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Heterogeneous Populations of Outer Membrane Vesicles Released from *Helicobacter pylori SS1* with Distinct Biological Properties

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Abstract

Gram-negative bacteria release outer membrane vesicles (OMVs) with great potential in a variety of biomedical applications. The study aimed to investigate nutritional growth supplements (Horse Blood Serum (HBS), Fetal Bovine Serum (FBS), β-Cyclodextrin (CD), and Cholesterol (Chlos)) and their impact on *Helicobacter pylori*'s (*H. pylori*'s) OMVs biological properties, which the obtained OMVs were named ExHp-HBS, ExHp-FBS, ExHp-CD and ExHp-Chlose according to the growth supplement used. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) showed a difference in protein components profile between all OMVs populations. Nuclear morphology for cells treated with 40 μg/mL of ExHp-CD displayed no change in nuclear morphology, while cells treated with ExHp-HBS, ExHp-FBS, and ExHp-Chlos showed chromatin condensation and nuclear fragmentation. Cell Counting Kit (CCK-8), lactate dehydrogenase (LDH), water-soluble tetrazolium salt (WST-1) assays showed no adverse effect for ExHp-CD in all used concentrations, while treated cells were negatively affected under higher concentrations for ExHp-HBS and ExHp-FBS (40 and 50 μg/mL) or in almost all the used concentrations of ExHp-Chlos. Obtained OMVs populations were phagocyted by macrophage RAW 264.7 cells leading to activate macrophages and produce cytokines. Interleukin 10 (IL-10) and Interleukin 4 (IL-4) were significantly ($p<0.001$) increased after stimulation with all obtained OMVs, while levels of Interferon gamma (IFN-γ) and Interleukin 12 (IL-12) were not significantly increased. All OMVs populations increased CD206 ratio over CD86 ratio, demonstrating M_2-phenotype polarization. Current findings revealed that growth supplements influenced OMVs biological properties while inducing Th_2-immune response. This knowledge could determine which type of OMVs population for the required biomedical application.

Keywords: Outer membrane vesicles, *Helicobacter pylori SS1*, Th_2-immune response, Macrophage RAW 264.7 cells, OMVs populations.

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1. Introduction

All organisms representing various life forms were found to release different extracellular vesicles naturally as part of their growth and all other physiological processes.[1,2] Both Gram-positive and Gram-negative bacteria release extracellular vesicles in a natural process necessary for interactions between inter-species and inter-kingdoms.[3] Bacterial membrane vesicles are involved in cellular crosstalk and possess unique targeting and packaging abilities and many other essential functions.[4,5] Recently, increased attention has recognized extracellular vesicles as a promising candidate for various biomedical applications such as immune modulation, drug delivery, cancer therapy, vaccine development, detoxification, phototherapy, and anti-bacterial agents.[6-14] Outer membrane vesicles (OMVs) from Gram-negative

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bacteria hold great importance. They compose of various components (Fig. 1) such as lipids, proteins (*e.g.* enzymes and structural proteins), carbohydrates, and genetic materials (*e.g.* DNA and RNAs).[15] Several factors were found to influence the production of OMVs, such as growth stage, infection settings, and biofilms.[4,16-19] These findings highlighted the crucial roles that OMVs might play in different biological processes and the different roles each OMVs population should participate in. Thus, the change of OMVs composition might be expected depending on the part that they are required to play. Moreover, the environmental conditions and its contents around the bacterial cells could influence OMVs production, such as antibiotics presence and temperature changes.[4,18,20,21] Thus, researchers should investigate further the variation of OMVs populations from a certain bacterial species and the different factors involved in this phenomenon. This could lead to finding the required OMVs population with the desired biological function. Thus, the specific required OMVs population could be used in the needed biomedical applications such as cancer immunotherapy, anti-bacteria adhesion agents, bacterial vaccine development, and drug delivery vehicles depending on the specific needed biological function.[22] Moreover, OMVs components could be analyzed and investigated if the need is to find novel components (*e.g.* antigens, functional proteins, biomarkers, enzymes, *etc*.) to be used in further applications.

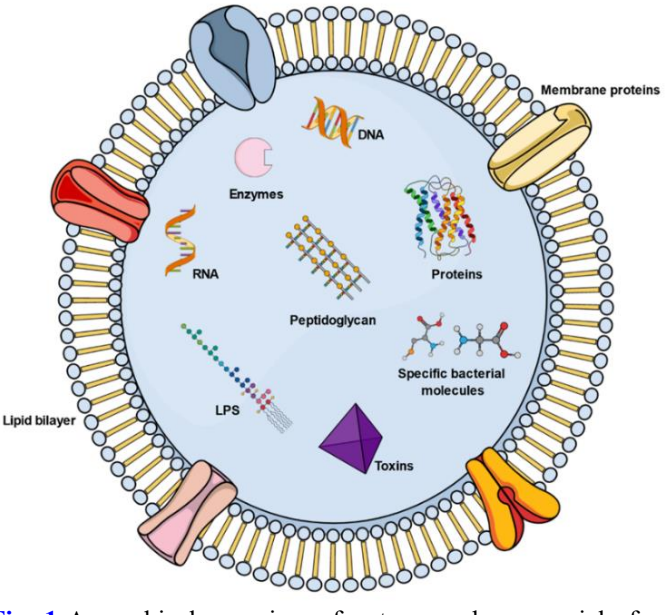

Fig. 1 A graphical overview of outer membrane vesicle from Gram-negative bacteria. OMVs are contained from a continuous lipid bilayer that is composed of a variety of cytoplasmic and membrane proteins, toxins, enzymes, nucleic acids, peptidoglycans, and biomolecules derived from their parental bacterial cell.

Helicobacter pylori (*H. pylori*), a gastric Gram-negative bacterium, is a widespread bacterium that infects half of the people worldwide, and in some countries, the infection rate reached 79% of the population.[23-25] Approximately 4.4 billion of the global population are infected with *H. pylori* worldwide.[25] *H. pylori* infection and its associated diseases remain persistent and hard to treat. This could be attributed to H. pylori resistance against antibiotics such as levofloxacin, clarithromycin, and metronidazole reaching to alarming level globally, which have a significant impact on the efficacy of *H. pylori* treatment.[26] Despite several treatment regimens that proposed (hybrid therapy, sequential therapy, concomitant therapy, probiotics supplemented triple therapy, *etc*.), the treatment choice is highly dependent on many factors such as the viability of susceptibility testing, local empiric therapy, the probiotic strain specificity, among many other factors.[23] Therefore, it is essential to find other effective measures to overcome *H. pylori* infection and its associated diseases.

H. pylori have various components with distinct biological functions. For instance, *H. pylori 's* outer membrane proteins (OMPs) own several biological functions that include maintaining the structure of the outer membrane, materials transportation, and contributing with interacting with the host.[27] OMPs expression in different strains is attributed to the *H. pylori* virulence. Moreover, OMPs pathogenicity could be achieved via the mechanisms of 1) adhesion, 2) bacterial cell-penetrating the defense barriers, and 3) immune system evasion.[28] Although some *H. pylori's* components (*e.g.* vacuolating cytotoxin A (VacA)) were found to stimulate the immune response, however, their cytotoxic effect limits their use in vaccine development.[29,30] Furthermore, they could alter host cells' functions in unfavorable ways and negatively impact their normal functions. Moreover, *H. pylori* observed to cause apoptosis in macrophages as well as epithelial cells through virulence factors/proapoptotic factors that include Lipopolysaccharides (LPS), VacA, Cytotoxin-associated gene A (CagA), Urease (Ure), γ-glutamyl transpeptidase and Fas ligand (FasL).[31-36] Besides, several outer membrane proteins of *H. pylori* were found to affect cell processes variously. For instance, LPS was observed to inhibit cell proliferation; meanwhile, glycine acid extract (GE), CagA, and UreA elevate cell proliferation.[37,38] Moreover, VacA was found to have a direct influence against T cells, epithelial cells, and phagocytes via inhibiting cell proliferation.[39-43]

Thus, the current work aimed to investigate the influence of the medium growth supplements on the variation of OMVs released from *H. pylori*. This could help in finding the specific OMVs population with the desired biological function for the required application.

2. Materials and methods

2.1 Cell culture

Macrophage RAW 264.7 cell line (RAW 264.7) (Procell life science & technology Co., Ltd., Wuhan, China) were used and primarily maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Gibco/Invitrogen, Carlsbad, CA) at 37 °C, 5% CO_2 and humid atmosphere. For several assays, serum-free-DMEM was used to avoid false results due to exosomes and proteins present in FBS.

2.2 *Helicobacter pylori* strain and growth conditions

Mouse-adapted *Helicobacter pylori* SS1 was thankfully provided by Prof. Longrui Pan, Department of Pharmacology, Hubei University of Medicine, Shiyan, China. Columbia blood agar supplemented with 10% defibrinated -whole blood from sheep, 10 µg/mL vancomycin, 2.5 µg/mL amphotericin B, 5 µg/mL trimethoprim, and 2.5 IU/mL polymyxin B. Plates inoculated with *H. pylori* were incubated for 72 h in a humidified atmosphere at 37 °C and 5% of CO_2. Brain Heart Infusion broth (BHI) supplemented with 10% FBS (Gibco/Invitrogen, Carlsbad, CA) and 10 µg/mL vancomycin were used for maintaining *H. pylori*, such as quantifying bacterial numbers and freezer stock preparations. However, for OMVs isolation, BHI broth supplemented with different nutritional supplements (β-Cyclodextrin, Cholesterol, and exosomes depleted Fetal Bovine Serum (FBS) and Horse Blood Serum (HBS)) and 10 µg/mL vancomycin were used.

2.3 OMVs isolation

Brain Heart Infusion broth (198 mL in 250 mL flask) was supplemented with 10 µg/mL vancomycin, and then different growth supplements were used separately, which were β-Cyclodextrin (1 g/L), 10% FBS, 10% HBS or Cholesterol (50 mg/L). Each supplemented BHI media was inoculated with 2 mL of *H. pylori* inoculum concentration of 1.5×10^8 CFU/mL (compared to a 0.5 McFarland turbidity standard) until the final concentration of 1.5×10^4 CFU/mL. The inoculated culture was incubated for 72 h until the OD_{600} value of the cultured medium reached approximately 1.0, which indicates reaching the logarithmic growth phase. Approximately 200 mL of *H. pylori* culture in BHI broth was collected for OMVs isolation using ExoBacteria™ OMV Isolation Kit (System Biosciences (SBI), Palo Alto, CA). Briefly, when desired OD is reached, bacterial culture was centrifuged at 5000 rpm for 20 min at 4 °C. To eliminate any cellular debris, the supernatant was transferred to a new tube and centrifuged again at 5000 rpm for 20 min at 4 °C. The supernatant was collected and filtered through a 0.45 µm filter. Then, the supernatant was filtered again using a 0.22 µm filter. OMV Binding Resin was mixed by shaking, and 1 mL of the OMV Binding Resin was added into the column. The column was equilibrated by adding the 1× Binding Buffer (10 mL), and the solution was allowed to completely flow through and further discarded. Clarified bacterial culture supernatant was added (30 mL) to the resin, and the column was capped and incubated on a rotating rack (30 min) to allow OMVs binding. The resin/supernatant mixture was allowed to flow through by uncapping the bottom and top of the column. The resin was washed with 15 mL Binding Buffer, and the flow-through was discarded. This washing step was repeated twice. After capping the bottom of the column, 1.5 mL OMV Elution Buffer was added, incubated for 2 min at room temperature with gentle shaking every 30 s. the column was uncapped at the bottom, and the eluate that contains the OMVs were collected in new microcentrifuge tubes. OMVs were washed using Amicon Ultra centrifugal Filter Device 3K MWCO. Protease inhibitor cocktail was added, and OMVs solution was aliquoted in microcentrifuge tubes (50µL) and stored at -80 °C until further use.

The protein concentration of OMVs was determined using BCA Protein Assay (Beyotime, Jiangsu, China). This was considered as the concentration of the obtained OMVs. The obtained OMVs were named ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos according to the growth supplement used.

2.4 Transmission electron microscopy (TEM)

Obtained OMVs were suspended in phosphate-buffered saline (PBS) in a concentration of 0.1 mg/mL. An amount of 10 µL of OMVs suspension solution was loaded on the grids of formvar carbon-coated 300 mesh for 30 min at 25 °C. The excess fluid was removed using filter paper by contacting filter paper lightly with the grid edge. The grids were partially covered using a Petri dish and left to dry overnight under room temperature. Finally, the air-dried grids containing OMVs were observed by TEM (HITACHI Transmission Electron Microscope, HT7700, Japan).

2.5 Dynamic light scattering (DLS)

OMVs were diluted with PBS to reach the concentration of 0.1 mg/mL. The size measurements were conducted using Zetasizer Nano Series (Malvern Instruments, Herrenberg, Germany).

2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein contents of different OMVs populations were analyzed using 12% SDS-PAGE. First, 20 µg of each OMVs population were boiled in SDS loading buffer (Beyotime, Jiangsu, China) for 5 min, and then were separated by 12% SDS-PAGE and followed by staining with Coomassie blue staining solution.

2.7 Macrophage RAW 264.7 viability assay

The viability assay was done using Cell Counting Kit (CCK-8), Dojindo Laboratories, Kumamoto, Japan. Briefly, different concentrations of each OMVs population was prepared using sterile PBS as 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL, respectively. The macrophage RAW264.7 cells were seeded in 96-well plates with a density of 3.5×10^5 cells/well and then were incubated at 37 °C under 5% CO_2 and humid atmosphere. After 24 h of incubation, cells were stimulated by replacing culture medium with fresh medium (100 µL) containing different concentrations of each OMVs population (previously mentioned) or PBS alone as controls (positive and negative). All the 96-well plates were incubated under the same previous conditions for 24 h. After the incubation period, the medium was replaced by 100 µL of fresh medium plus 10 µL of CCK-8 reagent, and plates were incubated for further 2 h. Finally, at 450 nm, the absorbance was measured using a microplate reader (Multiskan EX,

Thermo Fisher Scientific, USA). Cell viability of macrophage RAW 264.7 was calculated as the absorbance ratio of the stimulated cells to that of cells of the control groups as the following equation (I)*:

I = % Cells viability = (absorbance $_{sample}$ – absorbance $_{blank}$)/(absorbance $_{control}$ – absorbance $_{blank}$) × 100

2.8 Nuclear change of macrophage RAW 264.7 cells

Macrophage RAW264.7 cells were cultured at a density of 2.56×10^5 cells/mL in glass coverslips using a complete medium and were incubated for 4 h to allow cell attachment. After cell attachment, the medium was replaced with pre-warmed DMEM containing different OMVs populations (40 µg/mL). Cells incubated with DMEM medium only were used as controls. Afterward, coverslips were incubated at 37 ˚C, 5% CO_2, and humid atmosphere for 24 h. After incubation, media was discarded from the coverslips, and cells were washed using pre-warmed PBS three times. Cells were fixed with 4 % paraformaldehyde (PFA, v/v) for 10 min at room temperature. Three washing steps were followed using PBS for 10 min each time. Using 200 µL of DAPI solution (100 nM), cell nuclei were stained by covering the coverslips for 5 min. Cold PBS was used to wash coverslips three times. Fluorescence observation under confocal microscopy was conducted for nuclear segmentation and condensation, which was examined at 40× magnification using Olympus Ultra-Sensitive Confocal Microscope System, FV3000, Japan. One hundred cells were examined for each sample.

2.9 Hemolysis assay

One mL of fresh sheep blood (Hopebio, Qingdao, China) was washed three times by 2 mL of PBS solution. Then, the red blood cells (RBCs) pellet was dispersed in 20 mL of PBS solution. ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos were prepared using PBS (10 µg/mL). Triton × -100 and PBS were used as positive and negative controls, respectively. An amount of 60 µL of each OMVs population, Triton ×-100 or PBS, were added to 1140 µL of previously prepared RBC solution in Eppendorf tubes. Then, tubes were incubated at 37 °C and 100 rpm for 4 h. After incubation, the suspensions were centrifuged at 10000 rpm for 6 min. First, results were observed visually, then 200 µL of each supernatant was added to a 96-well plate. The absorbance was read at 550 nm using a microplate reader (MultiskanEX, Thermo Fisher Scientific, USA). The percent (%) of hemolysis was determined as the following (II):

II = Hemolysis (%) = (absorbance $_{Sample}$ – absorbance $_{PBS}$)/(absorbance $_{Triton\ ×\text{-}100}$ – absorbance $_{PBS}$) ×100.

2.10 LDH release assay

To test the cytotoxic effect of each OMVs population, lactate dehydrogenase (LDH) assay was performed according to the manufacturer's recommendations (Beyotime, Jiangsu, China). Macrophage RAW264.7 cells were seeded in 96-well plates with a density of 2.42×10^5 cells/well. After 24 h of incubation, 5, 10, 20, 30, 40, and 50 µg/mL of each OMVs population were added separately in each well set and were incubated for a further 48 h. RAW264.7 cells incubated with DMEM medium and DMEM medium alone without cells were used as positive and negative controls, respectively. An amount of 120 µL of culture supernatant from each well in each well set that belongs to each tested group was transferred into a new 96-well plate in corresponding wells. LDH released into the medium was tested by mixing the transferred culture medium with the reaction mixture (60 µL). At room temperature, the mixture was incubated in the dark for 30 min warped in aluminum foil, and placed on a horizontal shaker in low-speed shaking. Then, the absorbance was measured at 490 nm, and the LDH release was calculated.

2.11 WST-1 cell proliferation assay

The effect of different OMVs populations on metabolic activities and cell proliferation was monitored using the water-soluble tetrazolium salt (WST-1) assay of the mitochondrial dehydrogenase as instructed by the manufacturer (Beyotime, Jiangsu, China). Macrophage RAW264.7 cells were seeded in 96-well plates with a density of 3.2×10^5 cells/well. After 24 h of incubation, the medium was replaced with a DMEM medium containing 5 µg/mL, and 10 µg/mL of each OMVs population were added separately in each well set and further incubated for 24 and 48 h. RAW264.7 cells incubated without any agent and DMEM medium without cells were served as positive and negative controls, respectively. For each concentration, the experiment was repeated five times under the same conditions. After each incubation time, 10 µL of WST-1 solution (to 100 µL of culture volume) was added to each well. The 96-well plates were incubated further for 2 h in the dark at 37 ˚C and 5% CO_2. Then, the 96-well plates were placed on a shaker for 1 min to mix the system. A microplate (ELISA) reader was then used to measure the absorbance at 450 nm for samples and at 650 nm for reference, and the percentage of cell proliferation was calculated.

2.12 OMVs uptake by macrophage RAW 264.7 cells

OMVs labeling was done by using fluorescent dye 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, Beyotime, Jiangsu, China). Briefly, OMVs solution was prepared by suspending OMVs into sterile PBS to prepare the final concentration of 100 µg/mL. One mL of each OMVs population solution was incubated with 6 µL of DiO stock solution (10 µM) in the dark at 37 ˚C for 1 h with gentle agitation. The extra unbound dye was removed by using Nanosep centrifugal device MWCO of 3 K (Pall life sciences, Ann Arbor, MI 48103-9019 USA), and labeled OMVs were washed three times using sterile PBS. DiO-labeled OMVs were resuspended using DMEM. Aliquots of labeled OMVs were stored at -80 °C until further use.

RAW264.7 cells were cultured in glass coverslips at 4.3×10^5 cells/mL with complete medium and incubated at 37 ˚C, 5% CO_2, and humid atmosphere for 4 h to allow cell

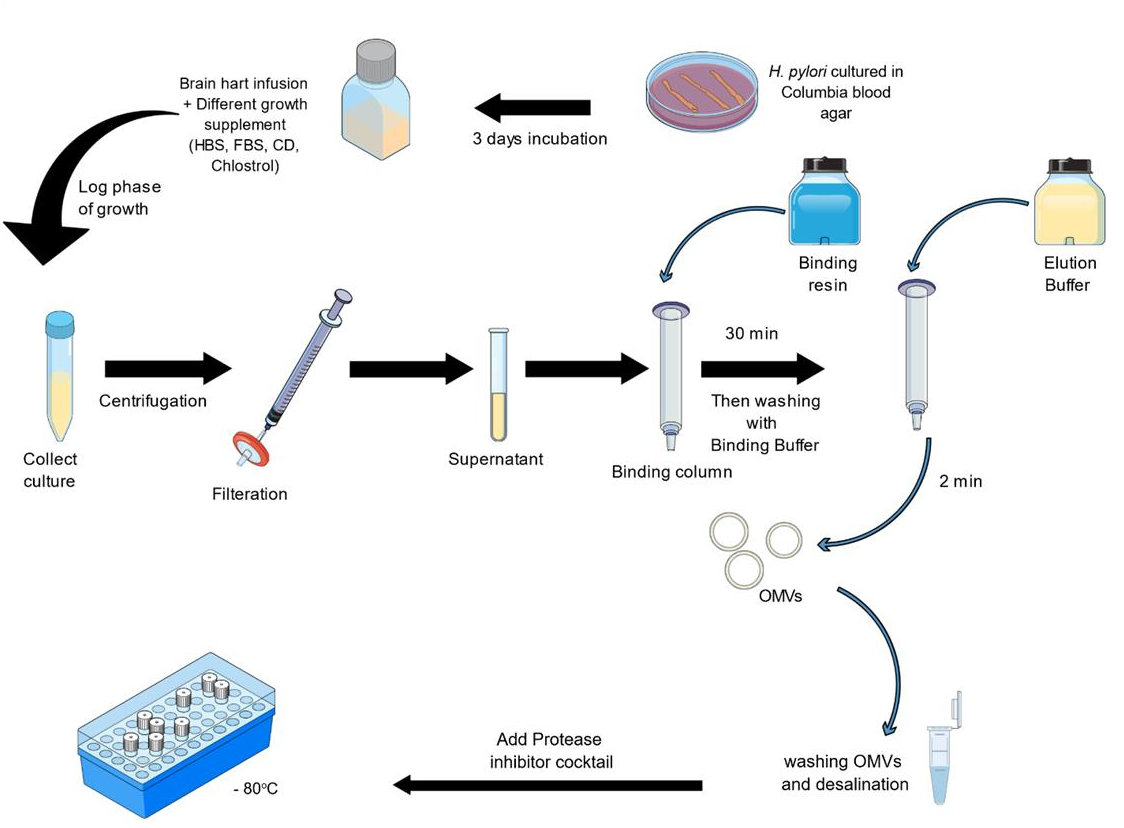

Fig. 2 Schematic illustration of OMVs isolation and preparation.

attachment. Then, the medium was replaced with pre-warmed DMEM containing 10 μg/mL of each DiO-labeled OMVs population, and then each glass coverslip was incubated further 24 h to allow OMVs uptake. Cells without the addition of DiO-labeled OMVs were used as controls. After incubation, cells were washed three times with PBS. Then, using 4% paraformaldehyde (PFA, v/v) the cells were fixed for 20 min followed by three times washing steps using PBS. To stain cell nuclei, 2-(4-Amidinophenyl)-6-indolecarbamidin dihydrochloride (DAPI, Beyotime, Jiangsu, China) was used. DAPI staining solution was added to cover the sample and left at room temperature for 3 ~ 5 min. DAPI staining solution was removed and washed three times with cold PBS. Finally, the cells were viewed using a confocal laser scanning microscope (Olympus Ultra-Sensitive Confocal Microscope System, FV3000, Japan).

2.13 Cytokines assay of macrophage RAW 264.7 cells

The macrophage RAW264.7 cells were seeded in 24-well plates with a density of 4.5×10^5 cells/well, and then were incubated for 24 h at 37 °C under 5% CO_2 and humid atmosphere. Then, the medium was replaced with a fresh serum-free DMEM medium that contains 10 μg/mL of each OMVs population. A fresh serum-free DMEM medium was used to avoid any cytokines production due to exosomes and proteins present in the Fetal Bovine Serum. A serum-free DMEM medium was added to control wells. After 24 h incubation, the cell-free supernatants from all groups were collected and analyzed for IFN-gamma (IFN-γ), interleukin-12 (IL-12), interleukin-4 (IL-4), and interleukin-10 (IL-10). The assays were determined by specific colorimetric sandwich ELISA assay kits according to the manufacturer's directions (Proteintech, Rosemont, IL 60018, USA).

2.14 Differentiation of macrophage RAW 264.7 cells

Immunofluorescence staining assay for stimulated macrophage RAW 264.7 cells was applied to evaluate macrophage polarization. Cells were seeded on glass coverslips at a density of 2.5×10^5 cells/mL, and then they were incubated at 37 ˚C, 5% CO_2, and humid atmosphere for 4 h. Following cell attachment, the cells were stimulated with 10 μg/mL of each OMVs population prepared in serum-free DMEM medium and further incubated under the same conditions for 24 h. Macrophage RAW 264.7 cells incubated only with serum-free DMEM medium were used as controls. The culture medium was discarded, and cells were washed with pre-warmed PBS three times. Cells were fixed with 4% PFA for 20 min at 4 ˚C. After fixation, cells were punched with 0.2% Triton ×-100-PBS for 5 min. Then, cells were blocked with 3% Bovine Serum Albumin (BSA)-PBS for 1 h at room temperature. Then, RAW 264.7 cells were incubated with 50 μL of the specific primary antibodies on the coverslips for 1 h at room temperature in a moist container. The primary antibodies against the M1 marker CD86 (1:100, CD86 polyclonal antibody, Proteintech, Rosemont, IL 60018, USA) and the M2 marker CD206 (1:100, CD206 polyclonal antibody, Proteintech, Rosemont, IL 60018, USA) were used. After 1 h incubation, coverslips were washed three times using

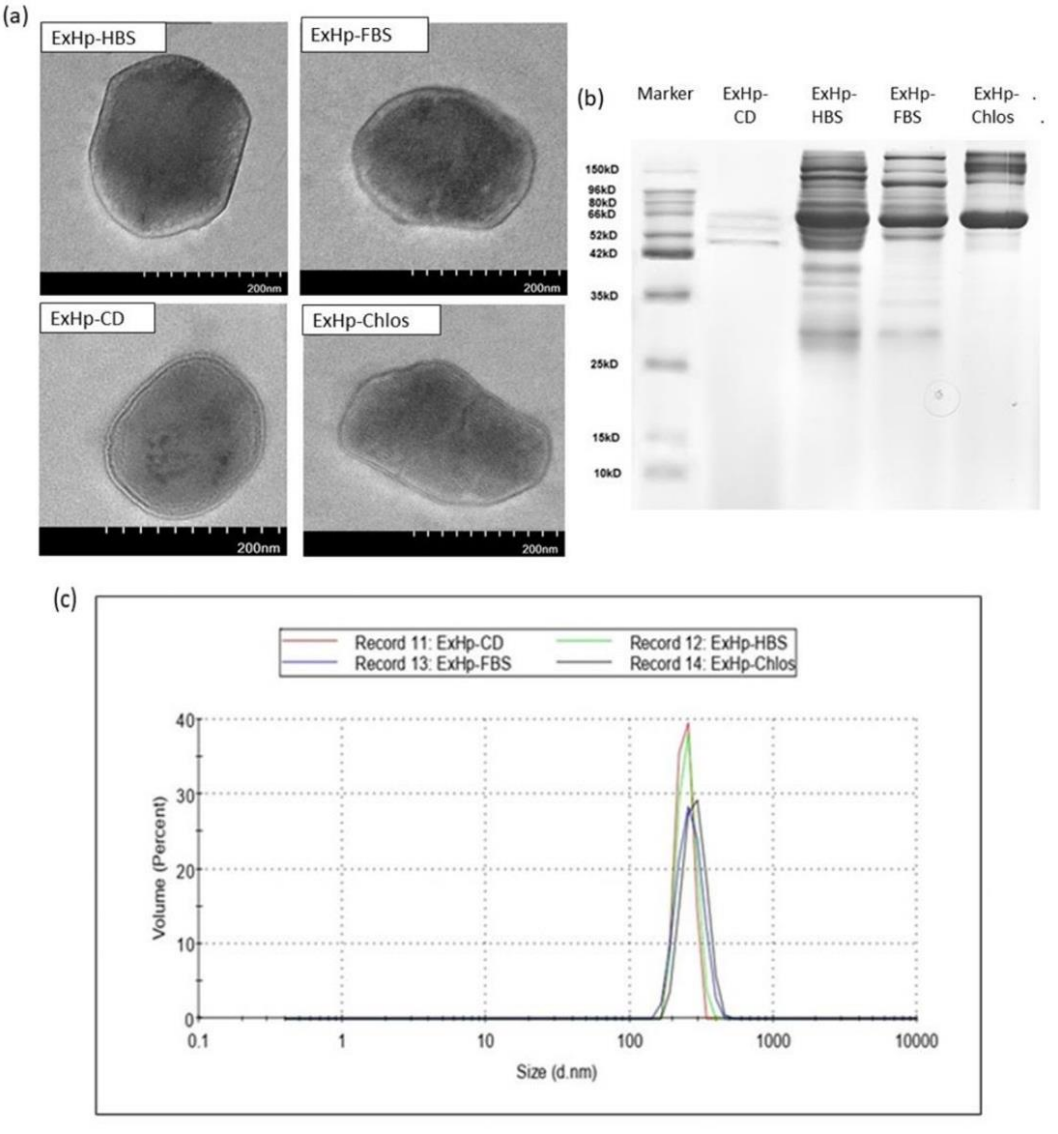

Fig. 3 Characterization of the different populations of OMVs. (a) TEM micrograph of the isolated OMVs revealed the spherical shape of OMVs with intact continuous bilayer membrane, Scale bar = 200 nm. (b) ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos proteins profile separated by SDS-PAGE 12% gel and stained with Coomassie blue (c) DLS analysis of the isolated OMVs.

PBS, followed by adding CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (50 μL, 1:100, Proteintech, Rosemont, IL 60018, USA) on each coverslip and incubation at room temperature in a dark-moist container for 1 h. Then, three times washing steps were done using PBS for 5 min each, followed by nucleus staining with DAPI (100 nM) for 5 min. Cold PBS was used to wash coverslips three times, and then coverslips were mounted in 15% glycerol. Fluorescence observation under confocal microscopy was conducted, and five different fields were randomly selected from each sample, and then DAPI, CD86, and CD206 were observed and analyzed.

2.15 Statistical analysis

Data were analyzed and presented using OriginPro 2018 software (Origin Lab, Northampton, MA 01060, USA). All data obtained were expressed as the mean ± standard deviation (SD) of at least five experiments. P-values of 0.05 or less carried out using a *t*-test were considered statistically significant.

3. Results and discussion

3.1 Identification and characterization of OMVs populations derived from *H. pylori*

Despite the importance of OMVs released as part of the normal growth of Gram-negative bacteria, the effect of the nutritional contents in the cultured medium on the OMVs production is not explored. In the current study, the impact of growth supplements on OMVs released from *H. pylori* SS was examined. All obtained OMVs were isolated, washed, and then stored for further evaluation (Fig. 2). Four OMVs populations were obtained from H. pylori SS in BHI medium supplemented with Horse Blood Serum (ExHp-HBS), FBS (ExHp-FBS), β-Cyclodextrin (ExHp-CD), or Cholesterol

(ExHp-Chlos). DLS analysis (Fig. 3c) showed the size of OMVs populations peaked at a mean diameter of 250 nm. TEM results showed that all obtained OMVs populations were observed to have intact continuous bilayer membrane and were spherical in shape Fig. 3a. There was no bacterial debris present in the micrograph, confirming the purity of the isolated OMVs (Fig. 3a). These results are confirming the findings of previous reports stating that OMVs derived from Gram-negative bacteria to be spherical bilayer membrane vesicles ranging from 20 to 500 nm.[44-48]

To explore the difference between the obtained OMVs populations, SDS-PAGE analysis was performed using 12% SDS-PAGE. Results showed a difference in protein components between all OMVs populations (Fig. 3b). The data revealed that all four OMVs populations contained various protein bands that appeared between 150 and 25 kDa.

3.2 Evaluation of RAW 264.7 viability towards OMVs populations

To determine the compatibility of OMVs populations, the effect of OMVs on the viability of macrophage RAW 264.7 cells were evaluated using CCK-8. As indicated in Fig. 4a, the ExHp-CD didn't affect the viability of the macrophage RAW 264.7 cells in all the tested concentrations (5 - 50 µg/mL) and the viability of cells was significantly increased compared to the control groups (5 µg/mL= 118.7 ± 8 %, 10 µg/mL= 125.7 ± 3.6 %, 20 µg/mL= 132.3 ± 7.7 %, 30 µg/mL = 133 ± 10.05 %, 40 µg/mL= 128.4 ± 2.7 % and 50 µg/mL= 126.8 ± 5.07 %). However, for ExHp-Chlos, results showed that the viability of cells was significantly reduced from concentrations ≥10 µg/mL (5 µg/mL= 100.2 ± 4.2 %, 10 µg/mL= 94.5 ± 4.8 %, 20 µg/mL= 69.7 ± 6.1%, 30 µg/mL = 60.4 ± 4.9 %, 40 µg/mL= 58.3 ± 3.2 % and 50 µg/mL= 56.9 ± 3.26 %) compared to untreated cells ($p<0.05$) suggesting major toxic effect on cell viability in these concentrations. Meanwhile for ExHp-HBS and ExHp-FBS, cell viability was decreased only in the higher concentrations (40 µg/mL and 50 µg/mL) compared to lower concentrations (5 to 30 µg/mL). This was confirmed via other experiments which were done in this study and was discussed in the discussion section.

Various biomedical applications require the used agent to be biocompatible and safe for clinical use. Although some bacterial virulence factors were used in biomedical applications, such as regulating immune response, several limitations have been raised against their cytotoxic effect, which hinders their use in clinical applications.[29] Thus, the biocompatibility of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos were evaluated. Cell viability was measured by testing the impact of the obtained OMVs populations on macrophage RAW 264.7 cells. Among all OMVs populations, ExHp-CD was found to be the most biocompatible population as the viability of cells was increased compared to other OMVs populations as well as the control groups in all the tested concentrations (5 - 50 µg/mL). Moreover, the rate of cell viability was increased significantly, which is an indication of the increase in cell numbers (Fig. 4a). This was confirmed by WST-1 cell proliferation assay, which was found that cell proliferation was increased (Fig. 6).

Results showed that ExHp-HBS and ExHp-FBS were biocompatible in lower concentrations (5 - 30 µg/mL), while higher concentrations were found to negatively affected the cell viability. This could suggest that these two populations could contain OMVs components that have cytotoxic effects under higher concentrations. Moreover, they could be considered to be used in lower concentrations. On the other hand, a significant cytotoxic effect on cell viability was observed when macrophage RAW 264.7 was treated with ExHp-Chlos in all used concentrations except the lowest concentration 5 µg/mL. This is an indication of the high cytotoxic effect of ExHp-Chlos that affected cell viability. This could be attributed to the composition of ExHp-Chlos that might contain cytotoxic components, which affected the cell viability even in lower concentrations.

SDS-PAGE revealed that protein contents differed in all obtained four populations (Fig. 3b). This suggests that the difference in OMVs components is responsible for the difference in the cytotoxic effect of isolated OMVs populations on macrophage RAW 264.7 cells. Thus, ExHp-HBS, ExHp-FBS, and ExHp-Chlos might compose biomolecules and virulence factors that can compromise various physiological functions, leading to negatively impacting the cells. Several *H. pylori*'s components were found to affect the host cells, such as macrophages and epithelial cells. These components include urease, VacA, lipopolysaccharide (LPS), CagA, and γ-glutamyl transpeptidase.[31-36] Thus, depending on the OMVs population composition, different effect on the cell is expected.

OMVs released from *A. nosocomialis* were found to reduce the viability of HEp-2 cells in concentrations higher than 20 µg/mL, which suggested the reduction of cell viability was caused by OMVs delivering cytotoxic factors.[49] Bacterial toxins such as cytolethal distending toxin (CDT), vacuolating cytotoxin A (VacA), ClyA cytotoxin, necrotizing factor type 1 (CNF1), and α-hemolysin, which are associated with several pathogenic Gram-negative bacteria are secreted by OMVs and were reported to negatively affect cell viability.[50-54] Moreover, multiple virulence factors could be delivered into the host cells all at the same time through OMVs to cause a simultaneous reduction in cell viability. For example, virulence factors, such as alkaline phosphatase, β-lactamase, hemolytic phospholipase C, as well as Cif were found to be delivered directly inside the host cytoplasm by OMVs, and then they were distributed rapidly to different subcellular sites leading to affect cell viability.[55]

Red blood cells could be negatively influenced by pathogenic bacteria and their related toxins. Various bacterial toxins and virulence factors can damage RBCs which cause intravascular hemolysis or could result in accelerating the damage of RBCs by the spleen and liver.[56,57] For instance, *Clostridium perfringens* α-toxin induces hemolysis in the

RBCs as a result of its lipolytic enzymatic activities (sphingomyelinase and phospholipase C). This leads to hydrolyze unsaturated phosphatidylcholine and sphingomyelin to toxic compounds, which damage the membranes of the RBCs causing intravascular hemolysis.[56] In addition, bacterial toxins can change the morphology and physical properties of the RBCs membranes resulting in impairing erythropoiesis and inhibiting erythroid differentiation as well as RBCs production.[58] The membrane of the RBCs is a target for a variety of pore-forming cytolysins produced by bacteria.[59] Moreover, *H. pylori* reported to possesses a few hemolytic proteins, and at least two putative hemolysin genes (HP1086 and HP1490) were sequenced in the genome of H. pylori 26695.[60,61] In the current investigation, Fig. 4b showed that there was no sign of hemolysis for all OMVs populations in the used concentration. These results are suggesting that ExHp-HBS, ExHp-FBS, Ex-Hp-CD, and ExHp-Chlos are hemocompatible in the used concentration.

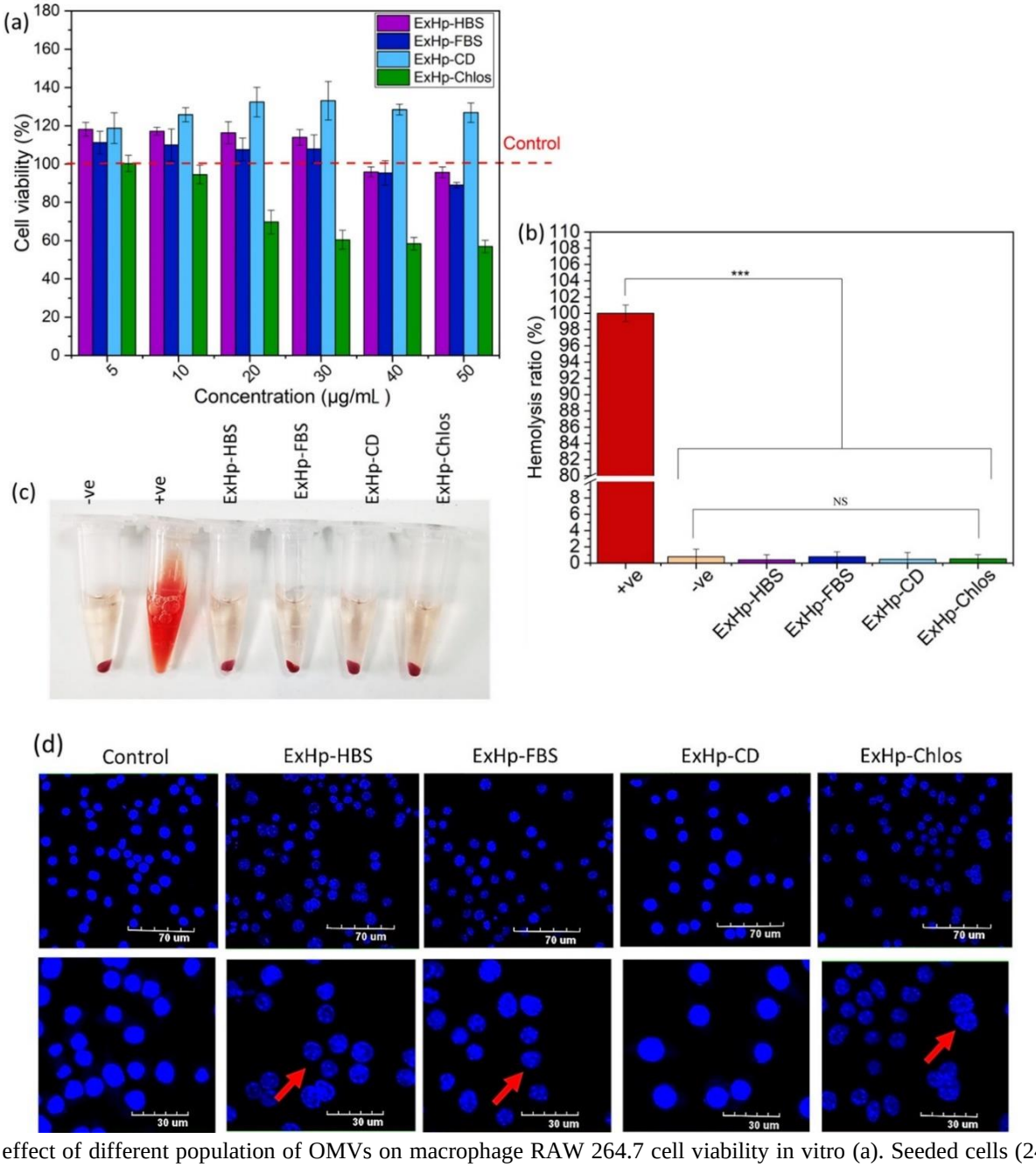

Fig. 4 The effect of different population of OMVs on macrophage RAW 264.7 cell viability in vitro (a). Seeded cells (24 h) were cultured with OMVs (5 - 50 µg/mL) for 24 h. (b) Hemolysis (%) assay of red blood cells (RBCs) treated with different populations of OMVs incubated for 4 h. Photo image of Eppendorf tubes showing the results of Hemolysis assay and confirmed by measuring the absorbance at 550 nm (c). (d) Nuclear change of macrophage RAW 264.7 cells treated with 40 µg/mL of OMVs populations for 24 h in comparison to control group cells. Chromatin condensation and nuclear fragmentation are indicated by the red arrows. (***) $P < 0.001$; (NS) Not significantly different (n=5).

In conclusion, OMVs contents should be highly considered as they could contain multiple virulence factors and biomolecules that simultaneously affect the viability of the host cells. Our data revealed that the same bacterial strain (*H. pylori*) that was stimulated with different growth supplements produced different OMVs populations. These different OMVs populations contained different compositions, which lead to different biological effects on the host cell (Fig. 3b & Figs. 4a & c). Moreover, the OMVs concentration used for stimulating the host cells should be determined in accordance with the amount of OMVs that could be applicable in the needed application but still can guarantee a low cytotoxicity rate. In this way, the full potential of OMVs could be achieved.

3.3 Effect of OMVs populations on the change in nuclear morphology

Fluorescence microscopy photos revealed the nuclear morphology changes, which were verified using DAPI staining on RAW 264.7 cells stimulated with 40 μg/mL of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos. Fig. 4c shows chromatin condensation and nuclear fragmentation in RAW 264.7 cells treated with 40 μg/mL of ExHp-HBS, ExHp-FBS, and ExHp-Chlos, which is a hallmark for apoptosis. On the other hand, nuclear morphology for RAW 264.7 cells with 40 μg/mL treated of ExHp-CD displayed the same nuclear morphology for those in untreated RAW 264.7 cells (control groups), which showed no sign of chromatin condensation and nuclear fragmentation. This demonstrates that ExHp-HBS, ExHp-FBS, and ExHp-Chlos induced apoptosis which is hallmarked by chromatin condensation and nuclear fragmentation. Meanwhile, ExHp-CD maintained to keep the cell nuclei unaffected, thus a sign of the biocompatibility of ExHp-CD under the used concentration. Comparatively, all these results suggesting better biocompatibility of ExHp-CD compared to all other OMVs populations. Despite all the potential that OMVs could offer, some OMVs that contain virulence factors could affect the host cells negatively by causing apoptosis.[62,63] For instance, OMVs from *E. coli* O104:H4 strain reported carrying virulence factors that caused apoptosis in the epithelial cells in the human intestine.[63] These virulence factors could directly target the nucleus of the host cells resulting in inducing cytotoxicity and causing apoptosis.[64] Similarly, *N. gonorrhoeae* produced OMVs that target PorB into the mitochondria leading to inducing apoptosis.[65] These findings clearly show that OMVs contained virulence factors have the potential to cause cell apoptosis in the host cells and change the nuclear morphology, while OMVs that lack those virulence factors could exhibit a better biocompatible effect. In the current work, four OMVs populations were isolated. Among these OMVs populations,

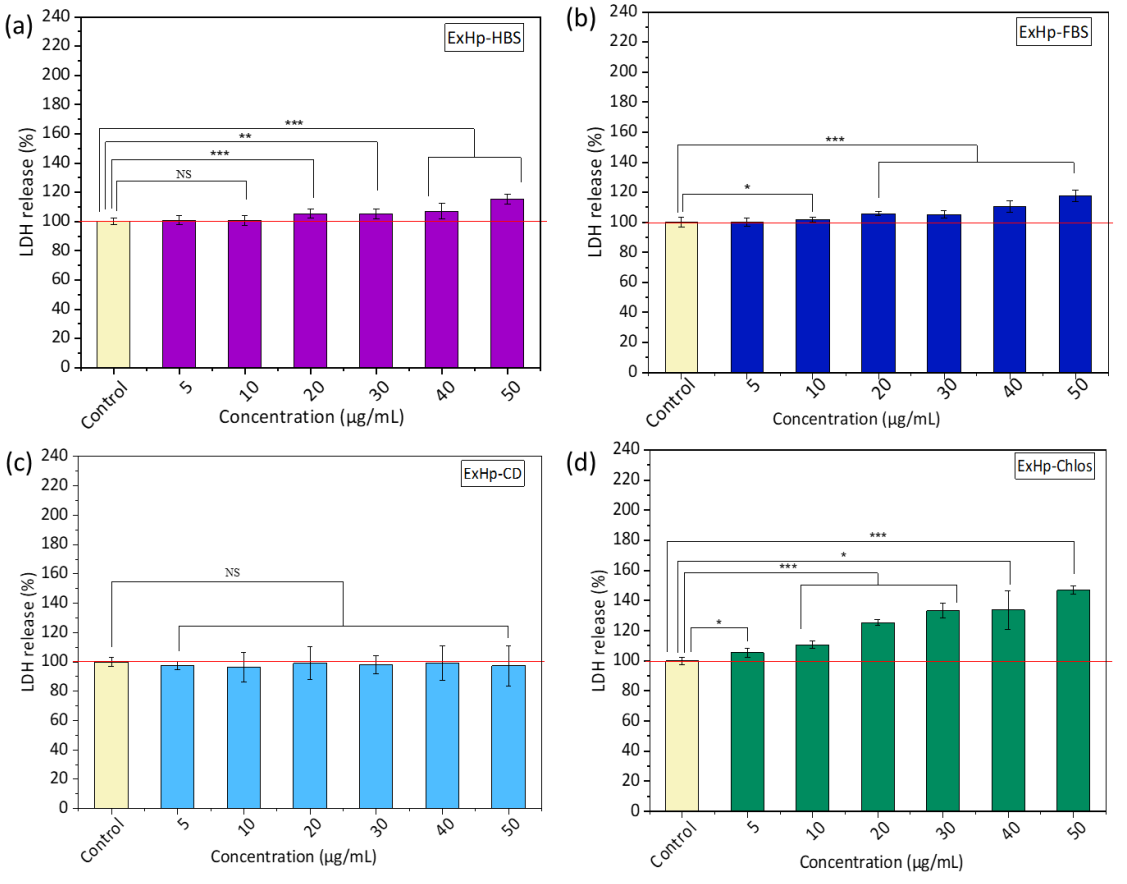

Fig. 5 LDH release assay for macrophage RAW 264.7 cells treated with 5 to 50 μg/mL of (a) ExHp-HBS, (b) ExHp-FBS, (c) ExHp-CD and (d) ExHp-Chlos for 48 h compared to untreated cells. (***) $P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$; (NS) Not significantly different.

three (ExHp-HBS, ExHp-FBS, and ExHp-Chlos) exhibited nuclear morphology in macrophage RAW 264.7 cells which could be attributed to their contents, while ExHp-CD didn't cause any chromatin condensation and nuclear fragmentation (Fig 3b & Fig. 4c).

3.4 Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) assay was performed to test the cytotoxic effect of ExHp-HBS, ExHp-FBS, ExHp-CD and ExHp-Chlos (5 to 50 µg/mL) on RAW264.7 cells from culture supernatants after incubation for 48 h. As shown in Fig. 5, increased levels of LDH were observed for RAW264.7 cells stimulated with ExHp-Chlos from 5 µg/mL to 50 µg/mL concentrations, which the increase in LDH levels were steadily increased with the increase of OMVs concentration. Meanwhile, for macrophages RAW264.7 cells stimulated with ExHp-CD results were in line with those of control groups indicating no cytotoxic effect that leads to an increase in LDH levels. On the other hand, LDH release from macrophages RAW264.7 cells stimulated with ExHp-HBS and ExHp-FBS were found to increase in the highest concentrations as it was 110.6 ± 3.8 % for 40 µg/mL and 117.8 ± 3.7 % for 50 µg/mL; meanwhile, it was 107.1 ± 5.5 % for 40 µg/mL, and 115.5 ± 3.36 % for 50 µg/mL for macrophages RAW264.7 cells stimulated with ExHp-HBS.

Lactate dehydrogenase (LDH), which is a normal living cell endoenzyme, and its release in cell culture supernatant reflects the compromised cell membrane integrity. Results showed that there was no significant change in LDH levels in all groups treated with ExHp-CD (5 to 50 µg/mL) and the control groups Fig. 5c. This is evidence that ExHp-CD in all used concentrations has no cytotoxic effect that leads to disturbing the cell integrity and release of intracellular contents. This was not observed with other OMVs populations, in which LDH levels were increased either only in higher concentrations (40 and 50 µg/mL for ExHp-HBS and ExHp-FBS) or in almost all the used concentrations (ExHp-Chlos), suggesting destruction in cell membrane integrity and release of the intracellular contents. These results are in line with the results from the cell viability test using CCK-8 (Fig. 4a). Previous studies reported that OMVs could induce cytotoxicity in the host cells, and that was attributed to the presence of cytotoxic components in the OMVs.[49,66,67] These cytotoxic components could affect cell membrane integrity leading to the release of the intracellular contents. For instance, OMVs from *E. coli* contained necrotizing factor type 1 (CNF1) that is a well-known toxin that could decrease the fluidity of the polymorphonuclear leukocytes membrane (PMNs).[52] Various virulence-associated proteins were found in OMVs released from *Acinetobacter nosocomialis* that exhibited cytotoxic effects against HEp-2 cells.[49] Similarly, the cytotoxicity of OMVs secreted from *Acinetobacter baumannii* against macrophages was linked to the cytotoxic impact of the outer membrane protein A that was packaged inside the obtained OMVs.[67] With regards to our current results, the cytotoxic effect of the highest concentrations of ExHp-HBS and ExHp-FBS (40 and 50 µg/mL) compared to the lower concentrations (5 to 30 µg/mL) were in agreement with a previous study that found OMVs from A. baumannii did not show any cytotoxic effect on the concentrations ≤ 15 µg/mL, while concentrations ≥ 20 µg/mL induced cytotoxic effect, moreover, cellular shrinkage, cell detachment and rounding of cells were observed.[66] This was speculated by the ability of OMVs to deliver bacterial effectors and cytotoxic biomolecules into the host cells, which lead to an increase in their cytotoxic effects with the increase of the concentrations of OMVs and their cytotoxic cargo. However, this cytotoxic effect was intense in the case of macrophages treated with ExHp-Chlos, which suggests a more cytotoxic effect of the OMVs and their cargo. Thus, this leads to conclude that ExHp-CD its cargo is the most biocompatible OMVs among all obtained OMVs populations in the current study.

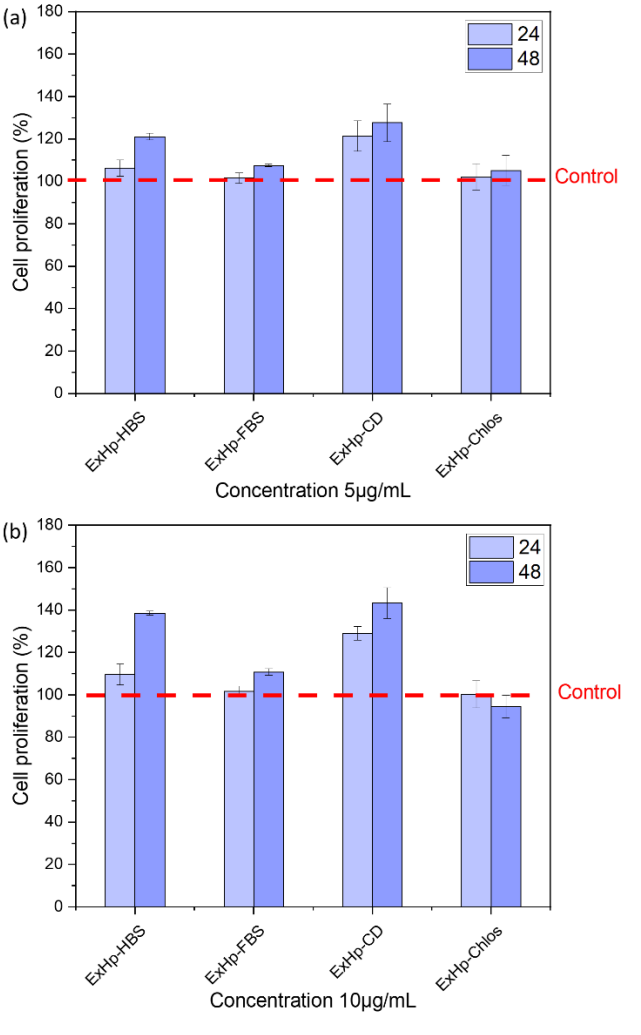

Fig. 6 WST-1 Cell Proliferation Assay for macrophage RAW 264.7 cells treated with 5 (a) and 10 (b) µg/mL of OMVs populations for 24 and 48 h. The WST-1 assay utilizes a cellular metabolism marker for cellular metabolic activity, which is an indication of an increase in cell proliferation. WST-1 assay was compared amongst treated and control groups. Values are means ± SD (n = 5).

3.5 The effect of OMVs populations on macrophage RAW 264.7 proliferation

The effect of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos (5 and 10 μg/mL) on the proliferation of RAW 264.7 cells was determined using WST-1 assay after 24 and 48 h incubation. Results showed that ExHp-HBS and ExHp-CD increased cell proliferation rate significantly compared to the control groups (Fig. 6). These results are an indication of the increase in cellular metabolic activity due to the increase in cell numbers. On the other hand, the cell proliferation rate was moderately increased when stimulating macrophage RAW 264.7 with ExHp-FBS compared to ExHp-HBS and ExHp-CD. On the contrary, cell proliferation was negatively affected and decreased when stimulating macrophage RAW 264.7 with 10 μg/mL of ExHp-Chlos after 48 h (Fig. 6). These results are in agreement with OMVs obtained from *A. baumannii* that affected cell proliferation negatively in concentrations higher than 20 μg/mL, which was evaluated by using WST-1 assay.[66] *H. pylori* have been associated with over-expression of the cycle-regulators of the host cells, which resulted in elevated levels of cell proliferation.[68] However, the various *H. pylori*'s components were found to differently affect cell proliferation. When the host cell stimulated with different *H. pylori*'s components some components could affect cell proliferation negatively while others could induce hyper cell proliferation. For example, LPS were found to affect cell proliferation negatively that leads to inhibit cell proliferation, meanwhile surface antigens (GE), CagA and UreA exhibited the ability to induce high levels of cell proliferation.[37,38] Moreover, VacA has a direct impact against T cells that inhibit its proliferation. This also was observed against other cell types like epithelial cells and phagocytes.[39-43] Our findings are constant with these reports as the different contents of ExHp-HBS, ExHp-FBS, ExHp-CD and ExHp-Chlos (Fig. 6) leads to different impact on cell proliferation. Thus, ExHp-CD that induced high levels of cell proliferation and better impact on macrophage RAW 264.7 deserves to be further investigated.

3.6 Uptake of OMVs populations by RAW 264.7 cells

The uptake of foreign antigens by macrophage cells leads to activate the macrophages and induce the immune response. Because OMVs contain several bacterial-derived antigens, so they have the ability to be taken up by macrophage cells and therefore induce immune response.[69,70] Thus, to determine whether obtained OMVs populations can be taken up by macrophage RAW 264.7 cells, ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos were prelabeled using DiO and then were incubated with RAW 264.7 cells for 24 h. After fixation and cell nuclei staining, cells were viewed using a confocal laser scanning microscope. In Fig. 7, the green fluorescence that was viewed in cytoplasms' represents the prelabeled-DiO-OMVs populations, which is an indication of the huge number of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos that were taken up by RAW 264.7 cells.

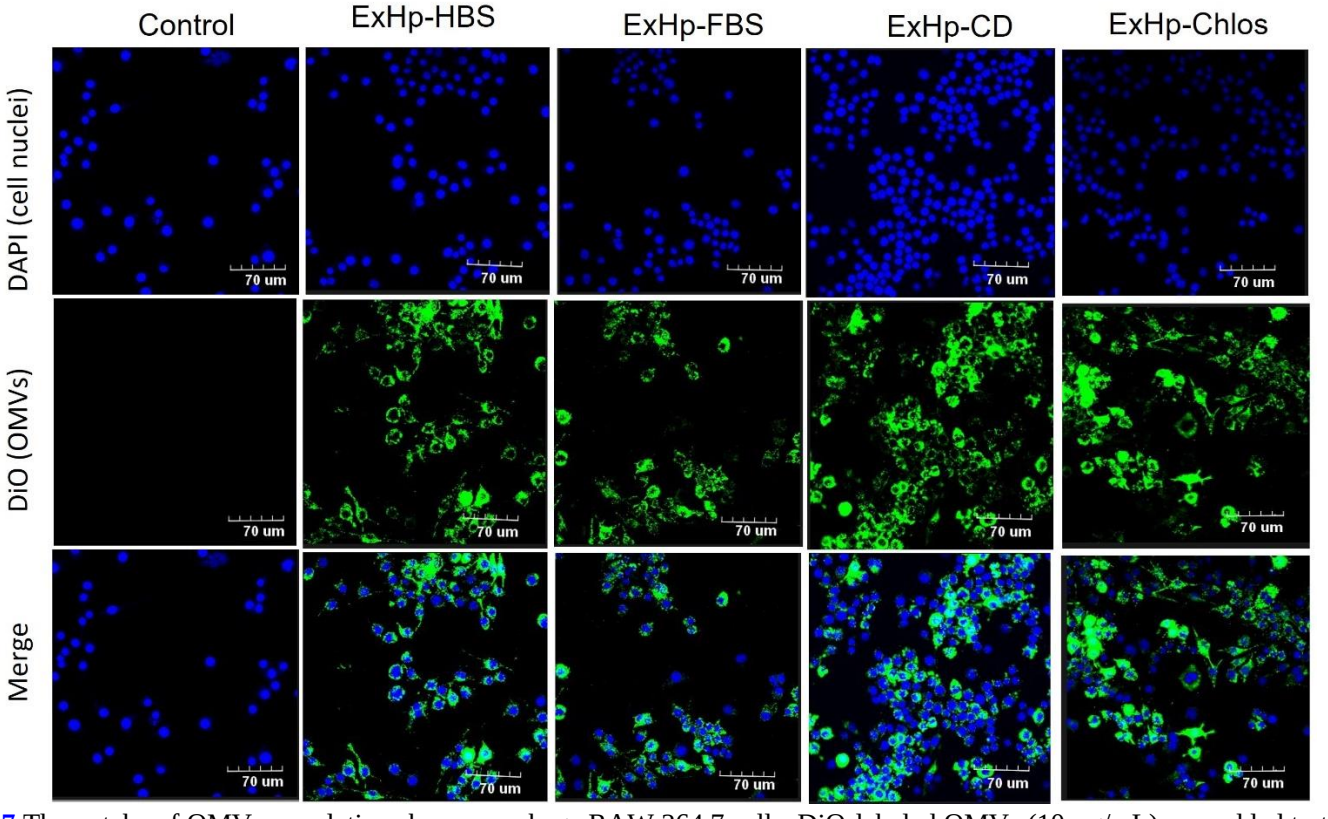

Fig. 7 The uptake of OMVs populations by macrophage RAW 264.7 cells. DiO-labeled OMVs (10 μg/mL) was added to the macrophage RAW 264.7 cells. Untreated cells were used as controls. Fluorescence microscopy photos showing green for OMVs (DiO) and blue for cell nuclei (DAPI).

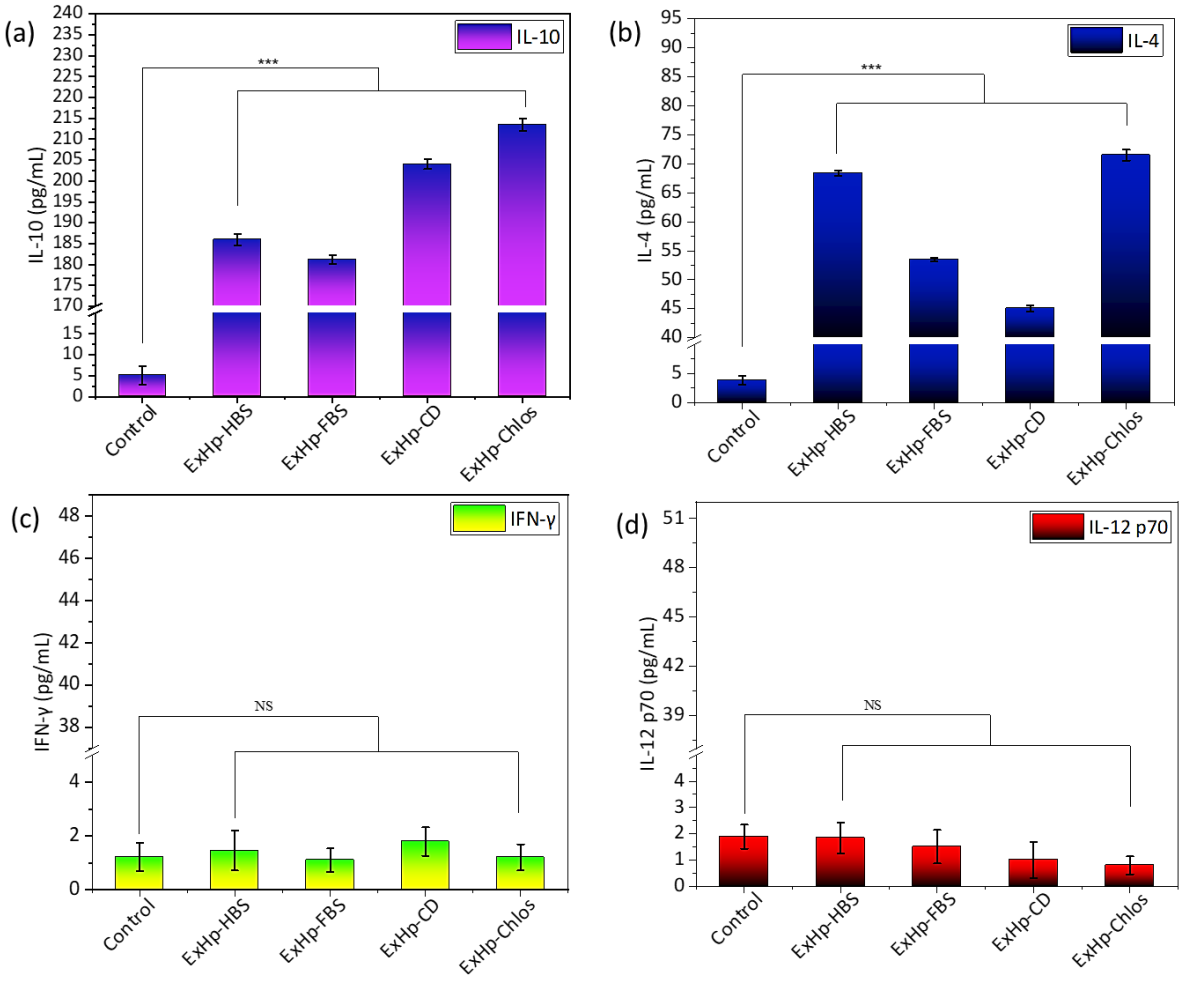

Fig. 8 Macrophage RAW 264.7 cytokines production by stimulation with OMVs (10 µg/mL) compared to control. RAW 264.7 cells supernatants were analyzed by specific colorimetric sandwich ELISA for (a) IL-10, (b) IL-4, (c) IFN-γ, and (d) IL-12 p70 after 24 h stimulation. The mean ± SD of five experiments is shown. (***) $P < 0.001$; (NS) Not significantly different.

Bacterial OMVs act as external foreign bodies to the host cells. This is attributed to their nature that composed of various components from their parental cell. Macrophages are classical immune cell types that induce potent immune responses once stimulated with OMVs. OMVs were found to be phagocyted by macrophages leading to activate macrophages and produce cytokines.[69,71] Moreover, OMVs phagocyted by macrophages were found to elect several immune responses.[72-74] Our current work revealed that ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos were phagocyted by macrophage RAW 264.7 cells (Fig. 7). Thus, they have the potential to induce an immune response.

3.7 Effect of OMVs populations on cytokines production

Cytokines production variations during ExHp-HBS, ExHp-FBS, ExHp-CD and ExHp-Chlos stimulation were examined using specific colorimetric sandwich ELISA to analyze IFN-γ, IL-12, IL-4, and IL-10 production levels. As shown in Figs. 8 a & b, the production of IL-10 and IL-4 were significantly ($p<0.001$) increased after stimulation with 10 µg/mL of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos compared to control. On the other hand, levels of IFN-γ and IL-12 were not significantly increased (Figs. 8 c & d).

Macrophages treated with OMVs from *P. gingivalis* were found to produce high levels of IFNβ, TNFα, IL-10, IL-12p70, and IL-6.[75] Moreover, OMVs isolated from *Legionella pneumophila* (*L. pneumophila*) have elected a pro-inflammatory response in macrophages.[76] Similar observations were found in the current work as the results showed the ability of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos to produce IL-4 and IL-10 that represents the election of anti-inflammatory response. These data suggested that ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos enforced macrophages to produce anti-inflammatory cytokines. Thus, all isolated OMVs populations evoked immune response in macrophage RAW 264.7 cells.

3.8 Differentiation of macrophage RAW 264.7 cells

Immunofluorescence staining was done to detect CD86 (M1 cells marker), and CD206 (M2 cells marker) for macrophage RAW 264.7 cells stimulated with 10 µg/mL of ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos. Compared to the control group, the groups stimulated with 10 µg/mL of all OMVs populations showed an increased ratio of CD206 and a

decreased ratio of CD86 (Fig. 9). The current findings demonstrated that stimulating macrophages with ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos had resulted in the polarization of M2-phenotype.

During infection, macrophages begin a transitional activation state between M1 and M2 canonical states. The activation of macrophages could establish an activated pro-inflammatory state known as M1 type and can be characterized by CD86. Meanwhile, M2-activated macrophages establish anti-inflammatory activation state that can be characterized by CD206.[71] The current results showed macrophage RAW 264.7 cells stimulated with ExHp-HBS, ExHp-FBS, ExHp-CD, and ExHp-Chlos exhibited polarization of macrophages into M2-phenotype, which confirmed the induction of a dominant Th2-immune response in the stimulated macrophages on account that IL-10 and IL-4 are related to type Th2-immune response (Figs. 8 a & b and Fig. 9).[77,78] Similar findings were reported when the response of macrophages towards OMVs derived from *Legionella pneumophila* was evaluated, which the expression level of CD206 (M2 marker) was increased.[71]

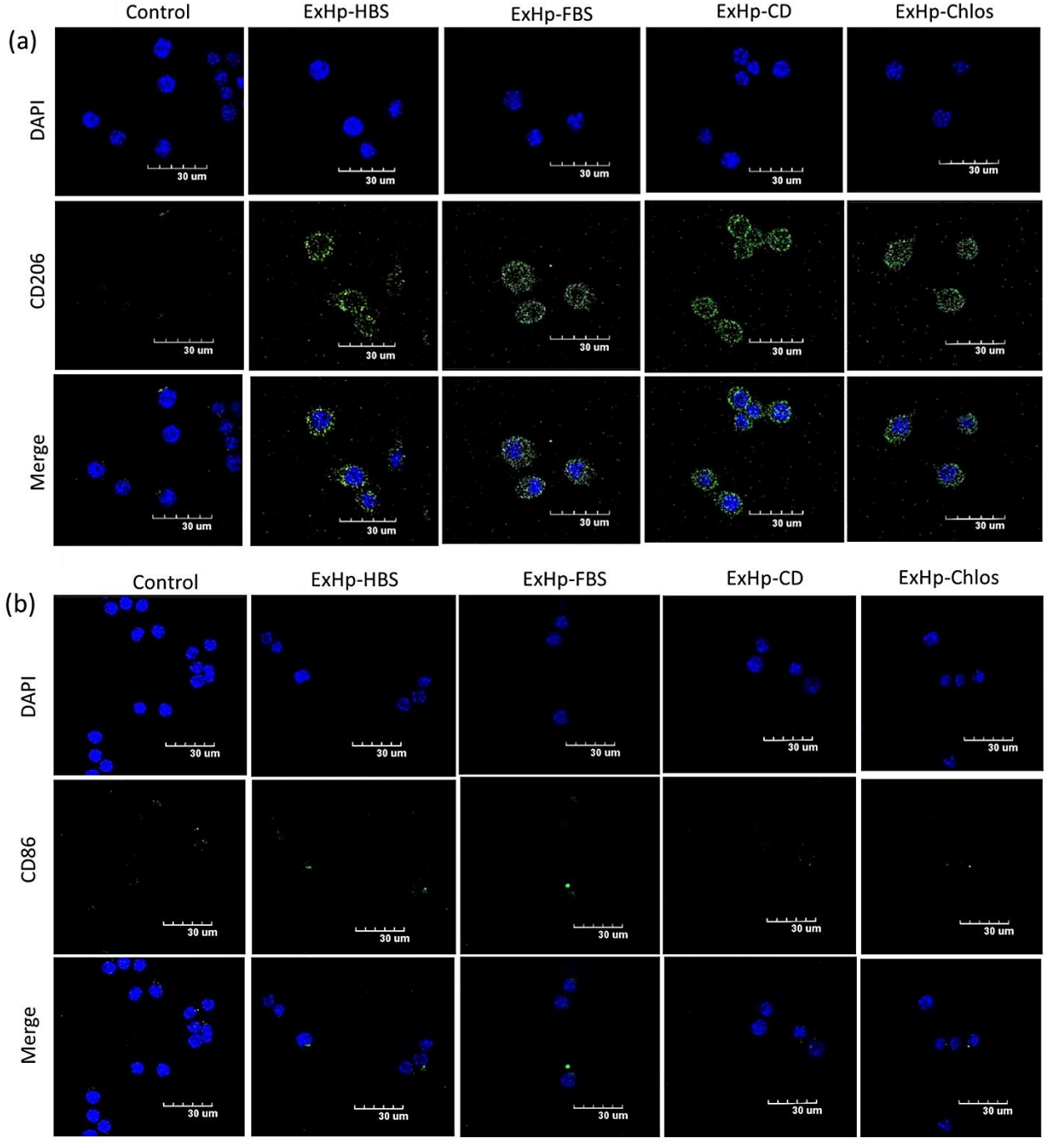

Fig. 9 Immunofluorescence staining of macrophage RAW 264.7 cells treated with 10 μg/mL of OMVs populations. M2 marker CD206 (a) and M1 marker CD86 (b) are showed in green and blue for cell nuclei (DAPI). Compared to the control, the groups stimulated with 10 μg/mL of OMVs populations showed an increased ratio of CD206 and a decreased ratio of CD86.

4. Conclusion

Outer membrane vesicles originated from bacteria contain several components such as bacterial antigens, pathogen-associated molecular patterns (PAMPs), and various proteins that attracted scientists to consider OMVs in various biomedical applications. Despite all these potentials, many challenges yet need to be investigated. In the current study, four different populations of outer membrane vesicles were obtained from *H. pylori SS1* that was cultured with HBS, FBS, β-Cyclodextrin, or Cholesterol. All the four OMVs exhibited distinct biological properties and OMVs contents. However, they all induced Th2 type immune response and produced anti-inflammatory cytokines by macrophage RAW 264.7 in vitro. In conclusion, obtained OMVs could be influenced by many factors that should be considered when working with OMVs. Our findings revealed that growth supplements in the culture medium influenced the production of OMVs and their biological properties, which is attributed to their contents. This knowledge determines which type of OMVs population to be chosen according to the required application. This could lead to finding the required OMVs population with the desired biological function. Thus, the specific OMVs population could be used in the needed biomedical applications such as in cancer immunotherapy, anti-bacterial adhesion agents, bacterial vaccine development, and drug delivery vehicles depending on the specific biological function. This study revealed that ExHp-CD and its associated cargo are the most biocompatible OMVs among all obtained populations, which can induce an immune response. Thus, it deserves to be further investigated. Moreover, this study concludes that β-Cyclodextrin induced the production of the most biocompatible OMVs derived from *H. pylori SS1*, while Cholesterol was found to induce the production of the more cytotoxic OMVs.

Conflict of interest

There are no conflicts to declare.

Acknowledgments

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Supporting Information

Not applicable.

References

[1] K. W. Kim, *Applied Microscopy*, 2018, **48**, 96-101, doi: 10.9729/am.2018.48.4.96.
[2] S. Gill, R. Catchpole, P. Forterre, *Fems Microbiol. Rev.*, 2019, **43**, 273-303, doi: 10.1093/femsre/fuy042.
[3] A. L. Leitão, F. J. Enguita, Non-coding RNAs and inter-kingdom Communication. Springer, 2016.
[4] L. Zavan, N. J. Bitto, E. L. Johnston, D. W. Greening, M. Kaparakis-Liaskos, *Proteomics*, 2019, **19**, 1-14, doi: 10.1002/pmic.201800209.
[5] L. Zavan, N. J. Bitto, M. Kaparakis-Liaskos, in Bacterial Membrane Vesicles. Springer, 2020, pp. 1-21.
[6] Z. Bolandi, N. Mokhberian, M. Eftekhary, K. Sharifi, S. Soudi, H. Ghanbarian, S. M. Hashemi, *Life Sci.*, 2020, **259**, 1-11, doi: 10.1016/j.lfs.2020.118218.
[7] Y. Li, Q. Li, D. Li, J. Gu, D. Qian, X. Qin, Y. Chen, *Life Sci.*, 2020, **264**, 1-8, doi: 10.1016/j.lfs.2020.118638.
[8] M. Yao, B. Cui, W. Zhang, W. Ma, G. Zhao, L. Xing, *Life Sci*, 2020, **264**, 1-13, doi: 10.1016/j.lfs.2020.118658.
[9] T. Chen, G. Zhang, L. Kong, S. Xu, Y. Wang, M. Dong, *Life Sci.*, 2019, **221**, 187-195, doi: 10.1016/j.lfs.2019.02.003.
[10] J. Shao, S. Li, Y. Liu, M. Zheng, *Life Sci.*, 2020, **240**, 1-8, doi: 10.1016/j.lfs.2019.117094.
[11] E. Bagheri, K. Abnous, S. A. Farzad, S. M. Taghdisi, M. Ramezani, M. Alibolandi, *Life Sci.*, 2020, **261**, 1-10, doi: 10.1016/j.lfs.2020.118369.
[12] Y. Liu, J. Luo, X. Chen, W. Liu, T. Chen, *Nano-Micro. Lett.*, 2019, **11**, 1-46, doi: 10.1007/s40820-019-0330-9.
[13] P. Dash, A. M. Piras, M. Dash, *J. Control. Release*, 2020, **327**, 546-570, doi: 10.1016/j.jconrel.2020.09.012.
[14] M. J. Uddin, J. Dawan, G. Jeon, T. Yu, X. He, J. Ahn, *Microorganisms*, 2020, **8**, 1-23 doi: 10.3390/microorganisms8050670.
[15] C. Schwechheimer, M. J. Kuehn, *Nat. Rev. Microbiol.*, 2015, **13**, 605-619, doi: 10.1038/nrmicro3525.
[16] S. Ayalew, A. W. Confer, B. Shrestha, A. E. Wilson, M. Montelongo, *Clin. Vaccine Immunol.*, 2013, **20**, 191-196, doi: 10.1128/cvi.00622-12.
[17] L. Turner, N. J. Bitto, D. L. Steer, C. Lo, K. D'Costa, G. Ramm, M. Shambrook, A. F. Hill, R. L. Ferrero, M. Kaparakis-Liaskos, *Front. Immunol.*, 2018, **9**, 1-10, doi: 10.3389/fimmu.2018.01466.
[18] A. J. Park, K. Murphy, M. D. Surette, C. Bandoro, J. R. Krieger, P. Taylor, C. M. Khursigara, *J. Proteome Res.*, 2015, **14**, 4524-4537, doi: 10.1021/acs.jproteome.5b00262.
[19] T. Pierson, D. Matrakas, Y. U. Taylor, G. Manyam, V. N. Morozov, W. Zhou, M. L. van Hoek, *J. Proteome Res.*, 2011, **10**, 954-967, doi: 10.1021/pr1009756.
[20] J. L. Kadurugamuwa, T. J. Beveridge, *J. Antimicrob. Chemoth.*, 1997, **40**, 615-621, doi: 10.1093/jac/40.5.615.
[21] K. J. McMahon, M. E. Castelli, E. G. Vescovi, M. F. Feldman, *J. Bacteriol.*, 2012, **194**, 3241-3249, doi: 10.1128/jb.00016-12.
[22] M. Li, H. Zhou, C. Yang, Y. Wu, X. Zhou, H. Liu, Y. Wang, *J. Control. Release*, 2020, **323**, 253-268, doi: 10.1016/j.jconrel.2020.04.031.
[23] Y. Hu, Y. Zhu, N. H. Lu, *Chinese Med. J.*, 2020, **133**, 335-343, doi: 10.1097/CM9.0000000000000618.
[24] O. Sjomina, J. Pavlova, Y. Niv, M. Leja, *Helicobacter*, 2018, **23**, 1-6, doi: 10.1111/hel.12514.
[25] J. K. Y. Hooi, W. Y. Lai, W. K. Ng, M. M. Y. Suen, F. E. Underwood, D. Tanyingoh, P. Malfertheiner, D. Y. Graham, V. W. S. Wong, J. C. Y. Wu, F. K. L. Chan, J. J. Y. Sung, G. G.

Kaplan, S. C. Ng, *Gastroenterology*, 2017, **153**, 420-429, doi: 10.1053/j.gastro.2017.04.022.
[26] A. Savoldi, E. Carrara, D. Y. Graham, M. Conti, E. Tacconelli, *Gastroenterology*, 2018, **155**, 1372-1382, doi: 10.1053/j.gastro.2018.07.007.
[27] A. J. F. Egan, *Mol. Microbiol.*, 2018, **107**, 676-687, doi: 10.1111/mmi.13908.
[28] C. Xu, D. M. Soyfoo, Y. Wu, S. Xu, *Eur. J. Clin. Microbiol. Infect. Dis.*, 2020, **39**, 1821-1830, doi: 10.1007/s10096-020-03948-y.
[29] M. R. Ki, I. H. Hong, J. K. Park, K. S. Hong, O. K. Hwang, J. Y. Han, A. R. Ji, S. I. Park, S. K. Lee, S. E. Yoo, K. S. Jeong, *Anticancer Res.*, 2009, **29**, 2393-2402.
[30] F. Fahimi, M. R. Tohidkia, M. Fouladi, R. Aghabeygi, N. Samadi, Y. Omidi, *Bioimpacts*, 2017, **7**, 59-71, doi: 10.15171/bi.2017.08.
[31] M. P. Tan, J. Pedersen, Y. Zhan, A. M. Lew, M. J. Pearse, O. L. Wijburg, R. A. Strugnell, *Infect Immun.*, 2008, **76**, 1289-1297, doi: 10.1128/IAI.00779-07.
[32] M. Hatakeyama, *P. Jpn Acad. B Phys.*, 2017, **93**, 196-219, doi: 10.2183/pjab.93.013.
[33] H. H. Cheng, G. Y. Tseng, H. B. Yang, H. J. Wang, H. J. Lin, W. C. Wang, *World J. Gastroentero.*, 2012, **18**, 34-43, doi: 10.3748/wjg.v18.i1.34.
[34] W. Gonciarz, A. Krupa, K. Hinc, M. Obuchowski, A. P. Moran, A. Gajewski, M. Chmiela, *PLoS One*, 2019, **14**, 1-27, doi: 10.1371/journal.pone.0220636.
[35] R. Kaebisch, R. Mejias-Luque, C. Prinz, M. Gerhard, *J. Immunol.*, 2014, **192**, 316-323, doi: 10.4049/jimmunol.1302476.
[36] M. Denic, E. Touati, H. De Reuse, *Helicobacter*, 2020, **25**, 1-5, doi: 10.1111/hel.12736.
[37] E. Mnich, M. Kowalewicz-Kulbat, P. Sicinska, K. Hinc, M. Obuchowski, A. Gajewski, A. P. Moran, M. Chmiela., *World J. Gastroentero.*, 2016, **22**, 7536-7558, doi: 10.3748/wjg.v22.i33.7536.
[38] N. Ito, H. Tsujimoto, H. Ueno, Q. Xie, N. Shinomiya, *J. Clin. Med.*, 2020, **9**, 1-11, doi: 10.3390/jcm9113699.
[39] R. L. Cassady-Cain, J. C. Hope, M. P. Stevens, *Infect. Immun.*, 2018, **86**, 1-11, doi: 10.1128/IAI.00683-17.
[40] C. Utsch, R. Haas, *Toxins (Basel)*, 2016, **8**, 1-10 doi: 10.3390/toxins8060190.
[41] M. S. Sundrud, V. J. Torres, D. Unutmaz, T. L. Cover, *P. Natl. Acad. Sci. USA*, 2004, **101**, 7727-7732, doi: 10.1073/pnas.0401528101.
[42] B. Gebert, W. Fischer, E. Weiss, R. Hoffmann, R. Haas, *Science*, 2003, **301**, 1099-1102, doi: 10.1126/science.1086871.
[43] M. Boncristiano, S. R. Paccani, S. Barone, C. Ulivieri, L. Patrussi, D. Ilver, A. Amedei, M. M. D'Elios, J. L. Telford, C. T. Baldari, *J. Exp. Med.*, 2003, **198**, 1887-1897, doi: 10.1084/jem.20030621.
[44] L. B. Jones, C. R. Bell, K. E. Bibb, L. Gu, M. T. Coats, Q. L. Matthews, *Biomedicines*, 2018, **6**, 1-12 doi: 10.3390/biomedicines6030079.
[45] Y. Kikuchi, N. Obana, M. Toyofuku, N. Kodera, T. Soma, T. Ando, Y. Fukumori, N. Nomura, A. Taoka, *Nanoscale*, 2020, **12**, 7950-7959, doi: 10.1039/c9nr10850e.
[46] Y. Hasegawa, H. Futamata, Y. Tashiro, *Front. Microbiol.*, 2015, **6**, 1-5, doi: 10.3389/fmicb.2015.00633.
[47] W. Zhang, X. Jiang, J. Bao, Y. Wang, H. Liu, L. Tang, *Front. Immunol.*, 2018, **9**, 1-12, doi: 10.3389/fimmu.2018.00090.
[48] M. Jarzab, G. Posselt, N. Meisner-Kober, S. Wessler, *Microorganisms*, 2020, **8**, 1-19, doi: 10.3390/microorganisms8091328.
[49] J. S. Nho, S. H. Jun, M. H. Oh, T. I. Park, C. W. Choi, S. I. Kim, C. H. Choi, J. C. Lee, *Microb. Pathog.*, 2015, **81**, 39-45, doi: 10.1016/j.micpath.2015.03.012.
[50] S. N. Wai, B. Lindmark, T. Söderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, B. E. Uhlin, *Cell*, 2003, **115**, 25-35, doi: 10.1016/s0092-8674(03)00754-2.
[51] C. Balsalobre, J. M. Silván, S. Berglund, Y. Mizunoe, B. E. Uhlin, S. N. Wai, *Mol. Microbiol.*, 2006, **59**, 99-112, doi: 10.1111/j.1365-2958.2005.04938.x.
[52] J. M. Davis, H. M. Carvalho, S. B. Rasmussen, A. D. O'Brien, *Infect. immun.*, 2006, **74**, 4401-4408. doi: 10.1128/IAI.00637-06.
[53] B. Lindmark, P. K. Rompikuntal, K. Vaitkevicius, T. Song, Y. Mizunoe, B. E. Uhlin, P. Guerry, S. N. Wai, *BMC Microbiol.*, 2009, **9**, 1-10, doi: 10.1186/1471-2180-9-220.
[54] H. Chang, D. Chen, B. Ni, Q. Zuo, C. Wang, R. Han, C. Lan, *Dig. Dis. Sci.*, 2016, **61**, 80-90, doi: 10.1007/s10620-015-3836-0.
[55] J. M. Bomberger, D. P. Maceachran, B. A. Coutermarsh, S. Ye, G. A. O'Toole, B. A. Stanton, *PLoS Pathog.*, 2009, **5**, 1-13, doi: 10.1371/journal.ppat.1000382.
[56] J. McCullough, *Hematology 2014, the American Society of Hematology Education Program Book*, 2014, **2014**, 404-409, doi: 10.1182/asheducation-2014.1.404.
[57] S. A. Jewell, R. W. Titball, J. Huyet, C. E. Naylor, A. K. Basak, P. Gologan, C. P. Winlove, P. G. Petrov, *Soft. Matter.*, 2015, **11**, 7748-7761, doi: 10.1039/c5sm00876j.
[58] T. Takagishi, M. Takehara, S. Seike, K. Miyamoto, K. Kobayashi, M. Nagahama, *Sci. Rep.*, 2017, **7**, 1-11, doi: 10.1038/s41598-017-05567-8.
[59] R. Benz, *Biochim. Biophys. Acta*, 2016,**1858**, 526-537, doi: 10.1016/j.bbamem.2015.10.025.
[60] M. C. Martino, R. A. Stabler, Z. W. Zhang, M. J. Farthing, B. W. Wren, N. Dorrell, *Infect. immun.*, 2001, **69**, 1697-1703, doi: 10.1128/IAI.69.3.1697-1703.2001.
[61] J. F. Tomb, O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, J. C. Venter, *Nature*, 1997, **388**, 539-547, doi: 10.1038/41483.
[62] S. Fulsundar, H. M. Kulkarni, M. V. Jagannadham, R. Nair, S. Keerthi, P. Sant, K. Pardesi, J. Bellare, B. A. Chopade, *Microb. Pathog.*, 2015, **83-84**, 12-22, doi: 10.1016/j.micpath.2015.04.005.

[63] L. Kunsmann, C. Rüter, A. Bauwens, L. Greune, M. Glüder, B. Kemper, A. Fruth, S. N. Wai, X. He, R. Lloubes, M. A. Schmidt, U. Dobrindt, A. Mellmann, H. Karch, M. Bielaszewska, *Sci. Rep.*, 2015, **5**, 1-18, doi: 10.1038/srep13252.
[64] C. H. Choi, S. H. Hyun, J. Y. Lee, J. S. Lee, Y. S. Lee, S. A. Kim, J. P. Chae, S. M. Yoo, J. C. Lee, *Cell. Microbiol.*, 2008, **10**, 309-319, doi: 10.1111/j.1462-5822.2007.01041.x.
[65] K. Koeppen, T. H. Hampton, M. Jarek, M. Scharfe, S. A. Gerber, D. W. Mielcarz, E. G. Demers, E. L. Dolben, J. H. Hammond, D. A. Hogan, B. A. Stanton, *PLOS Pathogens.*, 2016, **12**, 1-22, doi: 10.1371/journal.ppat.1005672.
[66] S. H. Jun, J. H. Lee, B. R. Kim, S. I. Kim, T. I. Park, J. C. Lee, Y. C. Lee, *PloS One*, 2013, **8**, 1-8, doi: 10.1371/journal.pone.0071751
[67] J. S. Jin, S. O. Kwon, D. C. Moon, M. Gurung, J. H. Lee, S. I. Kim, J. C. Lee, *PloS One*, 2011, **6**, 1-9, doi: 10.1371/journal.pone.0017027.
[68] V. V. Subhash, B. Ho, *Microbiology (Reading)*, 2015, **161**, 1150-1160, doi: 10.1099/mic.0.000066.
[69] Y. Li, Y. Liu, F. Xiu, J. Wang, H. Cong, S. He, Y. Shi, X. Wang, X. Li, H. Zhou, *Int J Nanomedicine*, 2018, **13**, 467-477, doi: 10.2147/IJN.S151110.
[70] J. D. Cecil, N. M. O'Brien-Simpson, J. C. Lenzo, J. A. Holden, W. Singleton, A. Perez-Gonzalez, A. Mansell, E. C. Reynolds, *Front. immun.*, 2017, **8**, 1017-1017, doi: 10.3389/fimmu.2017.01017.
[71] A. L. Jung, C. Stoiber, C. E. Herkt, C. Schulz, W. Bertrams, B. Schmeck, *PLoS Pathog.*, 2016, **12**, 1-26, doi: 10.1371/journal.ppat.1005592.
[72] X. J. Gao, T. Li, B. Wei, Z. X. Yan, N. Hu, Y. J. Huang, B. L. Han, T. S. Wai, W. Yang, R. Yan, *Drug. metab. dispos.*, 2018, **46**, 292-302, doi: 10.1124/dmd.117.079046.
[73] S. Mitra, R. Sinha, J. Mitobe, H. Koley, *Vaccine*, 2016, **34**, 1839-1846, doi: 10.1016/j.vaccine.2016.02.018.
[74] W. D. McCaig, C. L. Loving, H. R. Hughes, S. L. Brockmeier, *PLoS One*, 2016, **11**, 1-23, doi: 10.1371/journal.pone.0149132.
[75] A. J. Fleetwood, M. K. S. Lee, W. Singleton, A. Achuthan, M. C. Lee, N. M. O'Brien-Simpson, A. D. Cook, A. J. Murphy, S. G. Dashper, E. C. Reynolds, J. A. Hamilton, *Front. Cell. Infect. Microbiol.*, 2017, **7**, 1-15, doi: 10.3389/fcimb.2017.00351.
[76] A. L. Jung, K. Hoffmann, C. E. Herkt, C. Schulz, W. Bertrams, B. Schmeck, *J. Vis. Exp.*, 2017, doi: 10.3791/55146.
[77] R. Hu, H. Lin, J. Li, Y. Zhao, M. Wang, X. Sun, Y. Min, Y. Gao, M. Yang, *BMC Microbiol.*, 2020, **20**, 1-13, doi: 10.1186/s12866-020-01953-x.
[78] Q. Liu, X. Li, Y. Zhang, Z. Song, R. Li, H. Ruan, X. Huang, *Pathog. Dis.*, 2019, **77**, 1-13, doi: 10.1093/femspd/ftz050.

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