chitosan; HMW Chi-S, high molecular weight sulfated chitosan.



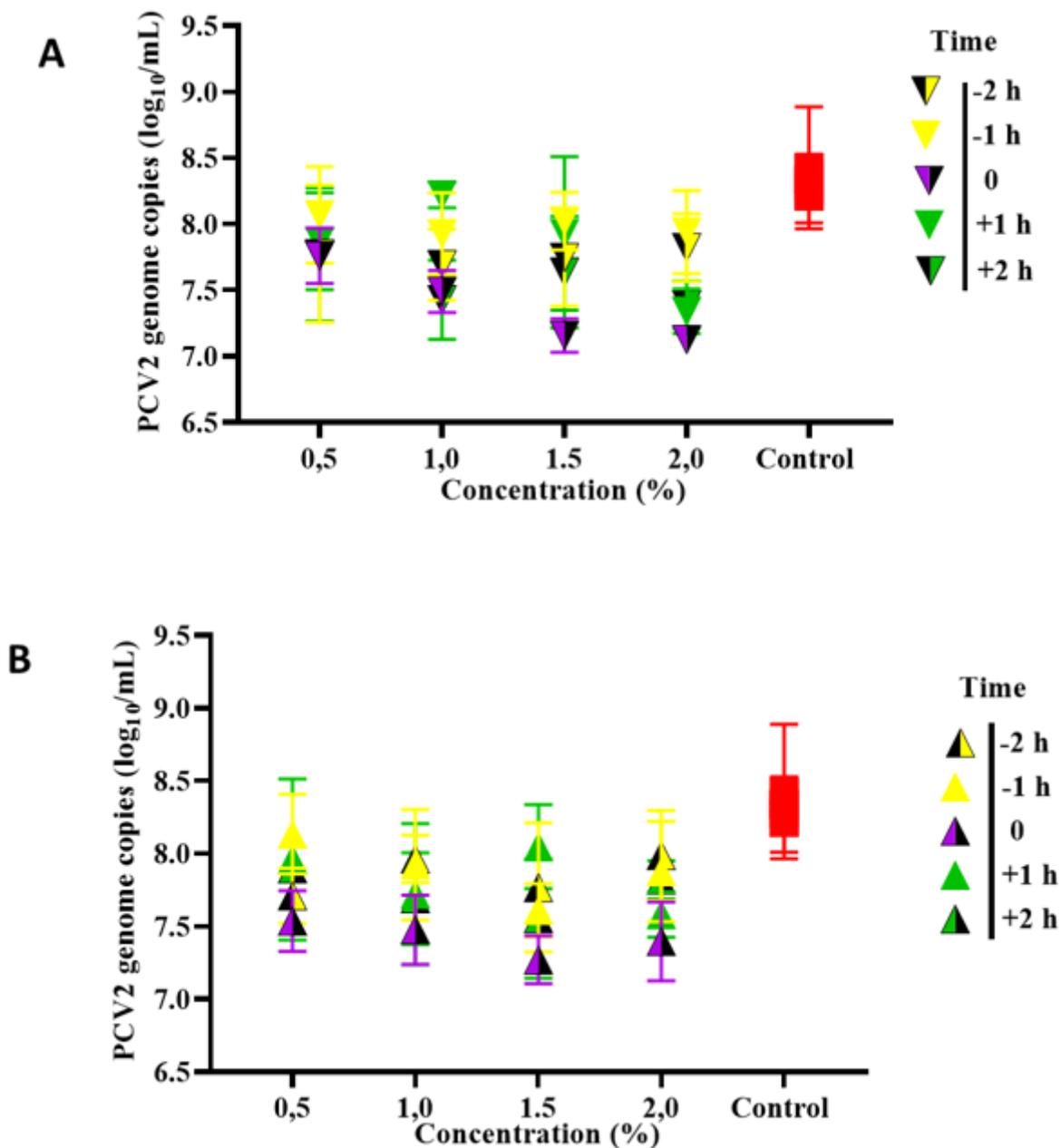
**Figure 2**

Surface morphology and micro-analysis (SEM-EDS) of Chi-C and Chi-S samples. LMW Chi-C (panel A, B); LMW Chi-S (panel C, D); LMW Chi-S (panel E, F) and HMW Chi-S (panel G, H). The colored represents elemental composition of analyzed region on the sample (green=oxygen; blue=nitrogen; red=carbon; pink=sulfur).



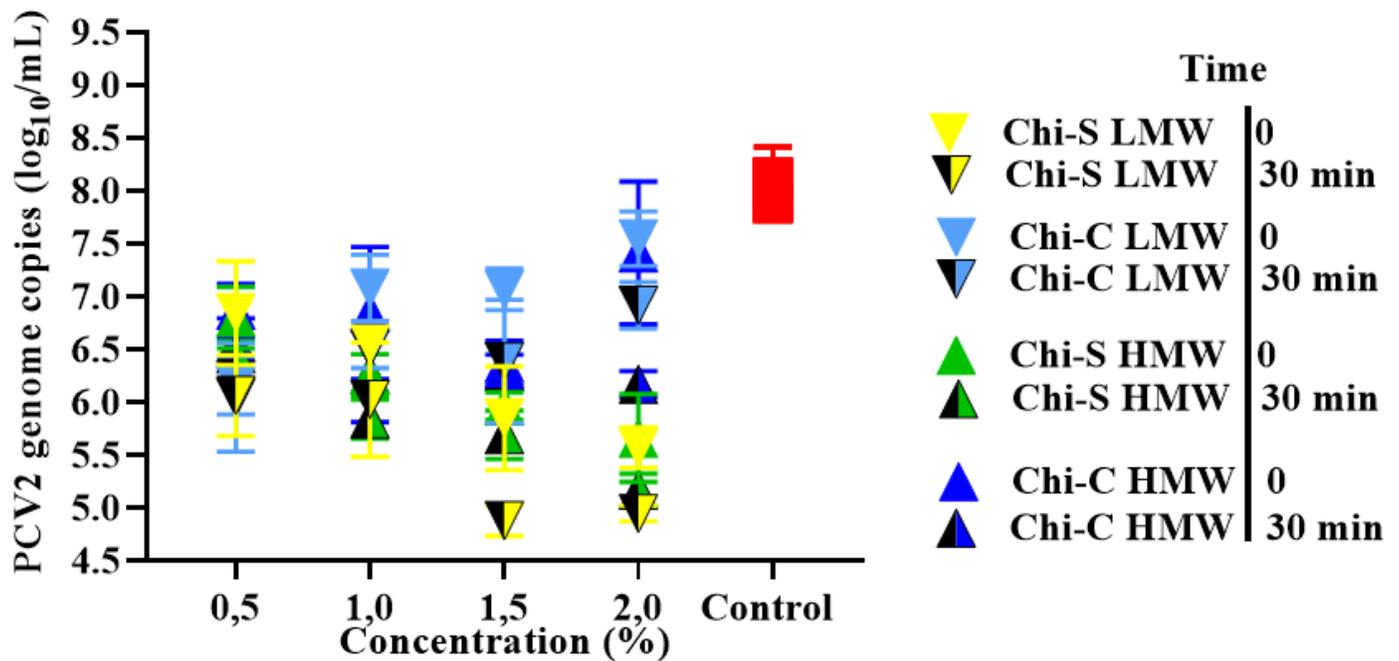
**Figure 3**

Antiviral effect of Chi-C and Chi-S samples. PK-15 cell monolayers were infected with 10  $\mu$ L of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>), after one hour incubation, the cells were exposed at four concentrations (0.5,1.0,1.5, and 2.0 %) of LMW Chi-C; LMW Chi-S; LMW Chi-S and HMW Chi-S. Red square represents virus control. The results are presented as log<sub>10</sub> viral DNA copies/mL and are expressed as the mean  $\pm$  SD generated from three replicates and a significant difference set to  $p \leq 0.05$  was considered



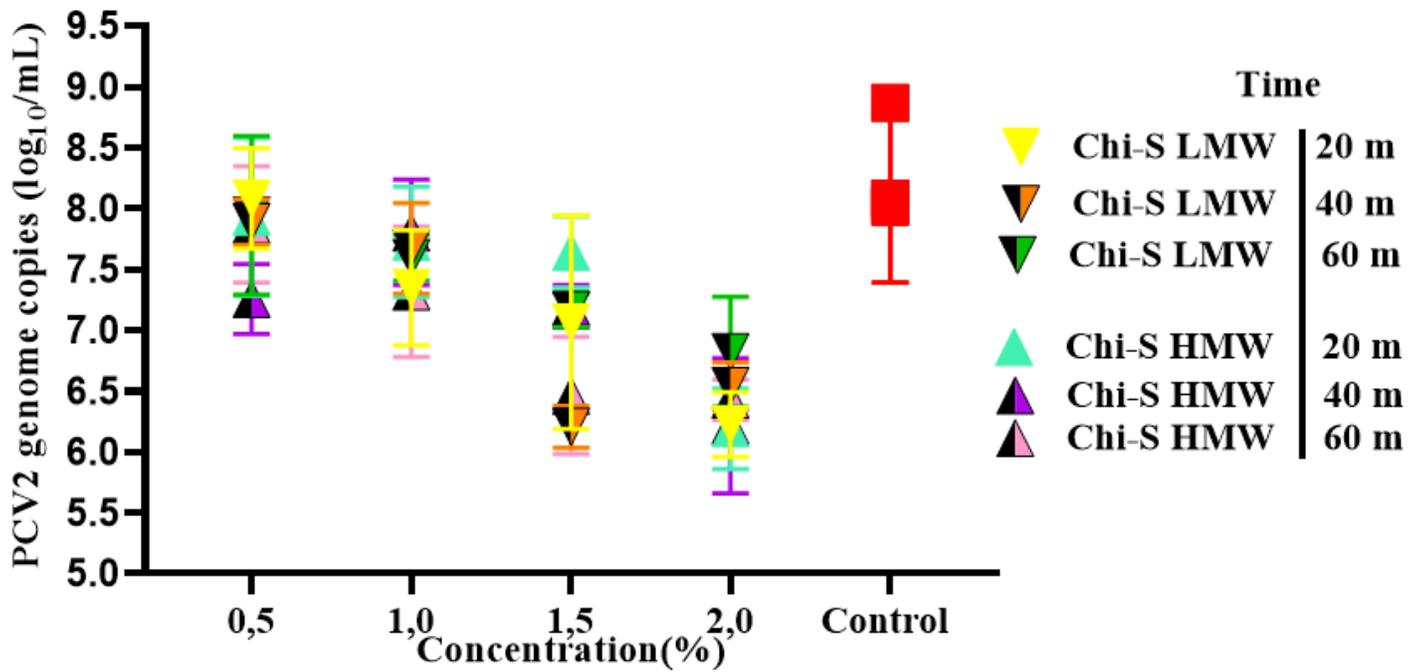
**Figure 4**

Time of addition assay. LMW Chi-S (graph A) and HMW Chi-S (graph B) at above-mentioned concentrations were added 1 and 2 h before infection of PK-15 cells (Preinfection), at the time of infection (Time 0) and 1 to 2 h post-infection. Red square represents virus control. The results are presented as log<sub>10</sub> viral DNA copies/mL and are expressed as the mean ± SD generated from three replicates and a significant difference set to  $p \leq 0.05$  was considered.



**Figure 5**

Viral attachment assay. Confluent PK15 cell monolayers were pre-cooled at 4 °C for 1 h followed by (i) Cellular infection with 10 µL/well of PCV2 suspension (10<sup>6</sup> TCID<sub>50</sub> ml<sup>-1</sup>), supplemented with 100 µl of MEM containing each polymer at above-mentioned concentrations (ii) infection with 10 µL/well with virus suspension (10<sup>6</sup> TCID<sub>50</sub> ml<sup>-1</sup>) that had been mixed and pre-incubated with 100 µL/well of each polymer at 4 °C for 30 min. Red square represents virus control. The results are presented as log<sub>10</sub> viral DNA copies/mL and are expressed as the mean ± SD generated from three replicates and a significant difference set to  $p \leq 0.05$  was considered



**Figure 6**

Viral penetration assay. PK-15 confluent cells were pre-cooled and infected with 10  $\mu$ L of PCV2 suspension ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) and incubated at 4 °C for 3 h to allow viral attachment. After this step, the cells were treated at room temperature with 100  $\mu$ L/well of each polymer using the same four concentrations (0.5,1.0,1.5, and 2.0%), followed by incubation at 37°C/5% CO<sub>2</sub> for 20, 40, and 60 min (separate plates) to allow the compound to interrupt penetration of virus into cells. Each time point was tested separately and independently. Red square represents virus control. The results are presented as log<sub>10</sub> viral DNA copies/mL and are expressed as the mean  $\pm$  SD generated from three replicates and a significant difference in  $p \leq 0.05$  was considered.