



# 4th International Electronic Conference on Medicinal Chemistry

1-30 November 2018

chaired by Dr. Jean Jacques Vanden Eynde

sponsored by



pharmaceuticals

## Antiviral activity of the exopolysaccharides produced by *Leuconostoc sp.* against HSV-1

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## Abstract:

Herpes viral infection is the most common human viral infection. According to the WHO data, about 80% of the world's population has the antibodies to herpes viruses. Modern chemotherapy of infectious diseases, caused by the herpes virus, is associated with the use of guanine-containing drugs, which are the modified acyclic nucleosides of acyclovir, penciclovir, cidofovir and others. The main disadvantage of these drugs, despite their high antiviral activity, is their toxicity and formation of drug resistance. This leads to the necessity for comprehensive study and discovery of the alternative and safe remedies for the prevention and treatment of various forms of herpetic lesions. The attention of researchers is focused on the compounds derived from natural sources. A promising approach for the treatment of diseases caused by herpes simplex virus is the use of lactic acid bacteria (LAB) and their metabolites.

Our work is related to the study of the anti-herpetic activity of exopolysaccharides produced by *Leuconostoc sp.*

**Keywords:** exopolysaccharides of lactic acid bacteria, herpes simplex virus 1 type, antiviral activity, cell cycle



## Introduction:

Lactic acid bacteria have shown great potential in the prevention of severe gastrointestinal disorders in human beings and animals. Although the mode of antiviral action of lactic acid bacteria has not been elucidated in details, they have shown significant ability to inhibit viral infections and/or replication either directly or indirectly caused by respiratory, gastroenteric, murine, influenza, herpes and Newcastle disease viruses. However, variations in the antiviral effect have been observed at species level based on the efficiency and biological properties of the test strain.

Exopolysaccharides are known as potentially useful and biologically active polymer substances for medicinal and pharmaceutical uses due to their versatile biological properties. Liu et al. reported that polysaccharide may prevent viral infection through blockage of virus adsorption onto the host cells by interacting either with virus particles or with the host cell. Some study confirmed that strong evidences on interaction of polysaccharide molecule and cell membrane should be occurred in order to confirm the proper blocking of receptor resulting in the adsorption of virus on the cell membrane. Inhibition of virus-cell adsorption onto the host cell is considered to be the first steps in viral infection process. It has been found that sulfated polysaccharides inhibit the virus-cell attachment and display antiviral effect against various types of viruses including hepatitis B virus, human cytomegalovirus, herpes simplex virus and influenza virus.

Hence, in the current scenario of antiviral research, lactic acid bacteria and their derived polymers or polysaccharides are considered potential candidates in antiviral therapy to prevent or treat viral infections in both human and animals with remarkable efficacy and might have significant contribution in medicine and pharmaceutical industries in future.



## Results and discussion

**Objects:** Cell culture BHK-21 (kidney of Syrian hamster) and herpes simplex virus 1 (HSV-1) were used. The strains of the *Leuconostoc sp* were isolated from fermented homemade vegetables: apples, tomato juice, and sauerkraut. Exopolysaccharides (EPSs) were isolated from the culture fluid.

### Source of *Leuconostoc sp.* isolation

code	fermented homemade vegetables
15a	pickled apples
48a	pickled apples
33a	pickled apples
2t	pickled tomato juice
43a	pickled apples
19s	sauerkraut
6s	sauerkraut

The first stage of the antiviral assay is necessary to determine the concentration of the compounds that is not toxic to the cells.

### Cellular toxicity:

Cellular toxicity of EPSs was tested *in vitro* according to a cell viability MTT-assay. Monolayers of BHK-21 cells in 96-multiwell plates were incubated with the compounds at concentration of 1500 – 370  $\mu\text{g/ml}$  for 72 h, then in a medium was added 20  $\mu\text{l}$  of a 5 mg/ml solution of MTT (Sigma, USA). The concentrations of EPSs that inhibit 50% of cell viability compared to control cells ( $\text{CC}_{50}$ ) were measured.

All EPSs at a concentration of 1500  $\mu\text{g/ml}$  exhibited little cytotoxic effect, and  $\sim 71 - 100\%$  of cells survived (data not shown) and their  $\text{CC}_{50}$  values were  $>3.5 \text{ mg/ml}$ .



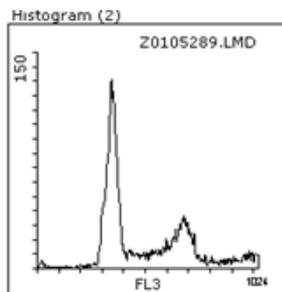
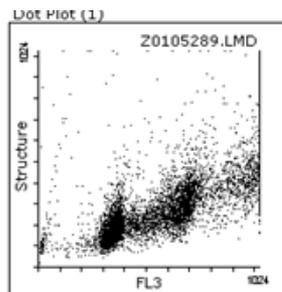
## Cell Cycle Analysis

Suppression of cell growth and proliferation frequently represents cell response to compound cytotoxicity and virus infection. Therefore, the influence of the EPSs on the cell cycle under normal conditions and the conditions of herpetic infection was analyzed. For the purpose cells ( $1 \times 10^6$ ) were harvested by centrifugation at 300 g (2000rpm) for 7 min, resuspended in 96% ice-cold ethanol, washed with PBS, resuspended in 500  $\mu$ l solution of PBS (Sigma) that contained RNase (100  $\mu$ g/ml) and propidium iodide (PI) (50  $\mu$ g/ml), and incubated at room temperature for 1 h. The cell fluorescence intensity was measured by a flow cytometer (Beckman Coulter Epics LX, USA) with laser wavelength 488 nm. Cell cycle profiles were analyzed with the program Flowing Software, version 2.5. As the intensity of the PI signal is directly associated with DNA content, the number of cells in a certain cell cycle phase and cells containing fragmented DNA (apoptotic cells), as well as cell structure, were estimated.

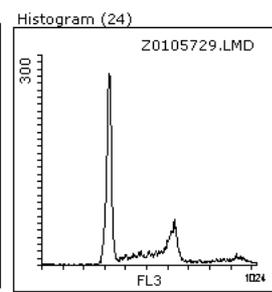
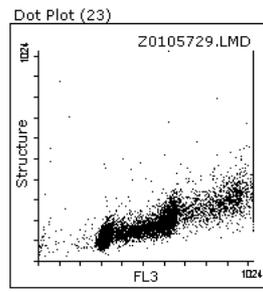
The effect of the EPSs on BHK-21 cells population is demonstrated on the following histogram and graphics. It was revealed that after 48 hours of growth 38% of BHK-21 cells remained in G1 phase, 10% were in S phase and 16% were in G2/M phase of cell cycle. Under the conditions of the EPSs treatment, the distribution of cells according to the structure and cell cycle phases was similar to control cells but not identical. Thus, depending on the used EPSs concentrations, 33 – 47% of cells remained in G1 phase, 8 – 13% were in S phase and 14 – 18% were in G2/M phase of cell cycle.



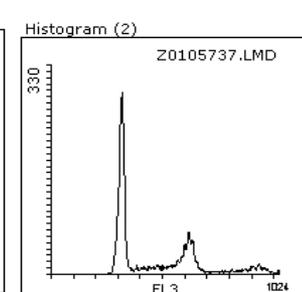
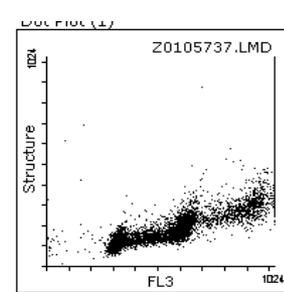
# Profiles of cellular cycles of BHK-21 cells treated with the EPSs



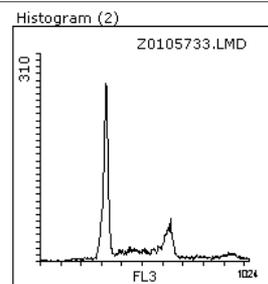
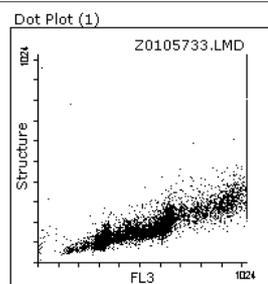
**BHK-21 (control cells)**



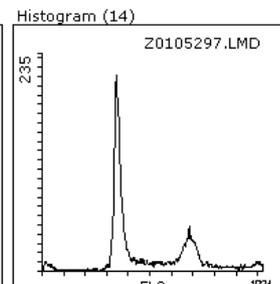
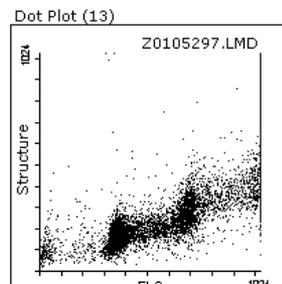
**15a (1500 µg/ml)**



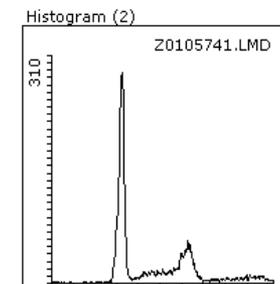
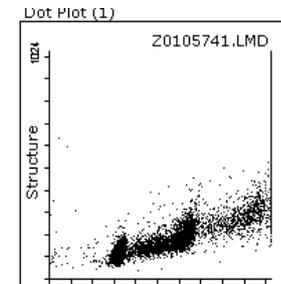
**48a (1500 µg/ml)**



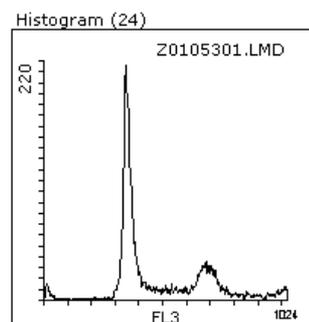
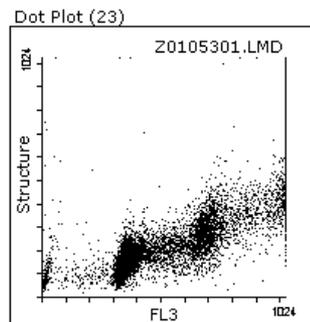
**33a (1500 µg/ml)**



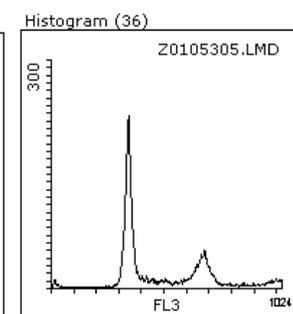
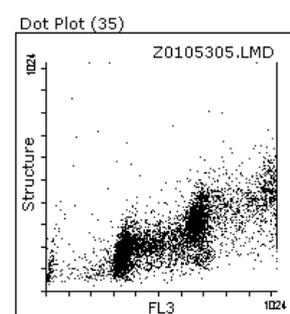
**2t (1500 µg/ml)**



**43a (1500 µg/ml)**



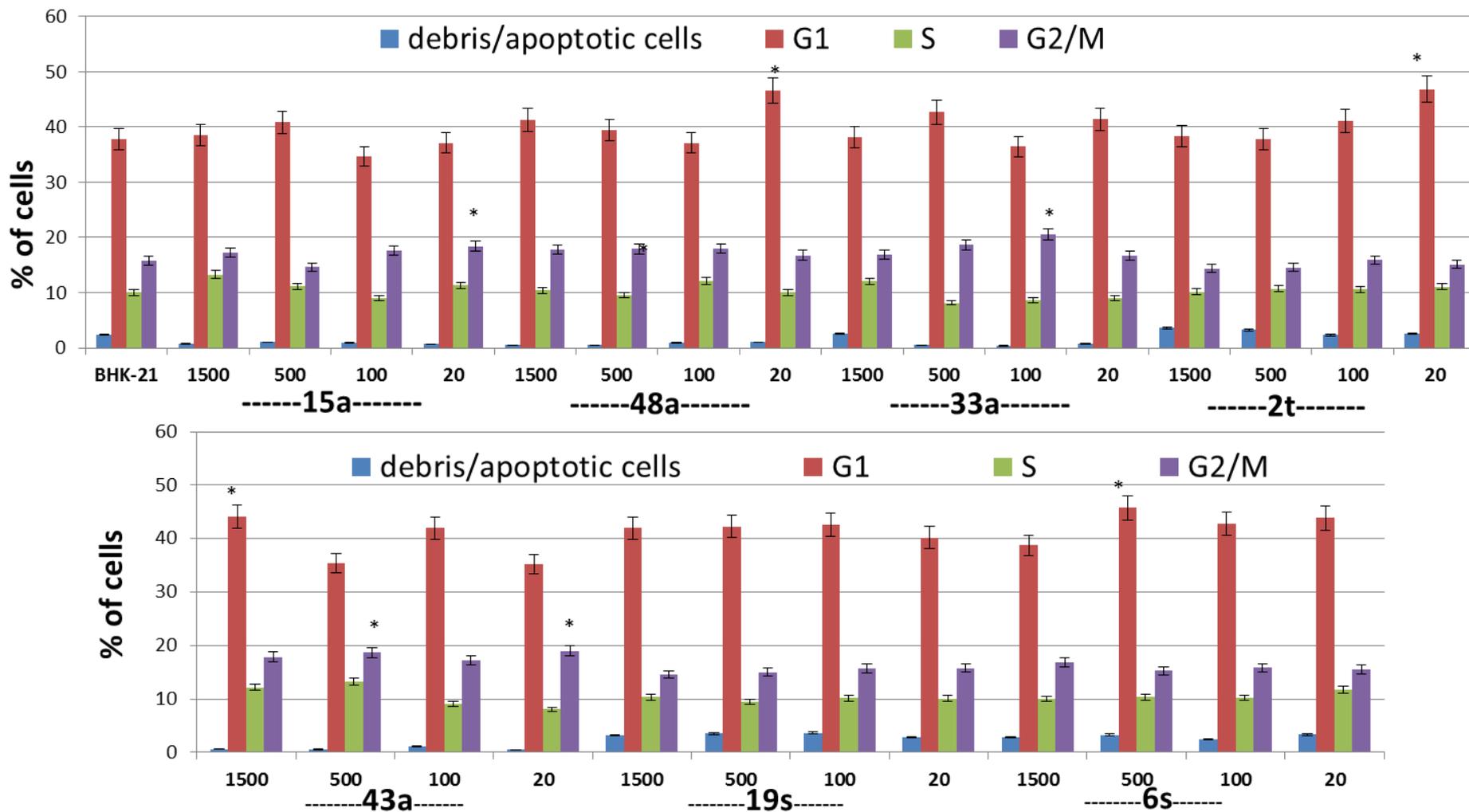
**19s (1500 µg/ml)**



**6s (1500 µg/ml)**



# Influence of the EPSs on the cell cycle of BHK-21 cells



Results corresponding to the percentage of cells in G1, S and G2/M phases of three independent experiments are presented as mean  $\pm$  S.D. \*Significant difference between control sample and treated cells ( $P < 0.05$ ).



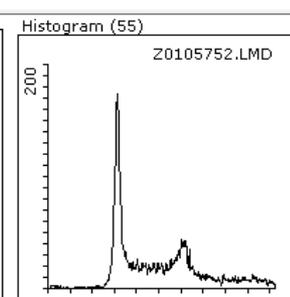
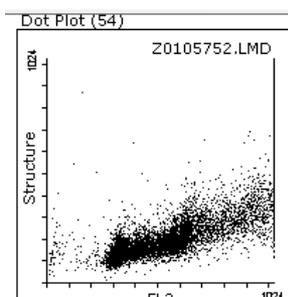
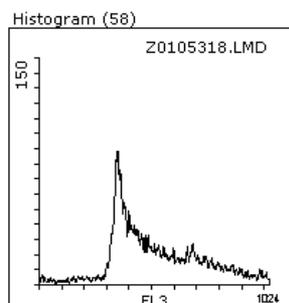
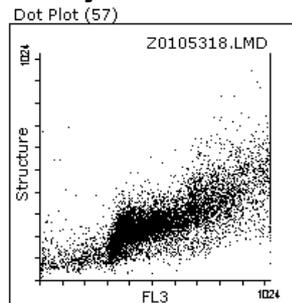
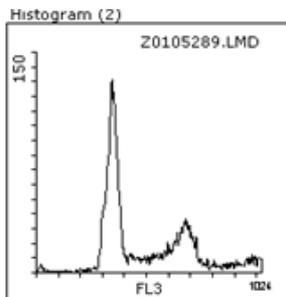
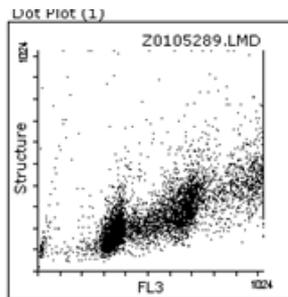
Virus infection frequently results in the disturbance of key cellular processes within the host cell. The subversion of cell cycle pathways is a well-established mechanism by which viruses create the most suitable environment for their replication. Notably, the induction of S-phase is either mandatory or at least advantageous for lytic replication of a number of viruses. The characteristic changes in DNA synthesis and content induced by HSV-1 infection allow the use of flow cytometry to detect not only an infection but also the potential antiviral activities.

The influence of the EPSs on the cell cycle under a condition of herpesvirus infection was studied and the result demonstrated on the following histogram and graphics. There was a significant number of cells in the S (23%) and G2/M (23%) phases of the cell cycle under herpetic infection. At a point when infected cells move into the S and G2/M phase of the cell cycle, the cells are producing viral DNA, late protein, and virions.

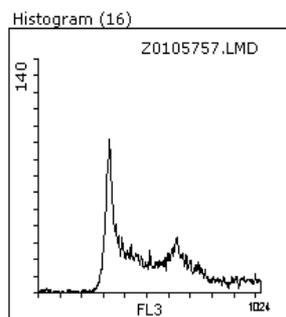
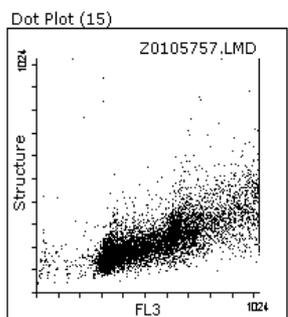
The normalization of the number of cells in all phases of the cell cycle compared with the profile of infected cells and the increasing number of cells in G1 phase by 17 - 79% and the decreasing number of cells in G2/M by 14 - 43% compared with the control values of viral infections were determined after using of EPSs.



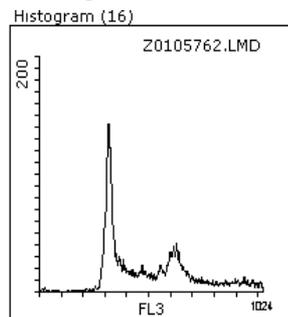
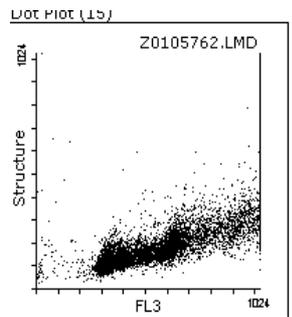
# Profiles of cellular cycles of infected BHK cells treated with the EPSs



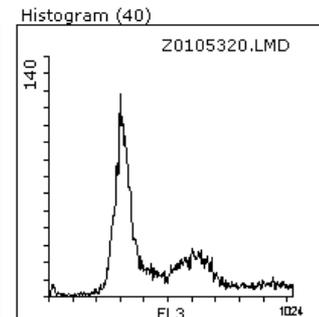
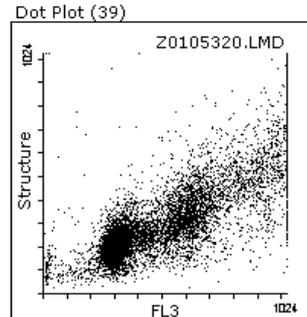
**BHK-21**



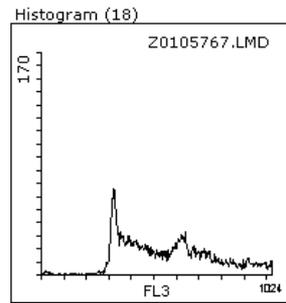
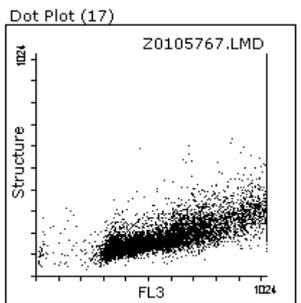
**BHK-21 + HSV-1**



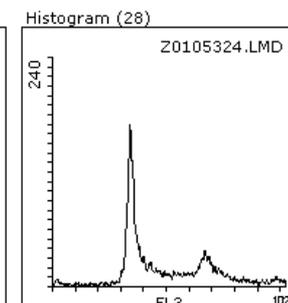
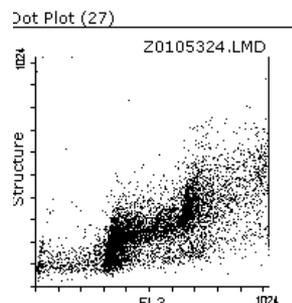
**15a (500 µg/ml)**



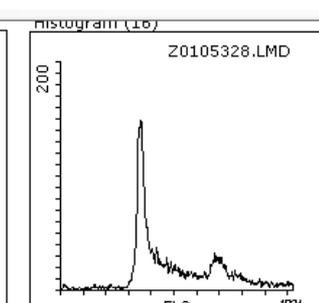
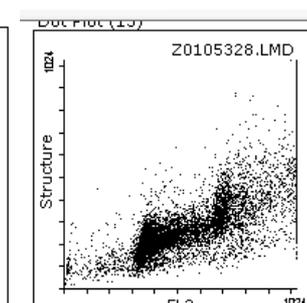
**48a (500 µg/ml)**



**33a (500 µg/ml)**



**2t (500 µg/ml)**



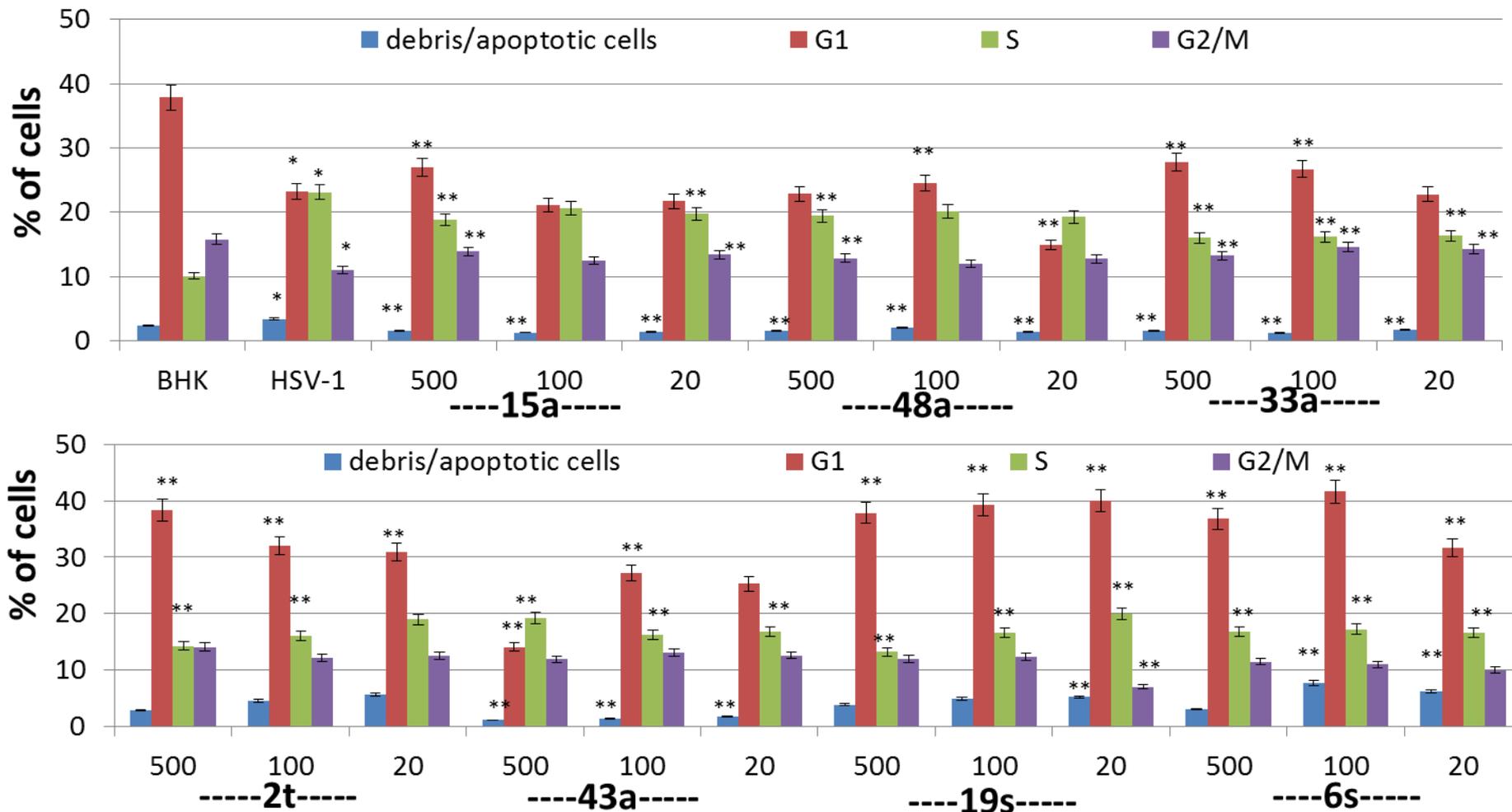
**43a (500 µg/ml)**

**19s (500 µg/ml)**

**6s (1500 µg/ml)**



# Influence of the EPSs on the cell cycle of infected cells treated with the EPSs



Results corresponding to the percentage of cells in G1, S and G2/M phases of three independent experiments are presented as mean  $\pm$  S.D. \*Significant difference between a test sample and control cells ( $P < 0.05$ ). \*\*Significant difference between test sample and control of infected cells ( $P < 0.05$ ).



## Antiviral Assay

We used a modification of an MTT-assay developed for screening of anti-HSV compounds. 50  $\mu$ l of virus suspension (a multiplicity of infection (MOI) of 3,2) was added to BHK-21 cells. After 2 hours, any unabsorbed virus was aspirated and 200  $\mu$ l of serial two-fold EPSs-containing medium was added to each well, and incubated at 37°C and 5% CO<sub>2</sub> for 2 days. The % of protection and the concentrations of the EPSs that inhibit virus reproduction at 50% compared with virus control (IC<sub>50</sub>) were measured.

It was determined, that only EPSs 2t, 19s, and 6s significantly inhibited HSV-1 reproduction; their IC<sub>50</sub> value equal 512±24, 242±9, 360±13  $\mu$ g/ml, and the selectivity index was 52, 49 and 39, respectively.

Herpes virus reproduction is characterized by a complex sequence of different steps with which antiviral agents might interfere. It is known that a protein bound microbial polysaccharide showed potent ability to reduce the viral titer of HSV-1 and HSV-2. The significant antiviral activity against herpes simplex viruses might be attributed to the following reasons including disintegration of the entire herpes simplex virus particles, solubilization of the virus envelope, modification in chemical composition, protein degradation, or by covering the essential proteins of virus envelope. Some study revealed that protein bound polysaccharide may exert its partial antiviral mode of action against HSV-2 via binding the glycoproteins of HSV-2 to protein bound polysaccharide which may interfere at the initial state of virus replication or release of any virions.

To determine the stage of HSV-1 infection that was inhibited by EPSs, virus and cells were treated with EPSs at various times before and after HSV-1 infections.



### ***Virucidal assay***

100  $\mu$ L of 1.5 mg/ml of the EPSs were mixed with 100  $\mu$ L of 2X HSV-1 in microfuge tubes and incubated at 37°C for 3 hours. Then, the BHK-21 cells were infected with 0.05 ml per well of ten-fold serial dilutions of the virus-containing suspension. The virus adsorption was carried out for 2 hours, then 0.15 ml of serum-free medium was added. After 3 days of incubation, virus titer (TCD<sub>50</sub>/ml) was calculated.

### ***Attachment assay***

The BHK cell monolayer was pre-chilled at 4°C for 1 h and then infected with 3,2 MOI HSV-1 in the absence or presence of serial dilutions of the EPSs. After 3 h of incubation at 4°C cell monolayer was washed with PBS three times and overlaid with medium containing 0.06% agarose. The infected cell monolayer was fixed and stained with 1% crystal violet in 20% ethanol. The antiviral activity of the EPSs was determined by the following formula:

$$\% \text{ Percentage inhibition} = (1 - \text{number of plaque (tested)} / \text{number of plaque (control)}) * 100\%$$

### ***Viral Penetration Assay***

The BHK-21 cell monolayer was pre-chilled at 4°C for 1 h, infected with 3,2 MOI HSV-1 and incubated at 4°C for a further 3 h to allow the attachment of HSV-1. After incubation, 50  $\mu$ L of 2X concentration of compounds were added. The infected cell monolayer was then incubated at 37°C to maximize the penetration of virus. At 45 min intervals, the infected cell monolayer was treated with PBS at pH 3 for 1 min to inactivate a non-penetrated virus. PBS at pH 11 was then immediately added to neutralize acidic PBS. The neutral PBS was removed and the cell monolayer was overlaid with overlay medium. After a further 48 h of incubation, the cell monolayer was fixed and stained. Plaques were counted as described above.



## Effect of EPSs on infectivity of viral offspring

Concentration ( $\mu\text{g/ml}$ )		EPSs present:			
		3 hour before adsorption	During adsorption	During penetration	After adsorption
		% inhibition of virus titer			
<b>2t</b>	150	91.34	96.42	91.11	72.29
	75		95.26	84.07	58.44
	38		93.47	84.81	30.07
	19		89.89	83.33	23.36
<b>19s</b>	150	86.73	87.88	97.48	88.24
	75		99.73	95.88	62.31
	38		98.77	67.41	80.12
	19		98.65	51.48	74.56
<b>6s</b>	150	97.04	99.24	96.33	99.82
	75		98.92	85.55	54.03
	38		97.16	74.07	50.56
	19		96.02	70.85	37.66



A various inhibitory effect of the EPSs was observed when there were added 3 h before adsorption, during virus adsorption and penetration, and after virus adsorption. The EPSs 2t, 19s, and 6s showed virucidal activity when were added to the virus before adsorption reduced HSV-1 infectivity by 86 – 97%. Our studies revealed that this EPSs were able to prevent the HSV-1 attachment to cells and penetration into cells in a different manner, reducing HSV-1 production by 70 – 99 %. It was shown that all EPSs reduced the titer of the virus obtained *de novo* by 23-99%.

### **Conclusions:**

Taken together, our results showed that the EPSs 2t, 19s, and 6s, isolated from the genus *Leuconostoc sp.*, in non-toxic concentrations possess the anti-HSV activity, which is realized via multiple mechanisms. The EPS 6s showed significant virucidal activity and reduces the viral adsorption, penetration, and yield of virus particles by 97 - 99%. Whereas the EPSs 2t and 19s block the viral adsorption to the cells and inhibits infectivity of viral progeny. Moreover, it was demonstrated that using the EPSs leads to the normalization of a life cycle of cells infected with herpes virus to the level of non-infected cells. Taking into account the promising anti-herpetic activity of the EPSs produced by *Leuconostoc sp.* herein reported further investigation is needed to explore the antiviral mechanism of these compounds in detail.

### **Funding:**

Publications are based on the research provided by the grant support of the State Fund for Fundamental Research (project F83).

