BACTERIAL EXTRAVESICULAR VESICLES DERIVED FROM *Streptrococcus parauberis* INTERNALIZATION IN TO RAW264.7 CELLS

E. H. T. Thulshan Jayathilaka*, Chamilani Nikapitiya and Mahanama De Zoysa
College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 34134
Email: thimira.thulshan.jayathilaka@gmail.com

ABSTRACT

Bacterial extracellular vesicles (BEVs) are small, membrane-bound particles released by both Gram-positive and Gram-negative bacteria, playing crucial roles in intercellular communication, pathogenicity, and the transfer of cargo between bacteria and hosts. *Streptococcus parauberis*, a Gram-positive pathogenic bacterium, causes significant mortality among various fish species. In our previous study, we isolated and characterized BEVs derived from *S. parauberis* (*Sp*EVs) and demonstrated their immunomodulatory activity. In this study, we focus on the interaction of *Sp*EVs with receptor proteins in Raw 264.7 cells and their mechanism of internalization.

qRT-PCR was conducted on Raw 264.7 cells treated with *Sp*EVs (5 and 10 μg/mL) to determine which receptors were activated. The results showed that the cell surface receptor *Tlr2*, peptidoglycan receptors *Nod1* and *Nod2*, and endolysosome receptors *Tlr3* and *Tlr9* interacted with *Sp*EVs. To confirm the internalization mechanism of *Sp*EVs, Raw 264.7 cells were pretreated with various EV internalization pathway inhibitors, including chlorpromazine (clathrin inhibitor), dynasore (dynamin inhibitor), methyl-β-cyclodextrin (lipid sequestrant), cytochalasin D (actin inhibitor), and 5-(N-ethyl-N-isopropyl)-amiloride (macropinocytosis inhibitor). The cells were then treated with fluorescence-labeled *Sp*EVs. The results showed that cells treated with chlorpromazine and dynasore significantly reduced fluorescence levels compared to negative control (NC). Finally, the internalization pathway was validated by performing qRT-PCR on known highly expressed genes in Raw 264.7 cells pretreated with the inhibitors and then treated with *Sp*EVs. A significant reduction in the fold expression of *Tnfα* and *Il10* was observed in chlorpromazine and dynasore-pretreated cells compared to the NC. This validates that SpEV internalization occurs through a clathrin and dynamin-dependent endocytic pathway.

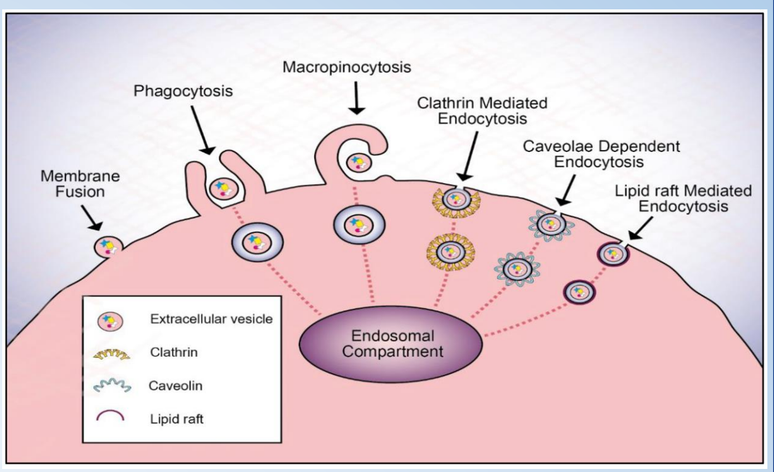
In conclusion, *Sp*EVs interacted with various cell receptors sensitive to BEVs membrane peptidoglycan and bacterial genetic materials. These interacted *Sp*EVs were internalized into Raw 264.7 cells through clathrin and dynamin-dependent endocytic pathway and subsequently induced immunomodulatory activity.

INTRODUCTION

Bacterial extracellular vesicles (EVs) are small, membrane-bound particles released by bacteria, playing key roles in intercellular communication and host-pathogen interactions. These vesicles carry a diverse range of biomolecules, including proteins, lipids, and nucleic acids, facilitating their transfer between cells. The internalization of bacterial EVs by host cells can occur through various pathways such as endocytosis, membrane fusion, and receptor-mediated uptake. Understanding these internalization mechanisms is crucial for unraveling the impacts of bacterial EVs on host immune responses and disease progression. Moreover, studying these processes opens new avenues for developing therapeutic strategies against bacterial infections.

EV internalization pathways

1. Membrane fusion
2. Phagocytosis
3. Macropinocytosis
4. Clathrin-mediated endocytosis
5. Caveolae-dependent endocytosis
6. Lipid raft mediated endocytosis



Lana Ginini et al., 2022

MATERIALS AND METHODS

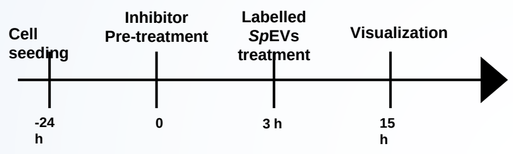
Interacting receptor determination → *Sp*EVs treatment on Raw264.7 cells → mRNA expression by qRT-PCR

*Sp*EVs internalization → *Sp*EVs labeling with Exosparkler kit → Raw cell treatment and fluorescence observation

*Sp*EVs internalization pathway determination → Raw264.7 cells were treated with inhibitors → Labeled *Sp*EVs treatment and fluorescence treatment

Treated inhibitors and inhibiting pathways

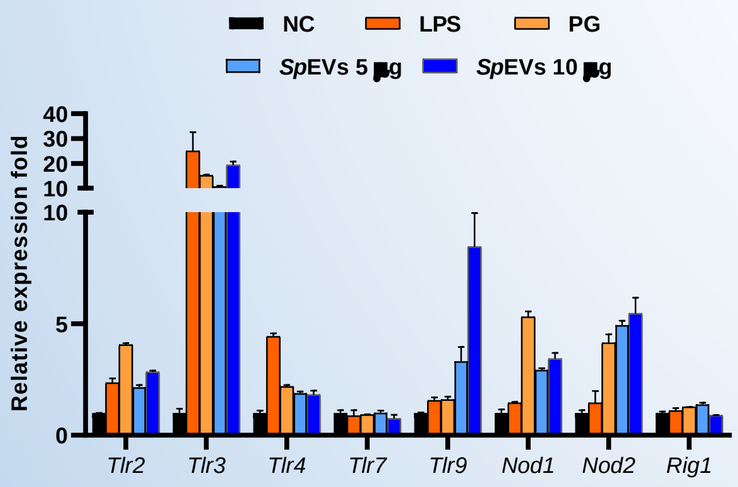
- Chlorpromazine - clathrin inhibitor
- Dynasore - dynamin inhibitor
- Methyl-β-cyclodextrin - lipid sequester
- Cytochalasin D - actin inhibitor
- 5(N-ethyl-N-isopropyl)-amiloride - macropinocytosis inhibitor



*Sp*EVs internalization pathway Validation → Raw264.7 cells were treated with inhibitors → mRNA expression of selected genes by qRT-PCR

RESULTS

*Sp*EVs interacting receptors



❖ **Mostly interacted receptors**

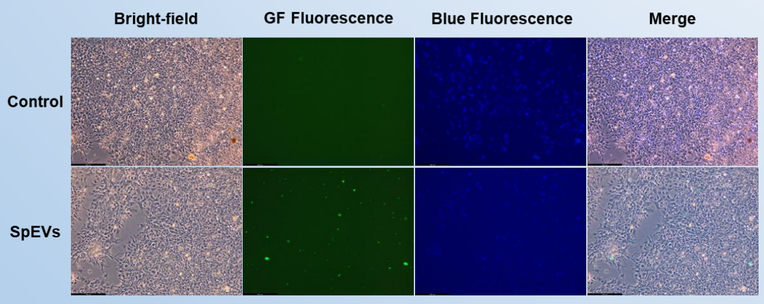
TLR3

TLR9

NOD1

NOD2

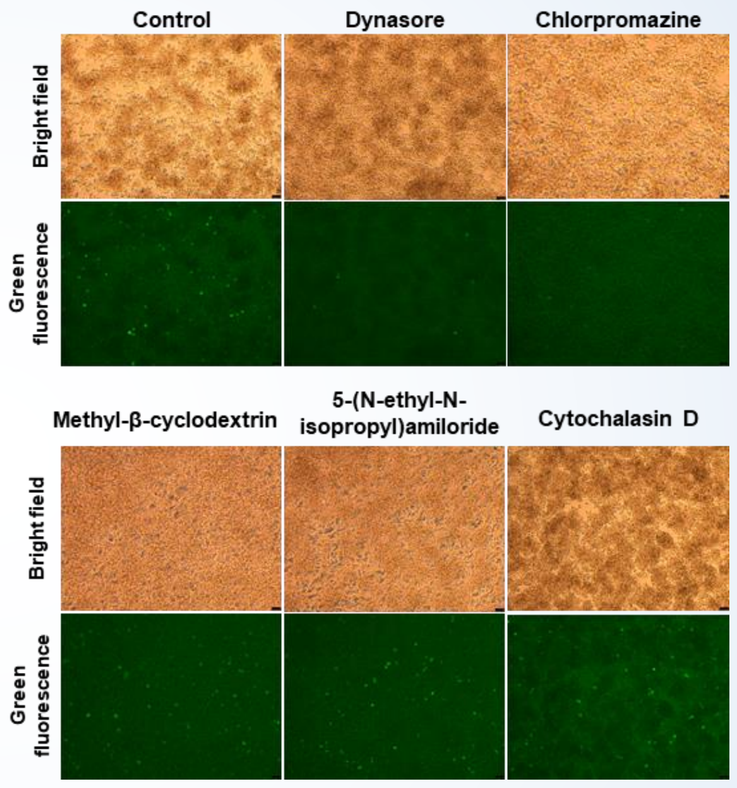
*Sp*EVs internalization

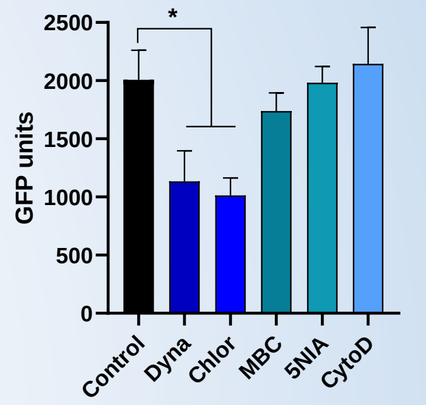


❖ Exo-sparkler labeled *Sp*EVs were internalized into Raw264.7 cells

RESULTS

Internalization inhibitors effect on *Sp*EVs internalization

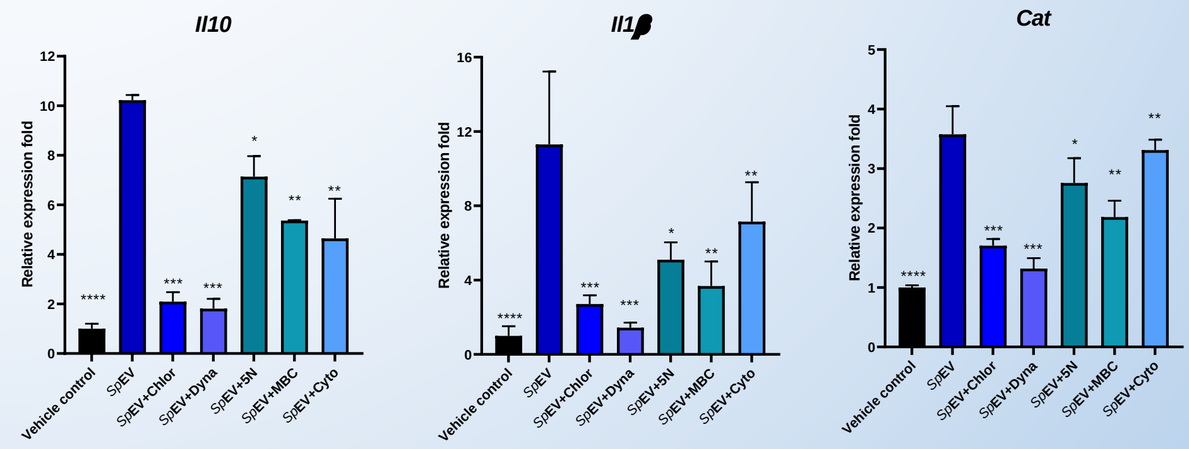




❖ Lowest fluorescence levels were determined in Chlorpromazine and dynozore-treated cells

❖ Confime *Sp*EVs were internalized through cavoline-clarithrine-mediated endocytosis

Anti-inflammatory activity of *Sp*EVs



❖ mRNA expression of *Il10, Il1β,* and *Cat* were significantly inhibited in Chlorpromazine and Dynozore treated cells

❖ Validate the *Sp*EVs were internalized through cavoline-clarithrine-mediated endocytosis

CONCLUSION

- ❖ *Sp*EVs interacted with Raw 264.7 cell receptors of TLR3, TLR9, NOD1 and NOD2 and showed the highest expression of their expression fold.
- ❖ Fluorescence labeled *Sp*EVs were internalized into Raw 264.7 cells after 12 h of incubation.
- ❖ *Sp*EVs internalization was mainly inhibited by Chlorpromazine and dynazore treatment confirmed the *Sp*EVs internalization involved in cavoline-clarithrine mediated endocytosis.

REFFERENCES

- Kang, C. S., Ban, M., Choi, E. J., Moon, H. G., Jeon, J. S., Kim, D. K., ... & Kim, Y. K. (2013). Extracellular vesicles derived from gut microbiota, especially Akkermansia muciniphila, protect the progression of dextran sulfate sodium-induced colitis. PloS one, 8(10), e76520.Bajic, S. S., Cañas, M. A., Tolinacki, M., Badia, J., Sánchez. (2020)
- Rai, A. K., & Johnson, P. J. (2019). Trichomonas vaginalis extracellular vesicles are internalized by host cells using proteoglycans and caveolin-dependent endocytosis. Proceedings of the National Academy of Sciences, 116(43), 21354-21360.
- da Silva, D. R., Gonzalez, C. F., & Lorca, G. (2023). Internalization of extracellular vesicles from Lactobacillus johnsonii N6. 2 elicit an RNA sensory response in human pancreatic cell lines. Journal of Extracellular Biology, 2(7), e101.

Acknowledgement

This work was supported by the National Research Foundation of Korea (NRF) grants, funded by the Korean government (MSIT) (2023R1A2C1006901).

충남대학교 의과대학 Chungnam National University

National Research Foundation of Korea

WORLD AQUACULTURE Society

AQUA 2024