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LPS-Induced Endometrial Cell–Derived Exosomes Suppress Probiotic *Lactobacillus* Growth

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Abstract

Spontaneous preterm birth (sPTB) has been increasingly associated with alterations in vaginal microbiota. While *Lactobacillus spp.*, which physiologically dominate the cervical microbiota, are considered protective with a lower risk of intra-amniotic infection and chorioamnionitis, other microbes such as *Gardnerella vaginalis* are associated with an increased risk of sPTB. Although this association is well documented, the host mechanisms that regulate the composition of vaginal microbial communities remain poorly understood. Emerging evidence suggests that host-derived exosomes may play a critical role in shaping the microbial environment. This study hypothesized that endothelial cell-derived exosomes may modulate the growth of probiotic *Lactobacillus spp.* through changes in microRNA cargo, thereby influencing the risk of sPTB. To investigate this hypothesis, HEC-1-A cells were stimulated with lipopolysaccharides (LPS), and exosomes were isolated from these cells. These exosomes were then applied to four *Lactobacillus* strains (*L. crispatus, L. gasseri, L. jensenii, L. reuteri*) to evaluate how LPS-induced alterations in exosomal microRNA content affect probiotic growth. The results indicate that exosomes derived from LPS-stimulated HEC-1-A cells inhibited the four bacterial strains and facilitate the expansion of the opportunistic pathogen *G. vaginalis* in a mixed-culture system. MicroRNA sequencing revealed that LPS stimulation increases the levels of miR-181d-5p and miR-181c in these exosomes, both of which may contribute to the suppression of *Lactobacillus spp.* growth. Taken together, these findings suggest a novel regulatory pathway in which host-derived exosomes influence the vaginal microbiota, suggesting that disruptions in this mechanism may contribute to vaginal dysbiosis and increase the risk of sPTB.

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Keywords: spontaneous preterm birth, vaginal microbiota, probiotic, exosome, microRNA

1. Background

Preterm birth is a critical health challenge affecting 9.9% of newborns worldwide in 2020 [1]. Premature infants are vulnerable to complications that may result in long-term health issues or even death [2]. In 2019, complications from preterm birth were the leading cause of death in children under 5 years old [3]. While multiple risk factors may contribute to preterm birth, most preterm births are spontaneous and have no identifiable cause. Notably, the role of inflammation and vaginal microbiomes in preterm birth is becoming increasingly evident, with intrauterine inflammation—often triggered by bacterial pathogens—driving prostaglandin-mediated uterine contractions and early labor [4, 5]. The role of lipopolysaccharide (LPS), derived from Gram-negative bacteria, in inducing preterm birth has also been well documented [6].

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The vaginal microbiota is crucial in pregnancy, with alterations in its composition linked to increased risk of preterm birth [7]. For instance, *Lactobacillus crispatus* dominance is considered a protective factor against preterm birth, whereas a more diverse and abundant vaginal microbiota, including increased abundance of *Gardnerella vaginalis* and several other taxa, are associated to increased risk [8-11]. Additionally, urinary tract infections [12, 13] and bacterial vaginosis [14] are recognized contributors to preterm birth, reinforcing the importance of maintaining a balanced vaginal microbiota throughout gestation.

Exosomes, small extracellular vesicles (30–150 nm) secreted by various cell types, are increasingly recognized as important mediators of intercellular communication [15]. Beyond host-to-host signaling, they play a critical role in host–microbiota interactions, as evidenced in the gut [16, 17]. However, while research has characterized the 'bottom-up' influence of microbial metabolites on host tissue, the reciprocal 'top-down' regulation of the microbiota by host-derived factors remains underexplored, particularly in the vaginal niche. Specifically, while exosomes in reproductive health are known to influence the local microenvironment [18], it remains unclear whether the host actively modulates the commensal-pathogen balance through exosomal cargo in response to inflammatory triggers relevant to preterm birth. Our previous work demonstrated that exosomes derived from the vaginal probiotic *Lactobacillus crispatus* protect placental cells from oxidative stress–induced senescence [19]. Building on this, the present study investigates the reverse interaction: whether endometrial cell–derived exosomes, released under LPS–induced inflammatory conditions, can influence vaginal probiotic bacteria, thereby impacting reproductive tract health and preterm birth risk.

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2. Methods

2.1. Cell Culture and Inflammation Induction

The HEC-1-A cervical cell was obtained from the Bioresource Collection and Research Center (BCRC60552; Hsinchu, Taiwan) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Life Technologies, Auckland, New Zealand), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 U/mL penicillin (HyClone Laboratories), and 50 μg/mL streptomycin (HyClone

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Laboratories). Cells were maintained at 37°C in a 5% CO_2 incubator and subcultured twice weekly. Inflammation was induced by incubation with various concentrations of lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MO, USA) for 48 h, and cell viability was subsequently assessed using the MTT assay (Sigma-Aldrich).

2.2. *Lactobacillus* Culture and Incubation with Exosomes

Lactobacillus crispatus BXB710, *Lactobacillus gasseri* BXB227, *Lactobacillus reuteri* BXB722, and *Lactobacillus jensenii* BXB112 were obtained from the BioX Biotech CO., Ltd (Taipei, Taiwan) and inoculated into MRS broth (Difco Laboratories; Detroit, MI, USA). The cultures were incubated at 37°C for at least 12 hours. Subsequently, 1% of the cultured bacterial suspension was transferred into fresh MRS broth (with or without exosomes) and incubated again at 37°C for 12 hours. Bacterial growth was then assessed by measuring optical density at 600 nm (OD_{600}) using a spectrophotometer as previously described [20].

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2.3. Enrichment and Characterization of Exosomes

The enrichment and characterization of exosomes from HEC-1-A cells were performed based on previously described methods [21]. Supernatant from HEC-1-A cell culture was harvested, and cell debris was removed by successive centrifugation at 3000 ×g for 15 min and 35,000 ×g for 60 min. The supernatant was then subjected to ultracentrifugation at 200,000 ×g for 60 min to pellet the exosomes, which were resuspended in PBS and passed through a 0.22-μm filter for purification. The concentration and size distribution of the exosomes were detected using the Nanosight nanoparticle tracking

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analysis system (LM10-HS, Malvern Instruments, Worcestershire, UK). For morphology observation, exosome (2 µL) were dried onto freshly 'glow discharged' 300 mesh formvar/carboncoated TEM grids (Ted Pella, Redding, CA, USA), negatively stained with 2% aqueous uracyl acetate, and observed under a transmission electron microscope (JEM-2100F, JEOL, Akishima City, Japan).

2.4. Exosome Cargo Quantifications

RNA concentrations were measured using the Qubit® RNA Assay Kit (Invitrogen) following the manufacturer's instructions. Briefly, exosomes (1–20 µL) derived from HEC-1-A cultured-medium with or without LPS induction were added to Qubit® Working Solution to achieve a final volume of 200 µL. The mixture was incubated at room temperature and analyzed using a Qubit™ Fluorometer to obtain total RNA and small RNA concentrations. DNA concentrations were measured using a microvolume spectrophotometer. Protein concentrations were determined using the Bradford assay.

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2.5. Exosome Uptake Analysis

Exosomes (10^{10} particles) derived from HEC-1-A cells cultured-medium with or without LPS induction were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) dye at 37°C in the dark with shaking for 1 hour. After the incubation, the exosomes were isolated by ultracentrifuge at 247,000 ×g at 4°C for 2 hours. The CFSE-stained exosomes were then resuspended in DPBS and incubated with bacteria cultures at 37°C for 2 hours. Following incubation, the bacterial cells were stained with the DRAQ5™ Fluorescent Probe (Abcam; Cambridge, UK). The samples were then observed using a confocal microscope (FV3000, Olympus,

Tokyo, Japan) [22].

2.6. Small RNA Extraction and Sequencing

Small RNA was extracted from the exosomes using the Quick-RNA™ MiniPrep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quality was then analyzed using a Bioanalyzer 2100, and the Small RNA Sample Prep Kit was employed for library preparation. After the library was constructed, quality control procedures were performed to ensure that the quality met the required standards. The libraries were sequenced using the Illumina HiSeq 2500 high-throughput sequencing platform to identify species-specific small RNAs. Base detection was performed to convert the raw images into sequence data, which was presented in FASTQ format along with the corresponding base quality scores. The sequences were further analyzed to identify the microRNAs present in the exosomes.

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2.7. Fermentation-Based Simulation System and Microbiota Profiling

Gardnerella vaginalis (BCRC17040) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in CASMAN'S MEDIUM BASE (Difco 0290) with 5% rabbit blood. Initially, *G. vaginalis* was mixed at a 1:1 ratio with four strains of lactobacilli (*L. crispatus, L. jensenii, L. gasseri,* and *L. reuteri* at a ratio of 1:1:1:1). The resulting bacterial suspension served as the 0-hour control group. Three bioreactor units maintained under strict anaerobic conditions were treated with either no sample (control), naïve HEC-1-A-derived exosomes, or LPS-induced exosomes (final concentration: 10^{10} particles/mL). The cultures were incubated for 48 hours, and microbial community analysis was performed at the end of the experiment.

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For microbial community analysis, the samples were immediately flash-frozen in liquid nitrogen and stored at –80 °C until further processing. Total genomic DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Full-length 16S ribosomal RNA gene sequencing was conducted using the LoopSeq™ 16S Microbiome SSC Kit and sequenced on the PacBio RS II Single Molecule Real-Time (SMRT) sequencing platform (Pacific Biosciences, Menlo Park, CA, USA). Circular consensus sequencing (CCS) was employed to improve sequencing accuracy. Amplicon sequence variants (ASVs) were inferred using the DADA2 pipeline, and taxonomic assignment was performed using the NCBI reference database.

2.8. Statistical Analysis

The data were expressed as mean ± standard deviation (SD) from three independent experiments and analyzed using the Statistical Analysis System (SAS Inc., Cary, NC, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) with Duncan’s multiple range tests or student’s t-test.

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3. Results

3.1. Impact of LPS on HEC-1-A Cells and Exosome Releases

HEC-1-A cells were treated with various concentrations of LPS (50, 100, 250, and 500 ng/mL) for 48 hours, and cell viability was subsequently assessed **(Figure 1)**. To balance robust inflammatory activation with cellular integrity, we selected an LPS concentration that maintained ≥80% cell viability, 250 ng/mL, for the subsequent LPS induction experiments. Exosomes secreted by

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HEC-1-A cells with or without LPS stimulation were then collected for analysis. Nanoparticle tracking analysis demonstrates that both samples had peak diameters between 100–130 nm, which is consistent with exosome size range. The particle concentrations **(Figure 2a)** and morphology **(Figure 2b)** of the exosomes appeared similar. The cargo of the exosomes was also examined. Results showed that LPS-induced cellular stress led to an increased amount of small RNAs in the exosomes, but the concentrations of long RNA, DNA, and proteins remained unaffected **(Table 1)**.

3.2. Exosomes Obtained from LPS-induced HEC-1A Cells Inhibited *Lactobacillus* Species Growth

We next examined whether the exosomes released from HEC-1-A cells have any impact on probiotics growth. Vaginal microbiota of health women is often dominated by beneficial *Lactobacillus spp.*, whereas the vaginal microbiota of individuals with bacterial vaginosis usually exhibits greater species diversity. Exosomes were labeled with CFSE, and bacterial cells were counterstained with DRAQ5. Confocal microscopy revealed CFSE fluorescence localized within DRAQ5-positive cells in all four *Lactobacillus* species (*L. crispatus* BXB710, *L. jensenii* BXB112, *L. gasseri* BXB227, and *L. reuteri* BXB722). The CFSE fluorescence appeared predominantly within the bacterial cell boundaries, consistent with possible internalization of exosomes derived from both naïve and LPS-treated HEC-1-A cells (**Figure 3**). Similar to the control group that were not incubated with exosomes, exosomes secreted from naïve HEC-1-A cells did not affect the growth of the four *Lactobacillus* species. In contrast, LPS-induced exosomes were found to inhibit the growth of all four *Lactobacillus* species **(Figure 4)**, with a particularly pronounced inhibitory

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effect observed on the growth of *L. gasseri* BXB227 and *L. reuteri* BXB722 **(Figure 5)**.

3.3. Exosomes from Naïve and LPS-Induced HEC-1A Cells Differentially Modulate Vaginal Microbiota Composition

The relationship between microorganisms and their hosts is characterized by intricate and evolving interactions. Although an overgrowth of opportunistic pathogens can be detrimental to the host, beneficial bacteria present in the environment have the potential to counteract the harmful effects of these pathogens [23]. We next examined whether exosomes derived from naïve and LPS-induced HEC-1-A would affect the composition of vaginal microbiota. In a fermentation-based simulation system, a mixed culture of the opportunistic pathogen *G. vaginalis* and four *Lactobacillus* species (control group) showed a reduction in the relative abundance of *G. vaginalis* from 0 to 48 hours **(Figure 6)**. This decrease was primarily attributed to the growth and competitive advantage of the *Lactobacillus* species, which suppressed the proliferation of *G. vaginalis*. On the other hand, the presence of exosomes (10^{10} particles/mL) derived from naïve HEC-1-A cells during the cultivation process significantly reduced the abundance of *G. vaginalis* while promoting the proliferation of the *Lactobacillus* species **(Figure 6)**. In contrast, exosomes from LPS-induced HEC-1-A cells demonstrated the opposite effect, increasing the abundance of *G. vaginalis* after 48 hours **(Figure 6)**.

3.4. LPS-Induced Exosomal miR-181d-5p and miR-181c Suppress *Lactobacillus* Growth

We further analyzed the microRNA content within the LPS-induced

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exosomes and found that LPS stimulation markedly increased the levels of miR-181d-5p and miR-181c **(Figure 7)**. Synthetic versions of these two microRNAs were subsequently applied to the four *Lactobacillus* species. The results demonstrated that both miR-181d-5p and miR-181c significantly inhibited the growth of these bacteria **(Figure 8)**.

4. Discussion

Previous studies in the gastrointestinal system have shown that exosomes released from intestinal epithelial cells can directly influence gut microbiota composition, for example by delivering antimicrobial peptides or regulatory RNAs into the lumen to suppress pathogens growth [24]. These studies align with our findings that exosomes secreted from the host endometrial cells play an important role in vaginal microbiota. Specifically, we observed that exosomes derived from LPS-induced (inflamed) endometrial cells not only inhibited the growth of probiotic *Lactobacillus* species (Figures 4 and 5) but also promoted the expansion of the opportunistic pathogen *Gardnerella vaginalis* (Figure 6). Such results indicate that inflammatory responses of the endometrium can alter vaginal microbiota, ultimately affecting pregnancy outcome.

In pregnancy models, systemic or intrauterine LPS activates uterine TLR4-dependent inflammation, elevates cytokines, induces cyclooxygenase-2 (COX-2)/prostaglandins, recruits leukocytes, and precipitates preterm labor [25-27]. Clinically, maternal biomarkers of endotoxin exposure such as LPS-binding protein (LBP) are elevated in pregnancies complicated by preterm premature rupture of membranes and intra-amniotic inflammation [28, 29]. Our data add to this body of evidence by showing that LPS-stimulated

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endometrial cells can alter the vaginal microbiota through exosome-mediated signaling, creating a dysbiotic environment. This is highly relevant given that bacterial vaginosis (BV) has been shown in a recent meta-analysis to increase the risk of preterm birth [14]. BV-associated gram-negative anaerobes, such as *Prevotella* and *Bacteroides* species, also produce LPS that can ascend to the endometrium [30], activate toll-like receptors (TLR) 4-mediated inflammatory pathways [31], and exacerbate adverse pregnancy outcomes, thus potentially creating a self-perpetuating cycle of inflammation and dysbiosis.

We further identified miR-181c and miR-181d-5p as microRNAs enriched in exosomes from LPS-induced endometrial cells (Figure 7). These two microRNAs belong to the miR-181 family, which is known to modulate multiple signaling pathways in mammalian cells [32, 33]. Intriguingly, there is currently no direct evidence demonstrating the impact of these microRNAs on bacterial growth, whereas our results show that synthetic miR-181c and miR-181d-5p significantly inhibited the growth of key vaginal *Lactobacillus* species, suggesting a direct microbial target (Figure 8). Taken together, these findings raise the possibility that targeted probiotic interventions aimed at restoring vaginal microecology and modulating miR-181 expression could help break the cycle of inflammation and dysbiosis in the reproductive tract. The interplay between vaginal microbiota, exosomal miRNAs, and host immune signaling represents a critical mechanistic axis warranting further investigation.

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5. Conclusion

This study identifies a novel host–microbe regulatory mechanism in which exosomes derived from LPS-stimulated endometrial cells suppress the growth of probiotic *Lactobacillus* spp. and promote the expansion of the opportunistic

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pathogen *Gardnerella vaginalis*. These effects are associated with increased exosomal levels of miR-181c and miR-181d-5p, suggesting that inflammation-driven changes in exosomal microRNA cargo can disrupt vaginal microbial homeostasis. Such dysbiosis may contribute to an elevated risk of spontaneous preterm birth, highlighting exosomal miRNAs as potential biomarkers and therapeutic targets for maintaining reproductive tract health.

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Declarations

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Competing interests: YCS is employed as a Research and Development Manager at BioX Biotech CO., Ltd., which supplied materials used in this study. The company had no role in the design, data collection, analysis, interpretation, or writing of the manuscript. The authors declare no other competing interests.

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Authors' contributions: LMW and YCS conceived the study and performed the formal analysis. BHL conducted the methodology, investigation, and visualization, with additional methodological input from EWL. CJT and LTL supervised the work. YCS, CHL, and LTL contributed to the writing. All authors reviewed the manuscript.

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Figure legends

Figure 1. Cell viability of HEC-1-A cells after LPS stimulation for 48 h. Data are shown as mean ± SD from three independent experiments.

Figure 2. Exosome of HEC-1-A cells after LPS stimulation was evaluated for (a) particle size by NTA and (b) morphology by TEM. Representative data from three independent experiments are shown.

Figure 3. Uptake of exosomes from HEC-1-A cells by different lactic acid bacteria (a) *Lactobacillus crispatus* BXB710, (b) *Lactobacillus jensenii* BXB 112, (c) *Lactobacillus gasseri* BXB227, and (d) *Lactobacillus reuteri* BXB 722. Bacteria were stained with DRAQ-5, and exosomes were labeled with CFSE. Representative data from three independent experiments are shown.

Figure 4. Effects of exosomes (10^{10} particles/mL) from HEC-1-A cells with or without LPS induction on the growth curve of (a) *Lactobacillus crispatus* BXB710, (b) *Lactobacillus jensenii* BXB 112, (c) *Lactobacillus gasseri* BXB227, and (d) *Lactobacillus reuteri* BXB 722. Data are shown as mean ± SD from three independent experiments.

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Figure 5. Growth inhibition of (a) *Lactobacillus crispatus* BXB710, (b) *Lactobacillus jensenii* BXB 112, (c) *Lactobacillus gasseri* BXB227, and (d) *Lactobacillus reuteri* BXB 722 after 36-hour treatment with exosomes from HEC-1-A cells with or without LPS induction. Data are shown as mean ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA with Duncan's multiple range tests (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Figure 6. Co-culture of *Gardnerella vaginalis* with four different *Lactobacillus* species (*Lactobacillus crispatus* BXB710, *Lactobacillus jensenii* BXB 112, *Lactobacillus gasseri* BXB227, and *Lactobacillus reuteri* BXB 722) followed by

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treatment with exosomes from HEC-1-A cells with or without LPS induction, to assess changes in microbial composition. Data are shown as mean from three independent experiments.

Figure 7. Differences in the abundance of microRNA species in exosomes derived from HEC-1-A cells after LPS stimulation. Data are shown as mean from three independent experiments.

Figure 8. The inhibitory effects of miR-181d-5p and miR-181c on the growth of (a) *Lactobacillus crispatus* BXB710, (b) *Lactobacillus jensenii* BXB 112, (c) *Lactobacillus gasseri* BXB227, and (d) *Lactobacillus reuteri* BXB 722. Data are shown as mean ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA with Duncan's multiple range tests (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$); all comparisons were made against the 'Control' group at the corresponding timepoint.

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Table

Table 1. **Changes in the cargo of exosomes from HEC-1-A cells after LPS stimulation.** Data are shown as mean ± SD (ng/10^8 particles) from three independent experiments. Statistical significance was determined by student's t-test.

Cargo	Blank exosome	LPS-induced exosome	P value
Long RNA	5.1 ± 0.45	4.21 ± 0.21	0.0361 (*)
Small RNA	4.76 ± 0.72	10.48 ± 0.52	0.0004 (***)
DNA	7.63 ± 1.05	9.23 ± 1.01	0.1299 (ns)
Protein	10.42 ± 1.23	8.74 ± 0.98	0.1379 (ns)

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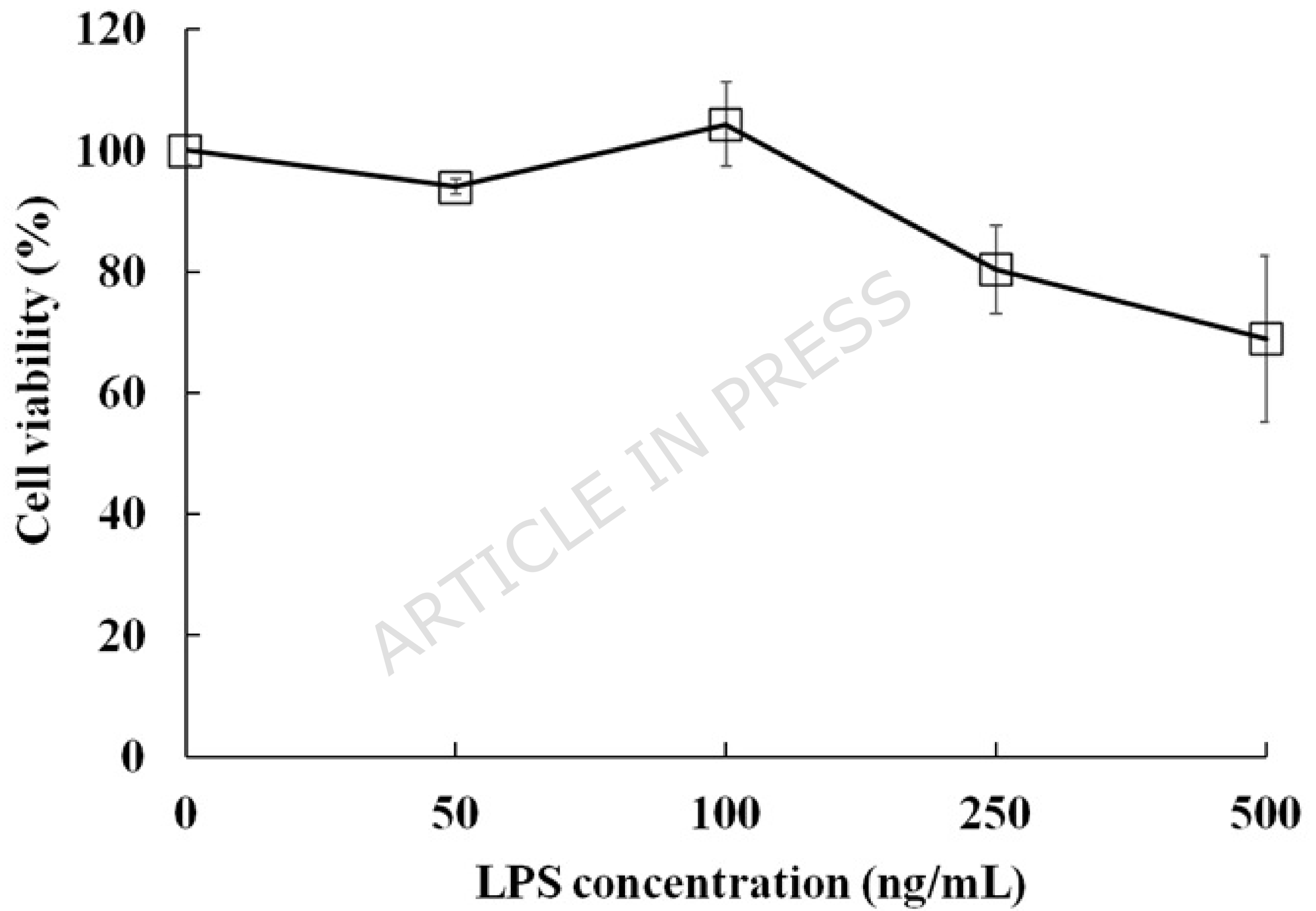

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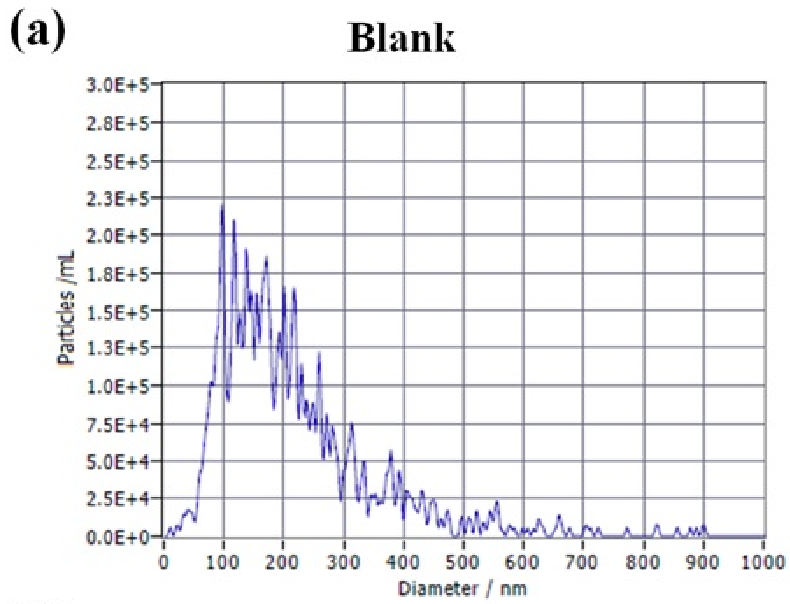

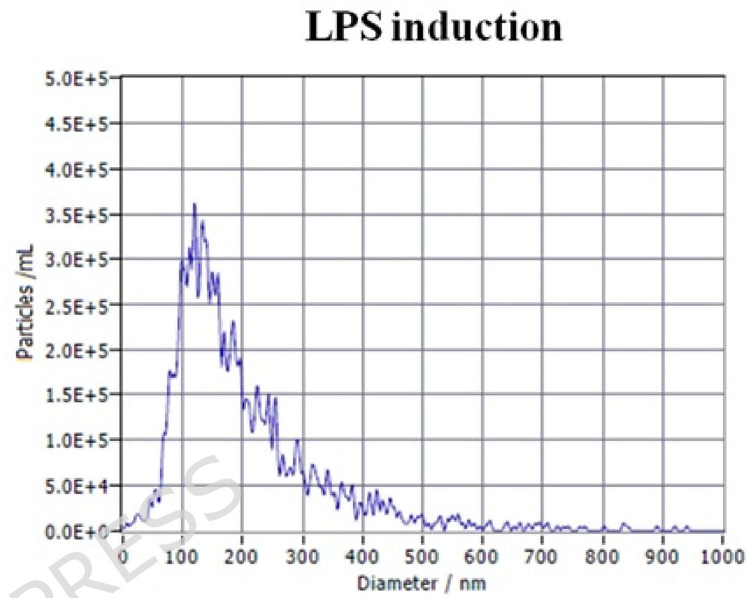

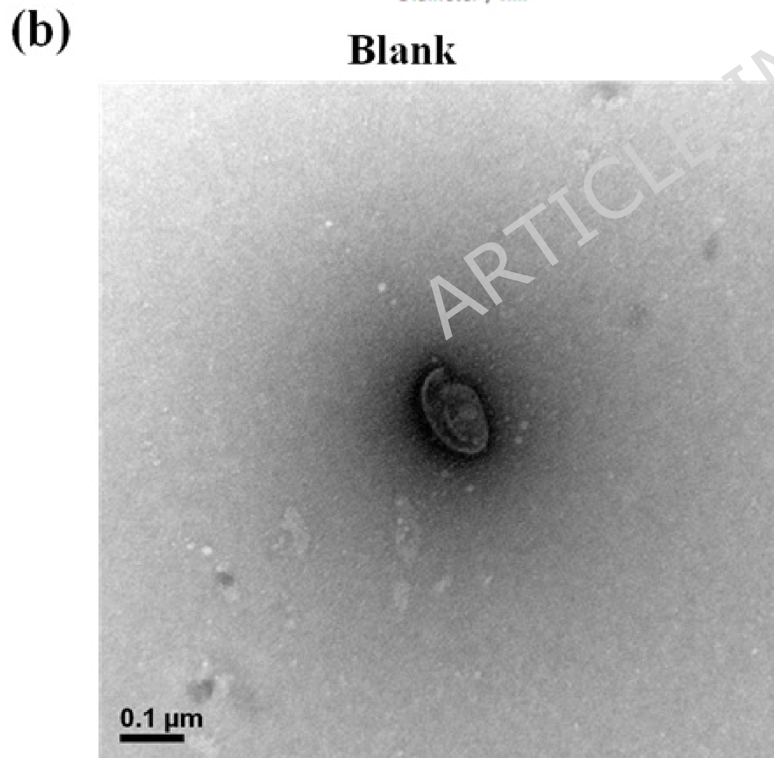

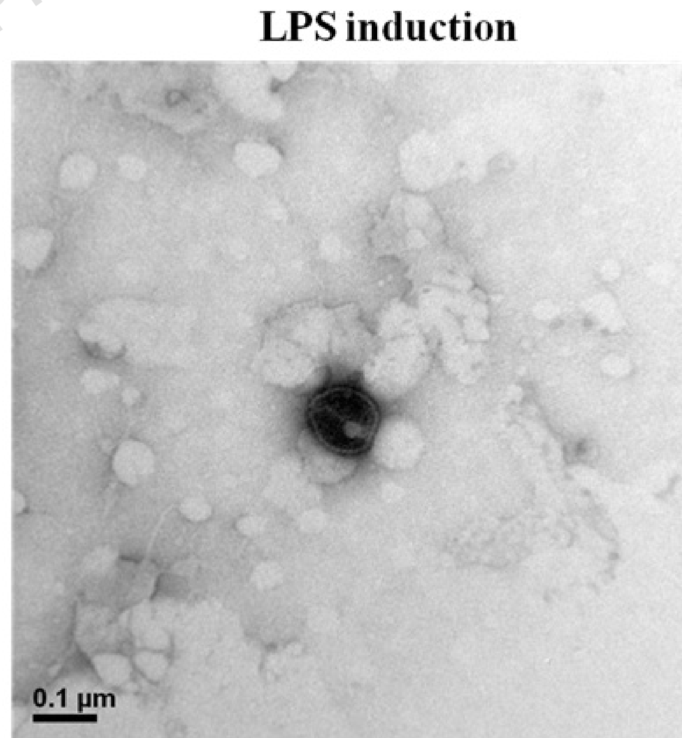

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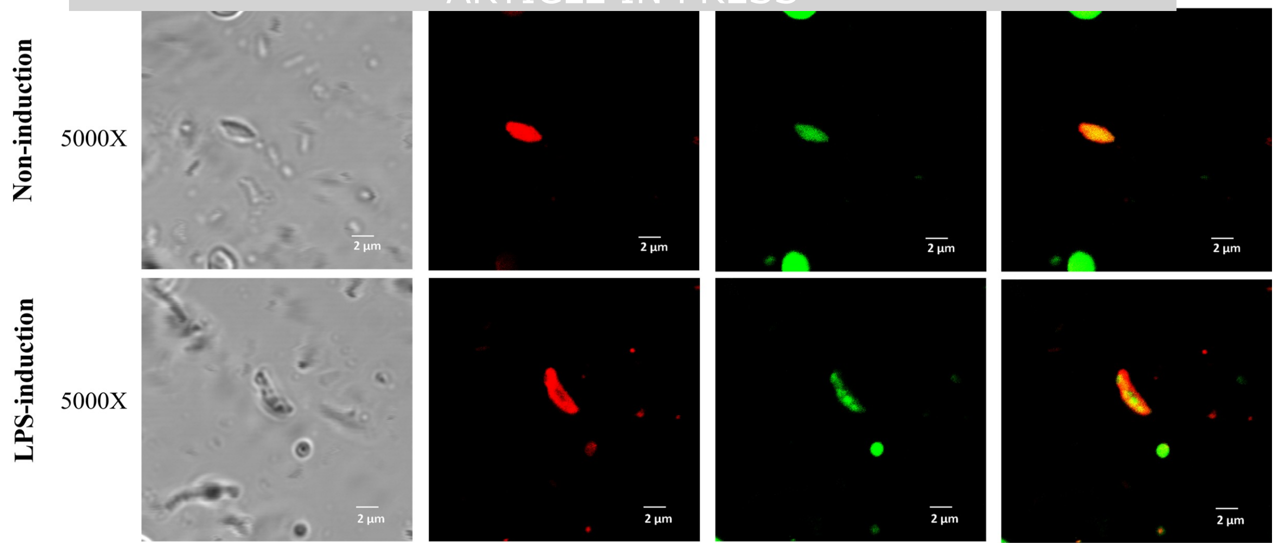

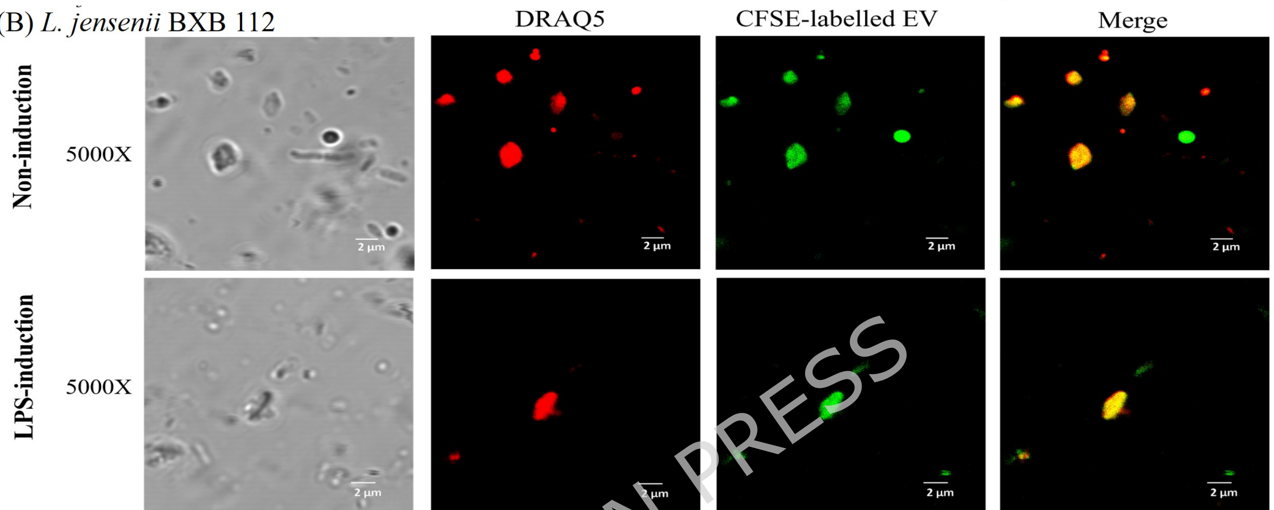

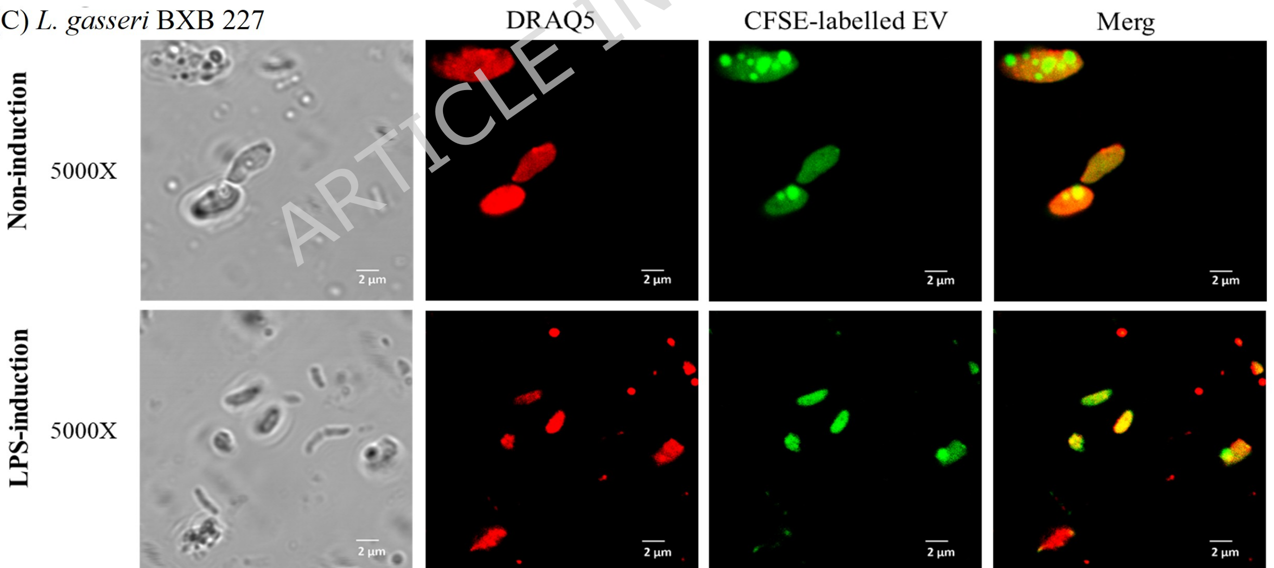

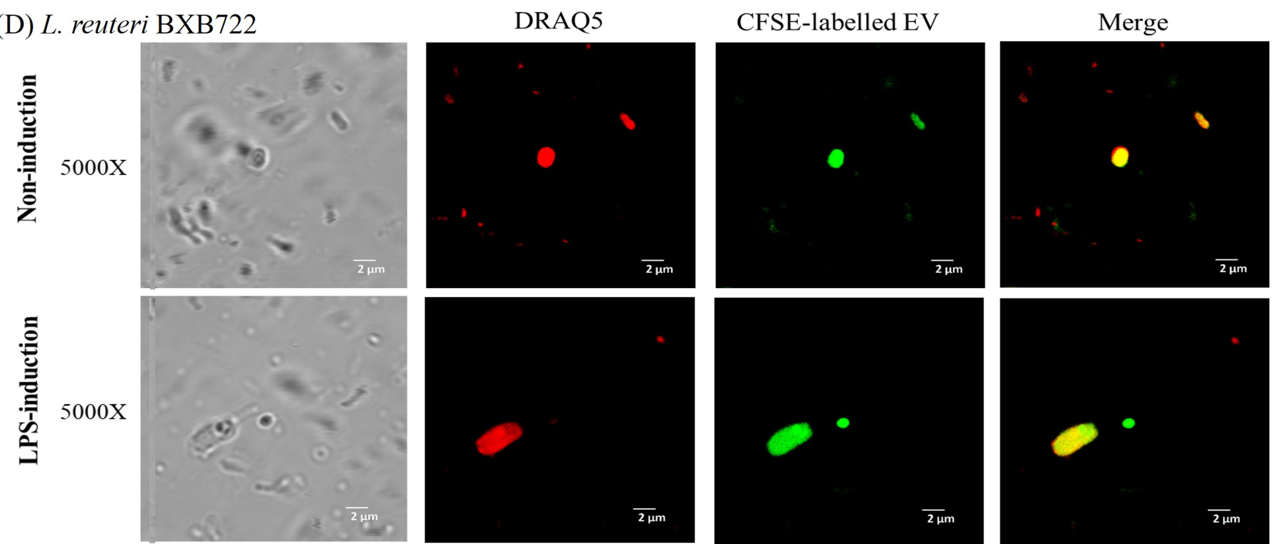

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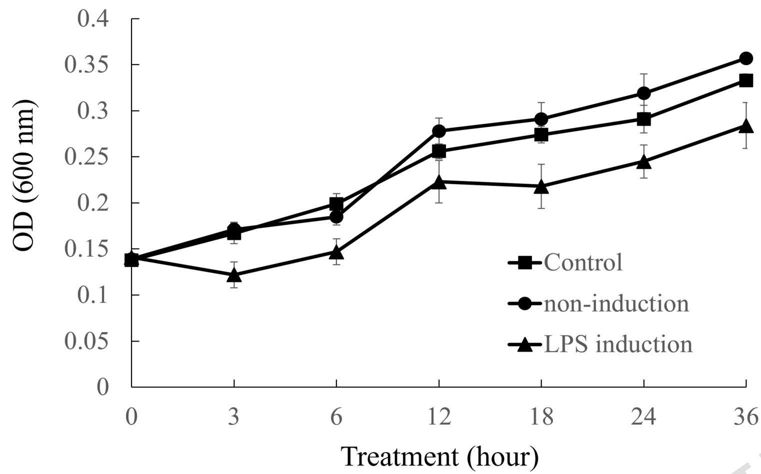

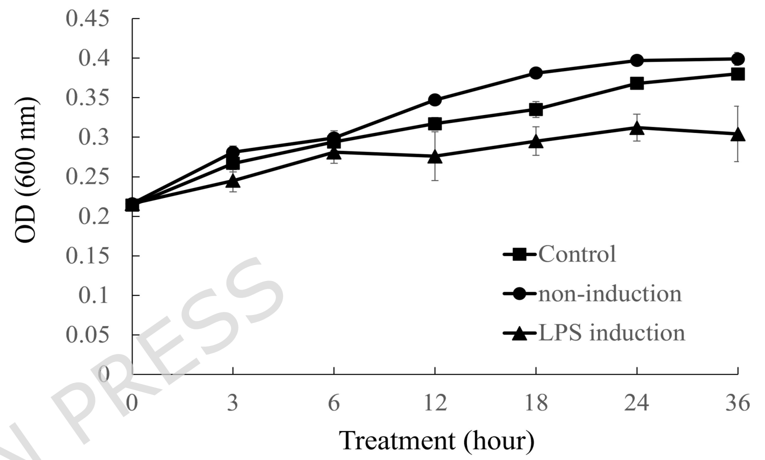

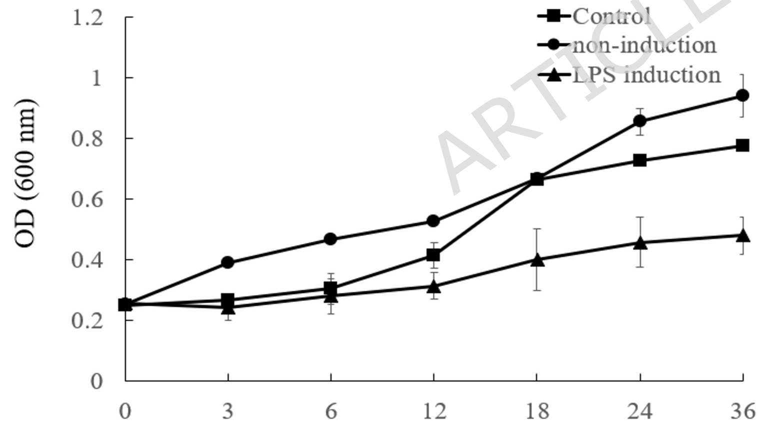

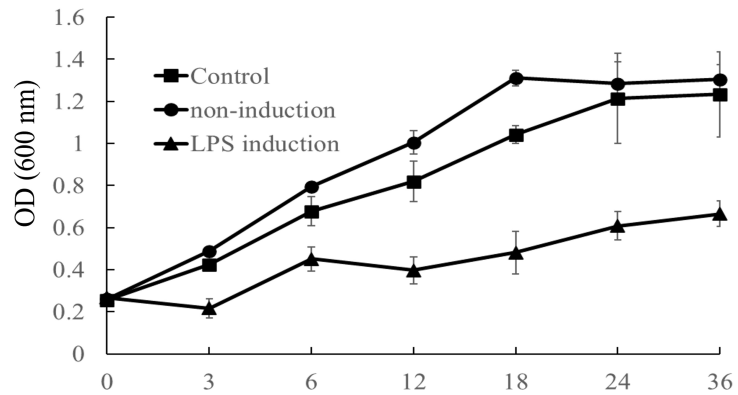

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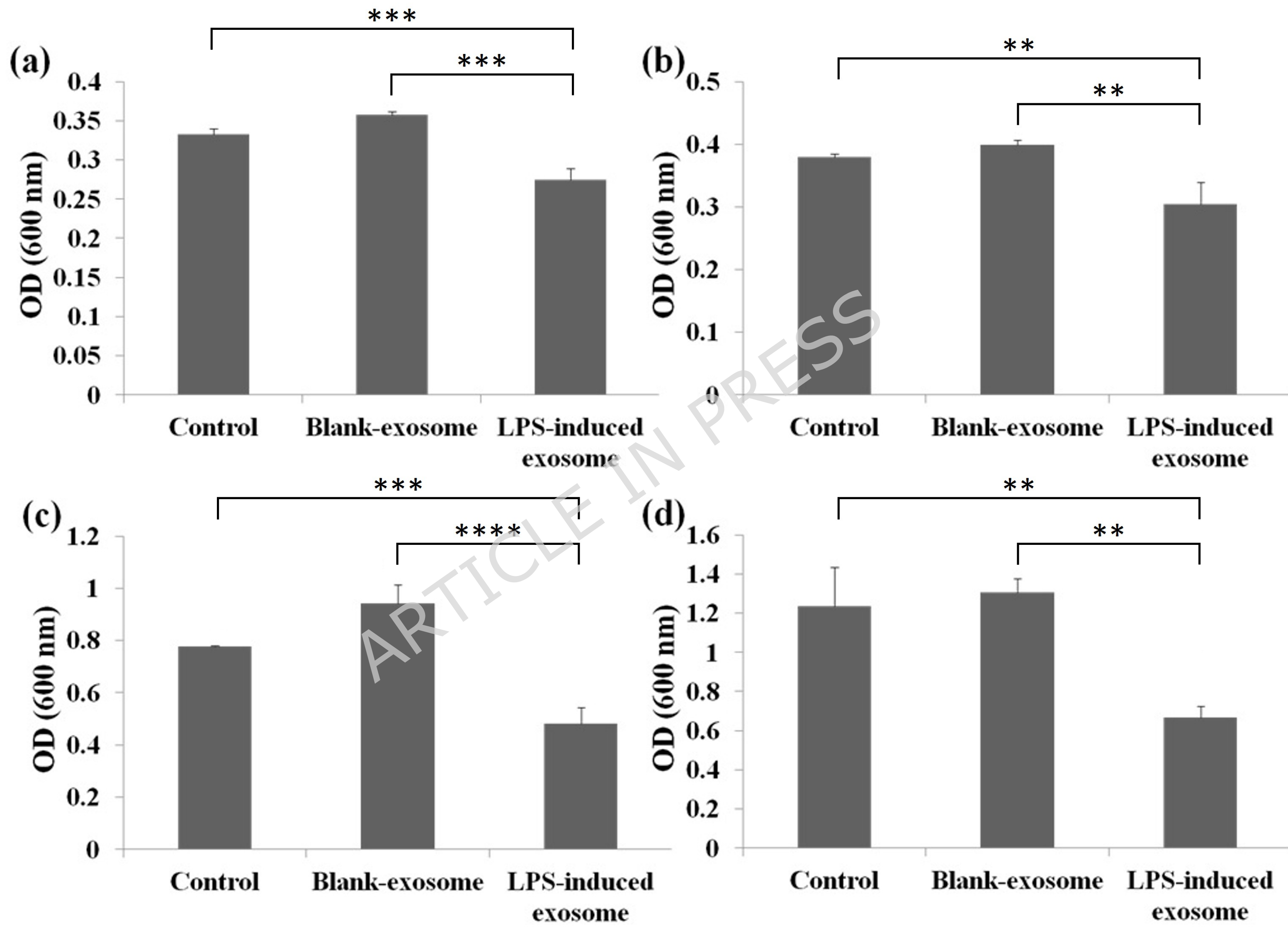

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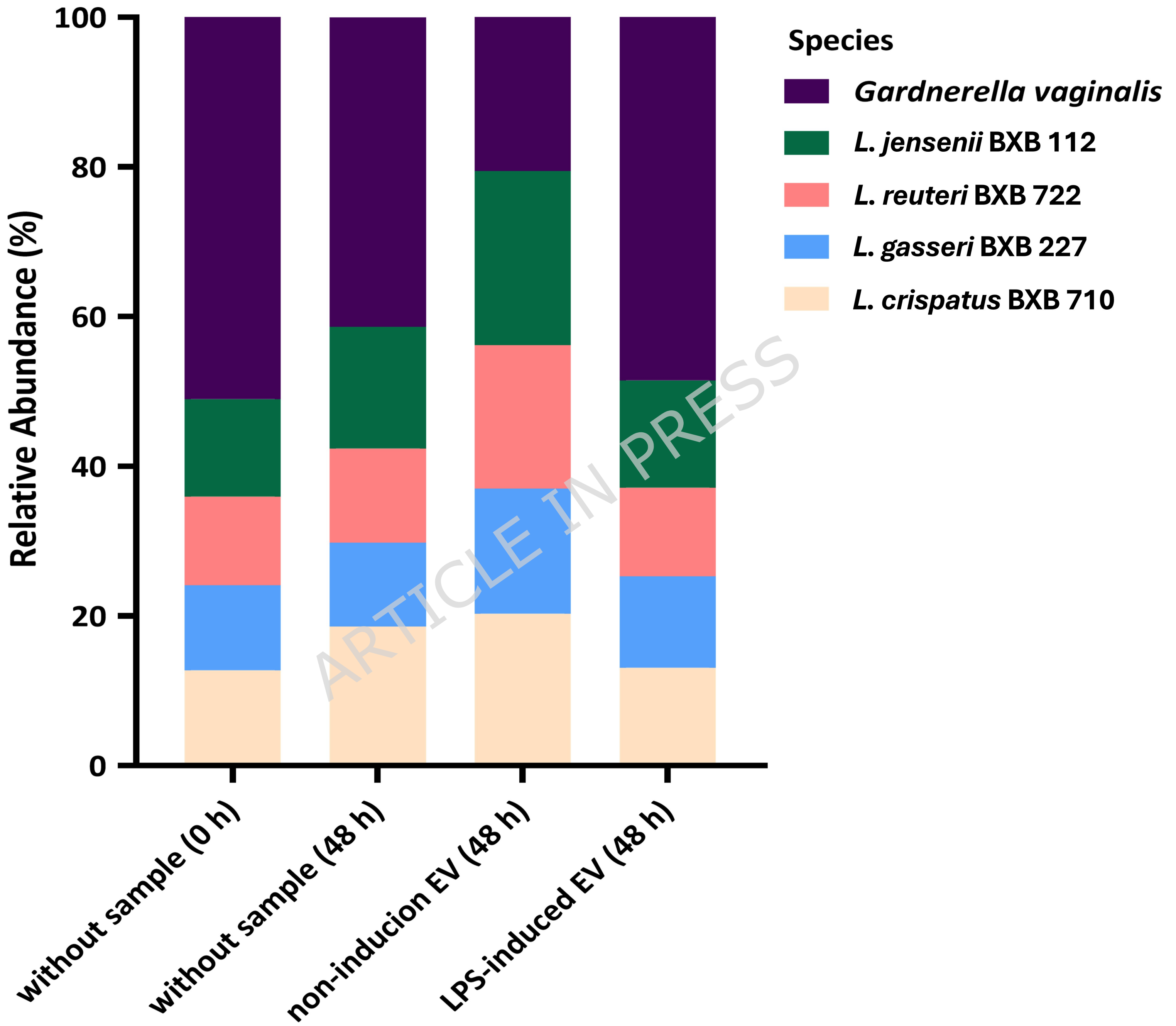

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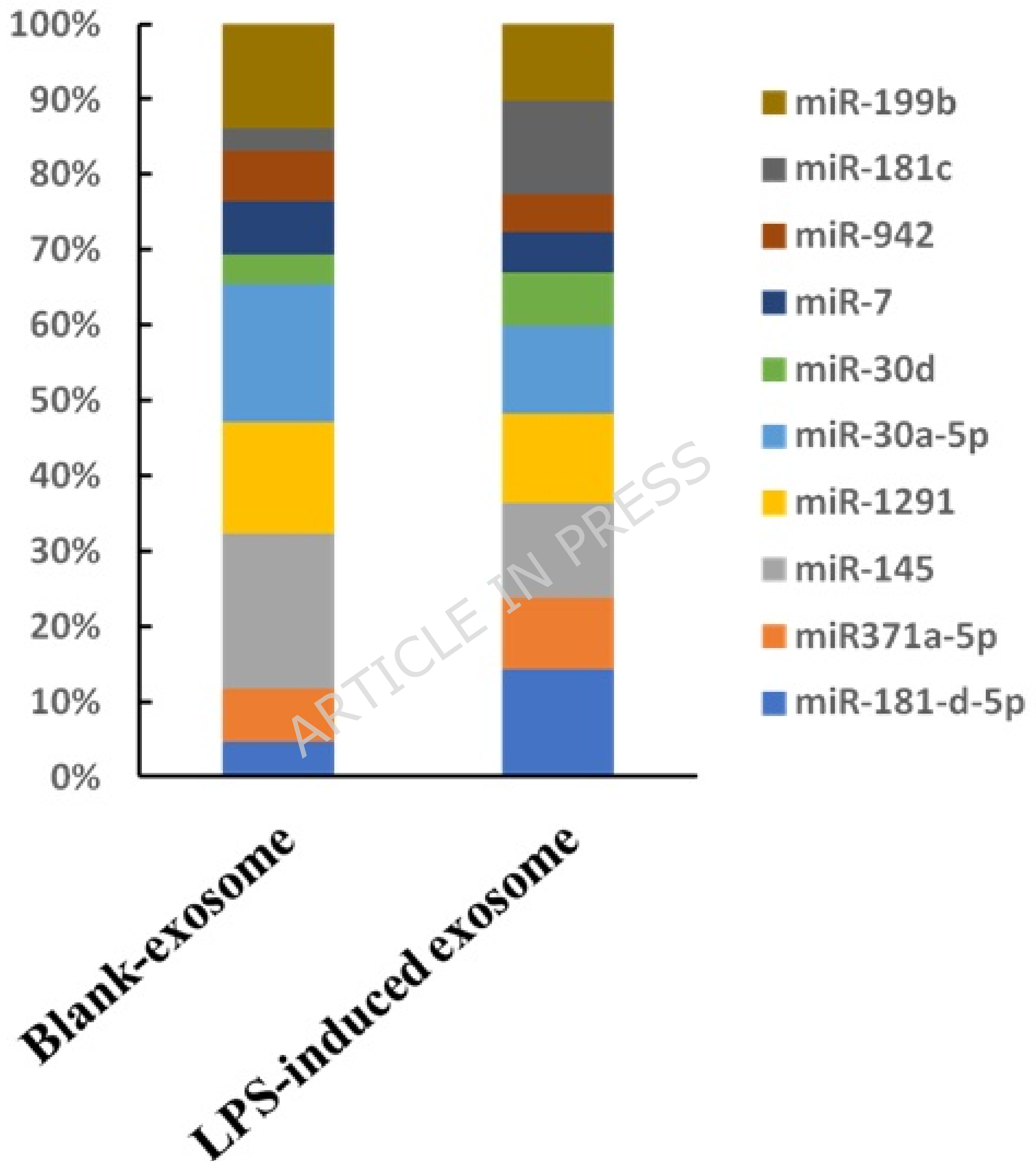

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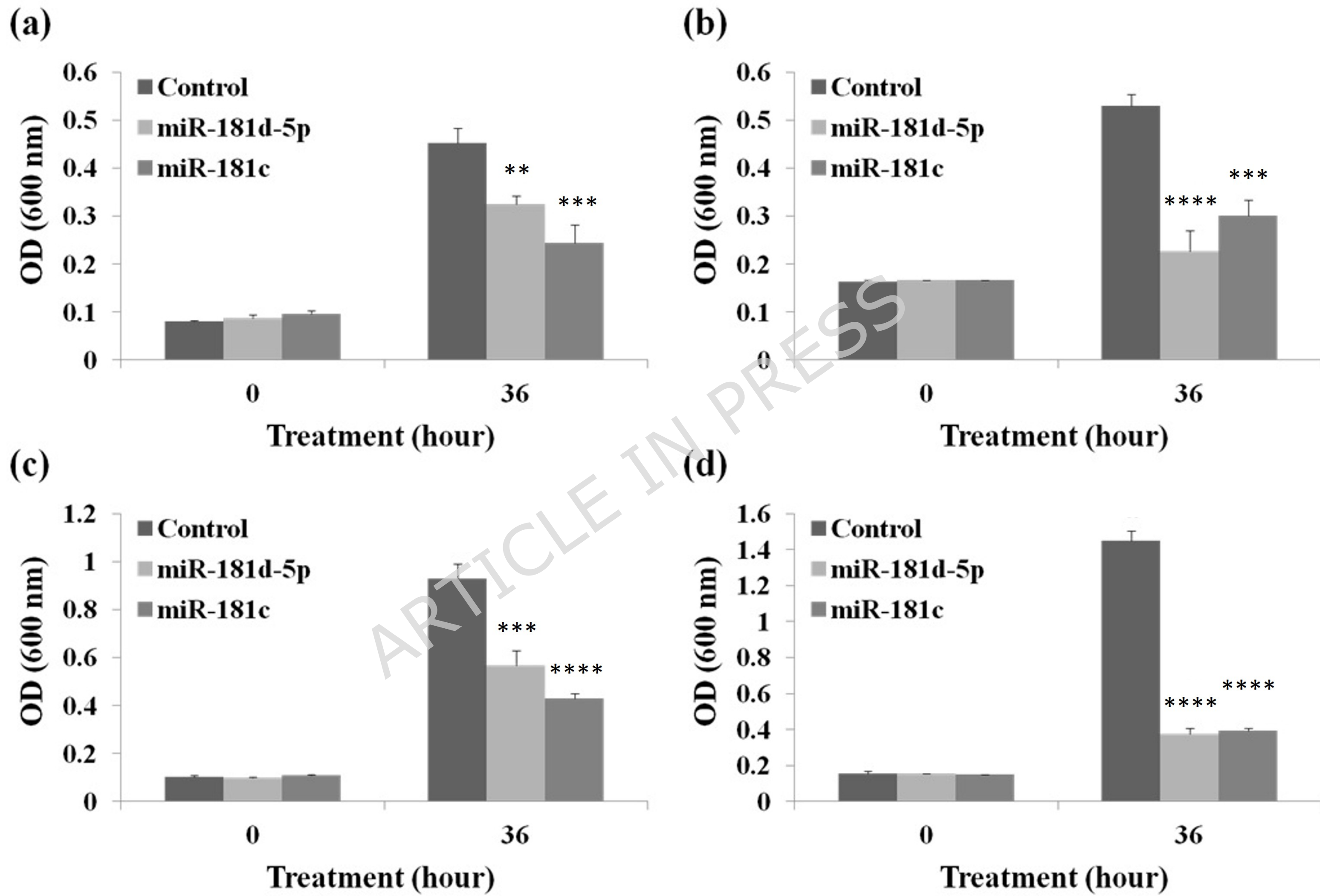

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