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“Isolation, characterization and evaluation of immunomodulatory effects of probiotic-derived extracellular vesicles”

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Abstract

In recent years, increasing attention has been directed toward extracellular vesicles (EVs), which are small particles released by all cell types both eukaryotic and prokaryotic ones. EVs mediate intercellular communication through the transfer of bioactive cargo. Among bacterial EVs, probiotic-derived extracellular vesicles (pEVs) have emerged as promising cell-free postbiotic candidates, owing to their capacity to modulate immune responses. While EVs from Gram-negative (Gram-) bacteria have been extensively studied, Gram-positive (Gram+) pEVs remain poorly explored. In this thesis, we aimed to isolate, characterize, and evaluate the immunomodulatory effects of pEVs derived from two Gram+ probiotic strains, namely *Lactiplantibacillus plantarum* (LP) and *Lacticaseibacillus paracasei* (LPC) were isolated by ultracentrifugation and purified by size-exclusion chromatography (SEC). Characterization of concentration, size distribution and morphology were analysed by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). Cell viability and immunomodulatory effects were evaluated in activated peripheral blood mononuclear cells (PBMCs) using MTT assay and ELISA, respectively. On the other hand, the impact on rheumatoid arthritis-derived fibroblast-like synoviocytes (RA-FLS) models was investigated under cytokine-induced inflammatory conditions in both two-dimensional (2D) cultures and three-dimensional (3D) models with assessment of cell viability and functional profiling.

Our results showed that LPC released a significantly higher number of EVs compared to LP, while both strains showed comparable size. Both LP-EVs and LPC-EVs were well tolerated by all tested cell types, showing no cytotoxicity. PEVs did not significantly modulate cytokine secretion in activated PBMCs, whereas in inflamed RA-FLS models, pEVs modulated lubricin expression specifically under 3D inflammatory culture conditions. Collectively, these findings reveal a strain- and model-dependent effect of pEVs on RA-FLS and support their further investigation as potential modulators of joint homeostasis.

Introduction

1. Extracellular vesicles (EVs): definition and classification

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles actively released by all cell types, including both eukaryotic and prokaryotic organisms¹. EVs represent a heterogeneous population of nanosized vesicles differing in size, biogenesis pathways, and molecular cargo². Once considered cellular waste products, EVs are now recognized as key mediators of intercellular communication and modulators of physiological and pathological processes^{2–4}. Depending on their cellular origin, EVs can be broadly classified into eukaryotic-derived and bacteria-derived vesicles³.

1.1 Eukaryotic-derived EVs

According to the minimal information for studies of extracellular vesicles (MISEV2023) guidelines, EVs can be classified using different criteria, including size, biogenesis, biochemical composition, and cellular origin. Although size-based classification remains one of the most widely used approaches, EV populations often exhibit overlapping size distributions and lack universally accepted size thresholds. Therefore, additional parameters should be considered to accurately define EV subpopulations⁵.

Based on their size and biogenesis, eukaryotic EVs can be categorized into exosomes, microvesicles (MVs), and apoptotic bodies². Exosomes originate from the endosomal pathway. Specifically, inward budding of the endosomal membrane leads to the formation of intraluminal vesicles (ILVs) which accumulate within multivesicular bodies (MVBs). When the MVB membrane fuses with the plasma membrane, the ILVs are released into the extracellular space and are then termed exosomes^{2,4,6}. Exosomes have a typical size range of 30–150 nm^{4,6}. MVs, also referred to as ectosomes⁷, are released through the outward budding of the plasma membrane, generating vesicles considerably larger than exosomes ranging in diameter from 150 to 1000 nm⁷. Apoptotic bodies represent the third class of EVs, being the largest in size (500–5000 nm) and are released during programmed cell death, known as apoptosis⁸.

Regardless of their differences in size and biogenesis, all EVs can encapsulate and transport various types of bioactive molecular cargos including proteins, lipids, and nucleic acids. The

composition of this cargo is influenced by the biogenesis mechanism, cell of origin, and physiological or pathological state of the parental cell^{2,4}.

1.2 Bacteria-derived EVs

Similar to eukaryotic cells, bacteria also release EVs (bEVs). They typically range from 20 to 400 nm in diameter and consist of a proteolipid bilayer enclosing bioactive molecules derived from the parental bacterium^{3,9}. Based on bacterial cell wall structure, bEVs are generally classified into two main groups: Gram-negative (Gram-) and Gram-positive (Gram+) bEVs (Figure 1).

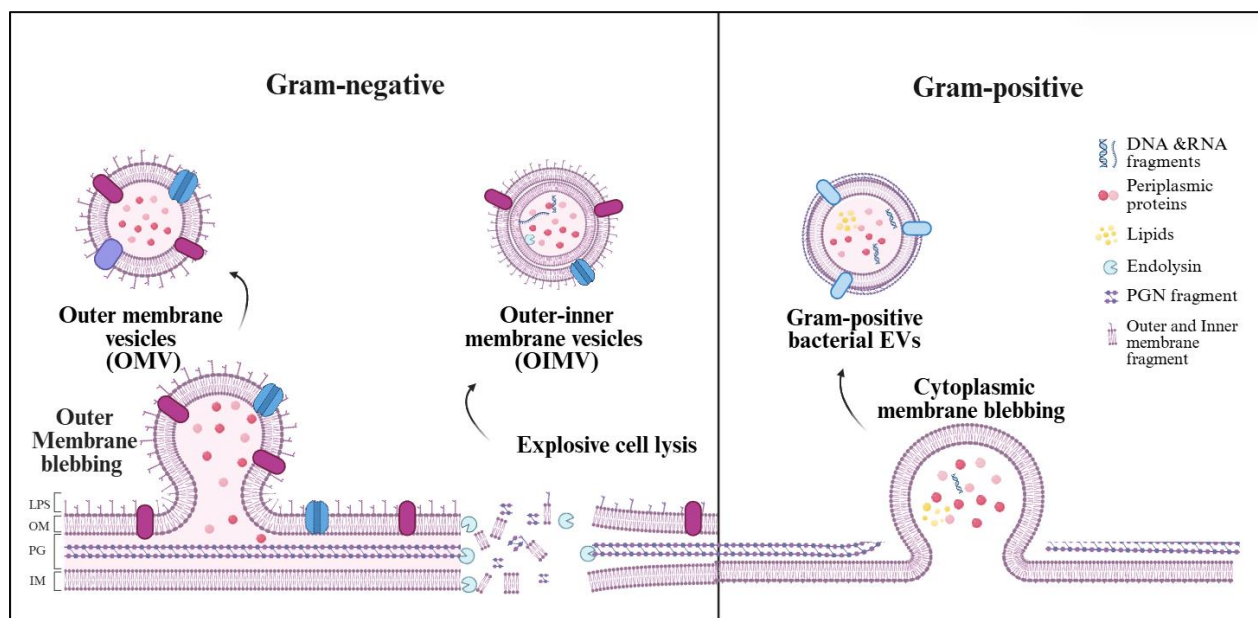


Figure 1) Biogenesis pathways of bacterial extracellular vesicles (bEVs). Gram-negative (Gram-) bacteria (left panel) generate outer membrane vesicles (OMVs) via outward blebbing of the outer membrane, driven by reduced lipoprotein anchoring to the peptidoglycan (PGN) layer or increased electrostatic repulsion between negatively charged lipopolysaccharide (LPS) molecules, and outer-inner membrane vesicles (OIMVs) via explosive cell lysis following PGN degradation by autolysins. Gram-positive (Gram+) bacteria (right panel) produce EVs through blebbing of the cytoplasmic membrane, where lipid-rich regions bulge outward to form vesicles^{2,3,16}. Figure created with BioRender.com.

Gram- bEVs are commonly referred to as outer membrane vesicles (OMVs). The first observation of OMVs was reported in 1965, when Bishop and Work identified these structures in *Escherichia coli* ATCC 12408^{10,11}. Despite extensive investigation, there are still no definitive models that

completely explain biogenesis and molecular mechanisms for their release⁹. However, two main mechanisms have been proposed: outer membrane (OM) blebbing and explosive cell lysis.

Regarding OM blebbing, two complementary models have been suggested. The first proposes that a reduced number of lipoproteins anchoring the OM to the peptidoglycan (PGN) layer weakens membrane stability, leading to localized bulging and OMVs release. Supporting this hypothesis, it was demonstrated that OMVs released from *E. coli* contain lower amounts of lipoprotein compared to the parental bacterium¹². The second model is related to the biophysical properties of lipopolysaccharides (LPS). An increased abundance of negatively charged LPS molecules enhances electrostatic repulsion within the OM, promoting membrane curvature and vesicle formation. For instance, OMVs produced from *Pseudomonas aeruginosa* under oxidative stress conditions are enriched in negatively charged LPS, supporting the role of membrane charge in vesiculation³.

The second proposed mechanism, explosive cell lysis, is associated with PGN remodeling mediated by autolysins. During PGN synthesis, localized activity of autolysins can generate cell wall destabilization and mechanical stress, which contributes to membrane rupture and initiation of vesicle formation. In fact, dysregulation or altered activity of autolysins involved in PGN remodelling has been shown to increase OMV production¹². During this process, weakening of the PGN layer allows the inner membrane to protrude into the periplasmic space, enabling the incorporation of cytoplasmic components into the forming vesicles. As a result, vesicles generated through this process contain material from both the inner and outer membranes and these vesicles are known as outer-inner membrane vesicles (OIMVs), as demonstrated in *P. aeruginosa*¹³.

OMVs contain a diverse range of biomolecules including components common to all bEVs, such as proteins and lipids, along with Gram- specific molecules including LPS and OM proteins. Supporting this, early research in 1998 detected muramic acid, a structural PGN component, in OMVs isolated from *Porphyromonas gingivalis*¹⁴. Furthermore, *P. aeruginosa* cultured under oxidative stress selectively packages negatively charged LPS into its OMVs. In contrast, OIMVs also encapsulate cytoplasmic molecules, including DNA and RNA fragments as first reported by Beveridge in 1995 for *P. aeruginosa*-derived EVs¹²⁻¹⁴.

Unlike Gram- bacteria, the ability of Gram+ bacteria to release EVs was recognized only later. For a long time, the presence of a thick and highly cross-linked PGN layer was considered a structural

barrier incompatible with vesicle release, leading to the assumption that Gram⁺ organisms were unable to generate EVs^{15,16}. However, subsequent studies have definitively demonstrated that these microorganisms actively release EVs, typically ranging from approximately 20 to 500 nm in diameter. The first Gram⁺ species in which EV production was described were *Staphylococcus aureus* and *Bacillus subtilis*³. Following these initial observations, EV release has been reported across a broad range of Gram⁺ bacteria including pathogenic ones as *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes* as well as probiotic strains belonging to *Lactobacillus* spp., and *Bifidobacterium longum*^{15,17}.

Despite increasing evidence, a definitive mechanism explaining also Gram⁺ bEVs formation is still lacking. Nevertheless, current models converge on a regulated membrane blebbing process originating from the cytoplasmic membrane. In this context, EV formation is proposed to initiate within lipid-enriched domains of the cytoplasmic membrane, where localized curvature leads to outward bulging of the membrane. As vesicle protrusion progresses, the surrounding PGN layer must be locally remodeled or weakened, a process often associated with the activity of autolysins and other cell wall-modifying enzymes. For instance, in *S. aureus*, reduced PGN cross-linking and exposure to β -lactam antibiotics greatly increase EV release. Nevertheless, proteomic analyses in Gram⁺ bacteria such as *Bacillus anthracis*, *L. monocytogenes*, *Streptococcus pyogenes*, *Propionibacterium acnes* and mycobacterial species have detected autolysins within EVs, further suggesting the active role of cell wall remodeling in vesicle formation¹⁸. This localized degradation enables membrane protrusions to traverse the rigid cell wall structure and eventually detach, resulting in the release of mature vesicles into the extracellular space¹⁵.

In contrast to Gram⁻ bEVs, Gram⁺-derived ones do not contain periplasmic components due to the absence of a defined periplasmic space in these microorganisms. Still, these vesicles transport a diverse repertoire of bioactive molecules derived from the parental cell, including fatty acids, phospholipids, cytoplasmic and membrane-associated proteins, lipoteichoic acids, PGN fragments, and nucleic acids^{13,15,19}. For example, vesicles from *S. aureus* have been shown to contain numerous cytoplasmic proteins, while DNA and RNA were detected in EVs from *Streptococcus sanguinis*, and *S. aureus*. Lipoprotein enrichment has also been reported in vesicles from *S. pneumoniae*, *S. pyogenes*, *B. subtilis*, and *Mycobacterium tuberculosis*. Accordingly, the composition of this cargo reflects both the cellular origin and the physiological state of the producing bacterium, contributing to the biological functions of Gram⁺ bEVs^{15,18}.

2. Isolation, purification and characterization of EVs

Currently, a wide range of methodologies is employed for the isolation and purification of EVs. However, as emphasized by the MISEV2023 guidelines, no single technique can be considered a universal gold standard due to the intrinsic heterogeneity of EV populations and the variability of biological samples⁵. Consequently, the selection of an isolation strategy should be guided by the specific EV source, downstream application, and the desired balance between purity, yield, and scalability. Among the most employed isolation approaches are ultracentrifugation (UC), size exclusion chromatography (SEC), tangential flow filtration (TFF), immunoaffinity (IA) capture, polymer-based precipitation, flow cytometry and microfluidic technologies (Figure 2). UC remains one of the most widely used methods and relies on sequential centrifugation steps to separate EVs according to their size and density. Alternatively, SEC separates particles based on hydrodynamic size, allowing efficient removal of soluble proteins and other low-molecular-weight contaminants. TFF has gained increasing attention due to its scalability and suitability for large-volume processing, whereas IA-based methods exploit specific surface markers to selectively enrich defined EV subpopulations^{20,21}.

More recently, microfluidic platforms have emerged as promising tools for rapid and highly controlled EV isolation, particularly in diagnostic applications. In addition, combined multi-step approaches integrating two or more isolation techniques are frequently employed to improve EV purity and recovery, thereby overcoming some of the limitations associated with individual methods^{22,23}.

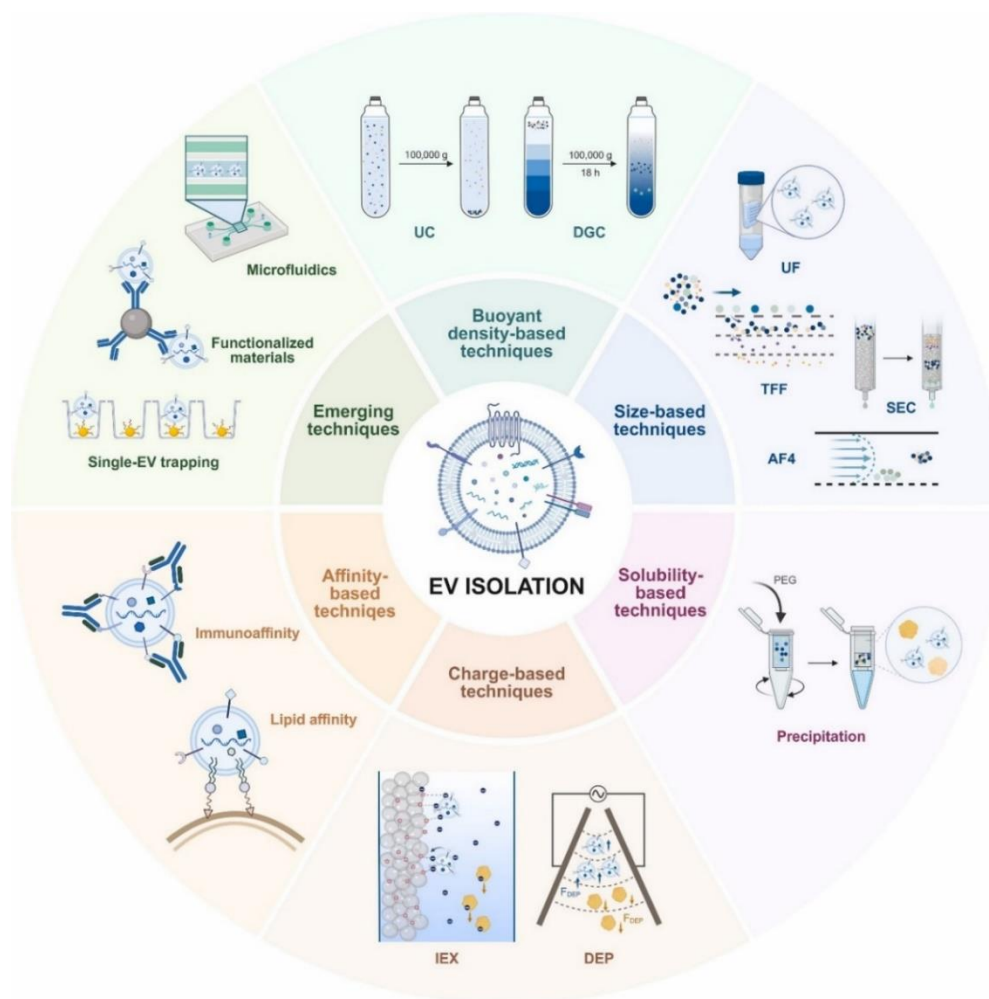


Figure 2) Overview of EV isolation techniques classified by the physicochemical properties they exploit. Isolation strategies are grouped into approaches based on buoyant density, particle size, solubility, surface charge, and affinity, alongside emerging techniques such as microfluidics, functionalized materials, and single-EV isolation methods²⁰.

Following isolation, EV characterization is essential to confirm the presence of vesicles and evaluate their physicochemical and biological properties. According to MISEV2023 recommendations, characterization should include the assessment of particle size, concentration, morphology, and molecular composition⁵.

Physical characterization is commonly performed using nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and resistive pulse sensing (RPS), which provide information regarding particle size distribution and concentration²³. Flow cytometry enables multiparametric analysis of EV populations through the detection of surface-associated markers and other phenotypic characteristics. Moreover, this technique allows the rapid analysis of large numbers of

particles, although its sensitivity remains limited for the detection of smaller EV populations²⁴. Morphological characterization is typically achieved through electron microscopy-based techniques. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and cryo-electron microscopy (cryo-EM) provide high-resolution visualization of EV structure and morphology, enabling confirmation of vesicular integrity and assessment of ultrastructural features²¹. Beyond physical characterization, the biological properties of EVs are largely determined by the study of their molecular cargo. Consequently, omics-based approaches, including proteomic, transcriptomic, metabolomic and lipidomic analyses are increasingly employed to identify potential functional biomarkers. Although these techniques provide comprehensive molecular profiling, their widespread implementation is currently limited by high costs, specialized instrumentation, and complex data analysis workflows²⁴.

Despite the availability of numerous isolation and characterization methods, each approach possesses inherent advantages and limitations. No single technique is capable of simultaneously achieving optimal EV recovery, purity, and preservation of biological activity across all sample types. Therefore, current consensus in the EV field supports the use of complementary and orthogonal methodologies, combining multiple isolation and characterization techniques to improve the reliability, reproducibility, and standardization of EV studies^{22,25,26}.

3. Gut microbiota and EVs

The human microbiota refers to the community of all microorganisms inhabiting the human body including not only bacteria, but also fungi, archaea, viruses, and protozoa²⁷. Although microbial communities are present across multiple body sites such as the skin, respiratory tract, and urogenital system, the gastrointestinal tract harbors the largest and most diverse microbiota. The gut microbiota is dominated by Gram+ genera including *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Streptococcus*, as well as Gram- genera such as *Bacteroides* and *Escherichia*²⁸. Its composition is highly dynamic and influenced by diet, lifestyle, age, and environmental exposure^{29,30}.

Beyond its metabolic and structural functions, the gut microbiota plays a central role in maintaining host immune homeostasis through the production of bioactive metabolites, such as

short-chain fatty acids (SCFAs) including acetate, propionate, butyrate, secondary bile acids, and tryptophan-derived metabolites^{27,28}. Many of these bioactive compounds are packaged into microbiota-derived EVs and can act locally or reach distal organs via EV-mediated transport. Microbiota-derived EVs represent a key mechanism of long-distance intercellular communication, enabling bacteria to influence host cells beyond their local environment and extending microbial signalling to peripheral tissues and immune compartments, thereby contributing to systemic host–microbe crosstalk³¹.

3.1 Gut microbiota-derived EVs in immune regulation

The gut microbiota is a fundamental regulator of immune system development and homeostasis, acting through both direct microbial-host interactions and EV-mediated communication. A central aspect of this crosstalk involves pattern recognition receptors (PRRs) and dendritic cells. These receptors recognize microbial-associated molecular patterns and activate downstream signaling pathways that regulate cytokine production and immune cell differentiation^{31–33}.

Beyond direct receptor engagement, bEVs serve as key carriers of bioactive microbial metabolites to host immune cells, mediating an additional layer of crosstalk between the gut microbiota and the host immune system. Among these cargo molecules, SCFAs (such as acetate, propionate, and butyrate), bile acids (BAs) and tryptophan-derived metabolites are key immunomodulatory effectors. While acetate is produced by most gut bacterial species, like *Bacteroides* spp., *Bifidobacterium* spp. and *Prevotella* spp., propionate and butyrate production is confined to specific bacterial species, such as *Bacteroidetes* and *Bacillota* phylum respectively³⁴. BEV-delivered SCFAs and BAs orchestrate both innate and adaptive immune responses by G protein-coupled receptors, nuclear receptors, and epigenetic pathways. These pathways induce regulatory T cells (Tregs) that secrete anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor beta (TGF- β), promote alternatively activated macrophages (M2) polarization, suppress nuclear factor- κ B (NF- κ B)-dependent inflammatory cytokines in macrophages and dendritic cells. Moreover, bEV-delivered SCFAs reinforce barrier integrity via inducing production of IL-22, and maintain balanced T helper 1 and 17 cell (Th1/Th17) activity^{35,36}.

3.2 Gut dysbiosis as a driver of pathological processes

The gut microbiota plays a fundamental role in maintaining host homeostasis through the regulation of nutrient metabolism, epithelial barrier integrity, and immune system maturation. Dietary shifts, aging, infections, antibiotic use, chronic inflammation, and genetic predisposition are different factors that lead to an unbalanced gut microbial community, referred to as gut dysbiosis. The most typical features of dysbiosis are a decrease in the microbial diversity, an overgrowth of pathobionts, and alterations in microbial metabolic activity³⁷. These alterations reduce stability of the mucosal barrier, increase epithelial permeability and facilitate the translocation of microbial products into the circulation, ultimately promoting systemic inflammation and immune dysregulation³².

Several studies have demonstrated that dysbiosis is associated with a reduction in beneficial SCFA-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia hominis*, two butyrate producers within the *Firmicutes* phylum whose decreases correlate with reduced fecal SCFA levels and increased disease activity in ulcerative colitis patients³⁸. These microorganisms contribute to intestinal homeostasis through the production of butyrate and other metabolites that support epithelial barrier function and regulatory immune responses. Conversely, dysbiotic microbiota often exhibits an increased abundance of potentially pro-inflammatory taxa. For example, expansion of *Enterobacteriaceae*, including adherent-invasive *E. coli*, is linked to the pathogenesis of Crohn's disease or ulcerative colitis³⁹.

The resulting imbalance promotes the activation of inflammatory pathways, disruption of mucosal tolerance, and chronic stimulation of the host immune system. Accumulating evidence suggests that many of the systemic consequences of dysbiosis are mediated through EVs. The enrichment of pathobionts during dysbiosis is associated with the release of EVs carrying increased levels of immunostimulatory molecules capable of activating PRRs, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors. Activation of these pathways promotes the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF- α), IL-1 β , IL-6, and IL-17, contributing to chronic systemic inflammation and loss of immune tolerance. Consequently, EV-mediated signaling has emerged as a critical component of the gut-organ axis linking intestinal microbial imbalance to pathological processes occurring in distant tissues. For instance, in mice fed with a high-fat diet, gut dysbiosis and intestinal mucosal

injury are accompanied by packaging of high mobility group box 1 (HMGB1) protein into exosomes, which have been proposed to deliver it to the liver and promote hepatic steatosis⁴⁰.

3.3 Gut dysbiosis and rheumatoid arthritis (RA)

Among the diseases associated with gut dysbiosis, rheumatoid arthritis (RA) has emerged as a well-characterized systemic autoimmune disorder in which alterations of the gut microbiota and host-microbe interactions are increasingly recognized as relevant to disease pathogenesis⁴¹.

RA is a chronic systemic autoimmune disease characterized by persistent synovial inflammation, pannus formation, cartilage destruction, and bone erosion²⁸. Several studies showed significant alterations in the gut microbiota of RA patients compared with healthy individuals. In particular, increased abundance of *Prevotella copri* has been consistently observed in naïve patients with early RA and has been associated with enhanced differentiation of Th17 cells and elevated IL-17 production²⁸.

Moreover, *Fusobacterium nucleatum*-derived EV enrichment was observed in fecal samples of RA patients. These bEVs activate TLR4 signaling in intestinal epithelial cells, upregulating downstream effectors such as extracellular signal-regulated kinase (ERK), cAMP response element-binding protein (CREB), and NF- κ B, which in turn promote the release of pro-inflammatory cytokines including IL-8 and TNF- α . Once released into the gut lumen, these vesicles can translocate to synovial tissues via systemic circulation, where they polarize macrophages toward the pro-inflammatory M1 phenotype (IL-1 β , IL-6, TNF- α), thereby contributing to joint inflammation through what is proposed as a gut-joint axis.

Similarly, *P. gingivalis*-derived EVs, originating in the oral cavity, trigger an even stronger inflammatory response than the bacterium itself, activating macrophages and epithelial cells to release TNF- α and IL-6. Notably, these bEVs carry the enzyme porphyromonas peptidylarginine deiminase (PPAD), which citrullinates host proteins, a key step driving the formation of anti-citrullinated protein antibodies (ACPAs) and immune complexes (ICs), both of which are hallmarks of RA autoimmunity.

Both *F. nucleatum*- and *P. gingivalis*-derived EVs are therefore proposed to translocate through the bloodstream to the joint, where local macrophage activation contributes to the synovial inflammation characteristic of RA, as shown in Figure 3^{41,42}.

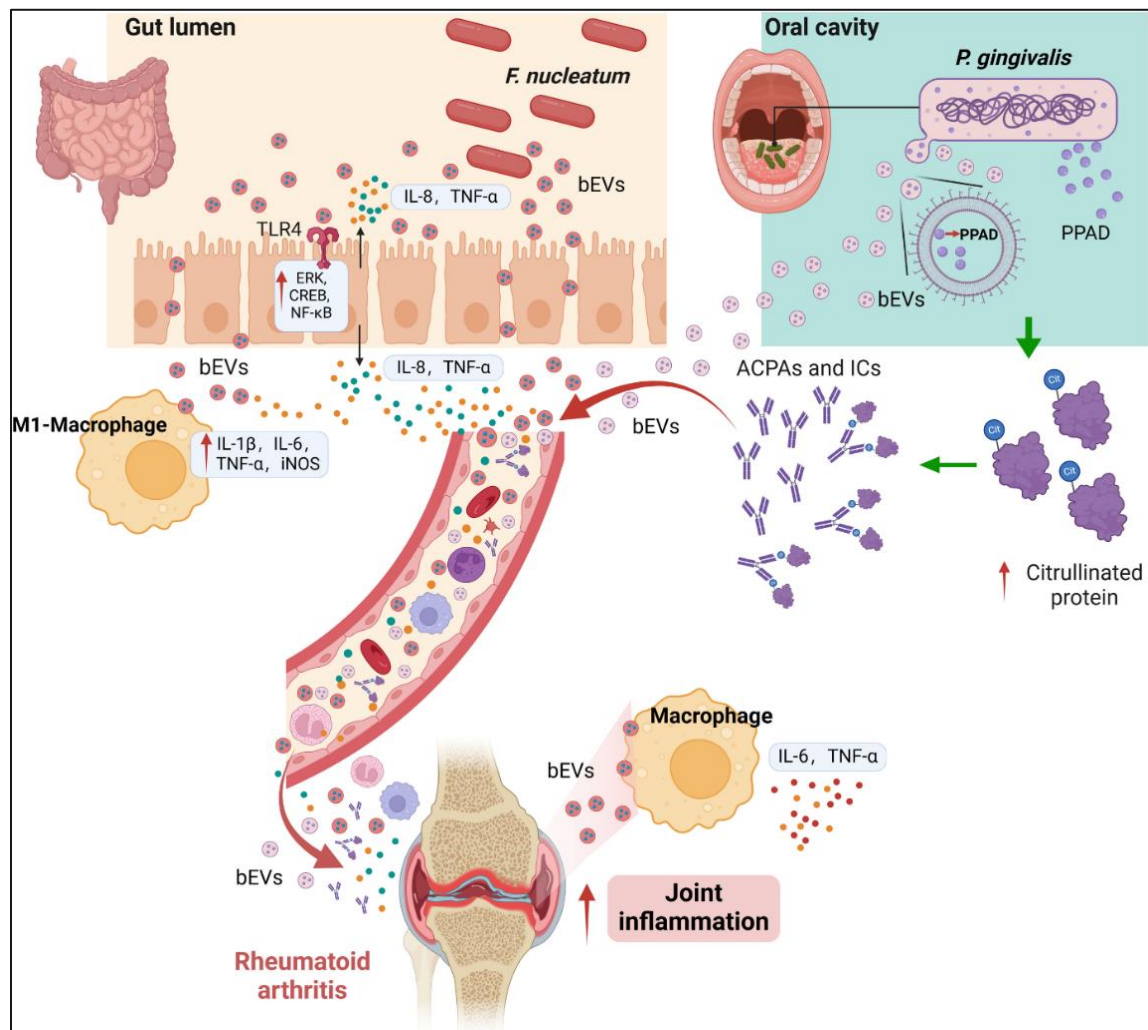


Figure 3) Proposed pathogenic roles of bacterial extracellular vesicles (bEVs) in rheumatoid arthritis (RA). Gut-derived *Fusobacterium nucleatum* bEVs activate toll like receptor (TLR) 4 signaling in intestinal epithelial cells, upregulating ERK, CREB, and NF-κB with release of IL-8 and TNF-α, then enter the circulation and polarize macrophages toward the pro-inflammatory M1 phenotype (IL-1β, IL-6, TNF-α), contributing to joint inflammation via the gut-joint axis. Oral *Porphyromonas gingivalis* bEVs carry peptidylarginine deiminase (PPAD), which citrullinates host protein and drives the formation of anti-citrullinated protein antibodies (ACPAs) and immune complexes (ICs). Both bEVs populations are proposed to reach the joint via the bloodstream, where macrophage activation (IL-6, TNF-α) contributes to synovial inflammation characteristic of RA⁴¹.

Conversely, RA patients frequently exhibit reduced abundance of beneficial bacteria with immunoregulatory functions, including *Bacteroides uniformis*, *Bacteroides plebeius*, *Bifidobacterium bifidum*, *Haemophilus* spp., alongside a decrease in *Faecalibacterium*, a well-characterized butyrate producer⁴³. Alterations in *Lactobacillus* spp. populations have also been reported in early RA, with *L. salivarius*, *L. iners*, and *L. ruminis* being the predominant species, while *L. mucosae* was exclusively detected in naive RA patients. However, whether these alterations contribute to RA pathogenesis or represent a compensatory host response remains unclear, warranting further investigation⁴⁴.

Despite these insights into the role of gut dysbiosis in RA, no definitive cure for this disease currently exists. Current therapeutic strategies, including nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs) like methotrexate (MTX), and biologic agents (bDMARDs) targeting TNF- α and other cytokines, mainly aim to reduce inflammation and slow disease progression⁴⁵.

However, a substantial proportion of patients do not achieve sustained remission or exhibit an incomplete response to these treatments. In addition, these therapies are associated with relevant adverse effects: long-term use of NSAIDs and corticosteroids increases the risk of gastrointestinal bleeding, metabolic disturbances such as diabetes, and generalized immunosuppression; conventional DMARDs require continuous monitoring due to hepatotoxicity and bone marrow suppression; and biologic agents, while highly effective in selected patients, are associated with an increased risk of serious infections and, in some cases, malignancies such as lymphoma⁴⁶. Importantly, several of these pharmacological interventions have also been associated with additional perturbations of gut microbial composition, further exacerbating dysbiosis and potentially contributing to treatment variability and reduced efficacy. For instance, MTX has a significant impact on the gut microbiome of RA patients according to its dose. While low doses increase microbial richness and diversity, high doses reduce intestinal bacterial diversity, mainly by decreasing anaerobic *Firmicutes* and increasing *Bacteroidetes*⁴⁷.

These limitations highlight the critical need for alternative therapeutic strategies aimed not only at suppressing inflammation but also at restoring immune and microbial homeostasis. In this context, probiotic-derived metabolites and EVs have emerged as promising candidates, as they can actively modulate host immune responses, reinforce epithelial barrier integrity, and counteract dysbiosis-

associated inflammatory signaling. PEVs represent a novel class of bioactive nanostructures capable of delivering immunomodulatory cargo to host cells, thereby offering a potential strategy to re-establish gut-immune balance and to complement or improve current therapeutic approaches in RA management.

4. Probiotic-derived EVs properties

In recent years, increasing attention has been directed toward the properties and potential of pEVs as key mediators of host-microbiota communication. The molecular cargo enclosed within these vesicles, including proteins, lipids, polysaccharides, and nucleic acids, is highly dependent on the producing bacterial strain and environmental conditions, and largely determines their biological activity. Beyond their well-established contribution to intestinal homeostasis, pEVs are now recognized as systemic regulators capable of modulating immune responses at both local and distal sites, thereby extending the functional impact of probiotic bacteria beyond the gut lumen⁴⁸.

PEVs exhibit immunomodulatory properties through coordinated mechanisms on both innate and adaptive immunity. In innate immunity, pEVs interact with PRRs, such as TLRs on macrophages, dendritic cells, and epithelial cells, reducing pro-inflammatory signaling cascades such as NF- κ B and the mitogen-activated protein kinase (MAPK), downregulating cytokines including TNF- α , IL-6, and IL-1 β , and promoting anti-inflammatory cytokines such as IL-10 and TGF- β . For instance, EVs from *Lactobacillus paracasei* suppress TNF- α secretion in macrophages and promote the human acute monocytic leukemia (THP-1) cell differentiation toward the anti-inflammatory M2 phenotype, and inducing anti-inflammatory effects in human skin^{49,50}. In adaptive immunity, pEVs induce Tregs and suppress differentiation of pro-inflammatory Th1 and Th17 subsets, contributing to the control of chronic inflammation⁴⁹. For example, exposure of dendritic cells to EVs from *Bifidobacterium bifidum* induces Treg cells and increases anti-inflammatory cytokine secretion⁵¹.

Beyond these mechanisms, pEVs exhibit similar immune-mediated effects in several models. In colorectal cancer, *Akkermansia muciniphila* OMVs polarize macrophages toward the tumor-killing phenotype to limit cancer progression, while *Lactocaseibacillus rhamnosus* EVs in addition to anti-programmed cell death protein 1 (anti-PD-1) therapy increase butyrate levels and enhance CD8⁺ T-cell cytotoxic activity in tumor tissue. In skin wound healing, *Lactiplantibacillus*

plantarum EVs attenuate chronic inflammation by inducing M2 macrophage polarization, accelerating tissue repair⁵².

5. AIM of the thesis

The aim of this thesis is to isolate and characterize EVs derived from two probiotic bacterial strains and to evaluate their immunomodulatory and anti-inflammatory potential *in vitro*. Specifically, the effects of pEVs on activated peripheral blood mononuclear cells (PBMCs) and fibroblast-like synoviocytes (FLS) from patients with RA are investigated using bidimensional (2D) and three-dimensional (3D) culture models.

Materials and methods

1. Bacterial cultures

Probiotic bacteria *Lactiplantibacillus plantarum* PBS067 (LP) and *Lacticaseibacillus paracasei* LPC1114 (LPC) were kindly provided by Synbalance s.r.l. Bacterial cultures were prepared from frozen stocks that were thawed and initially inoculated at 1% (v/v) in 10 mL of standard MRS medium (VWR, Radnor, Pennsylvania, USA) for overnight growth at 37°C. On the next day, 1% (v/v) of the culture was inoculated into 30 mL of ultracentrifuged custom made MRS medium (EV-free medium) and incubated at 37 °C for 24 hours.

2. Isolation, purification and characterization of EVs

After incubation, bacterial cultures from each strain were centrifuged at 3,000 rpm for 15 minutes at 4°C to pellet bacterial cells. The supernatants were filtered using 0.22 µm cellulose acetate syringe filters (ClearLine, Dominique Dutscher SAS, France) to eliminate any remaining bacterial cells. EVs were isolated by ultracentrifugation at speed of 100,000 g for 4 hours at 4°C using a swing rotor (Sorvall™ WX+ Ultracentrifuge Series; Thermo Scientific™; Massachusetts, U.S).

The vesicle pellets were resuspended in 500 μ L of freshly filtered sterile phosphate buffered saline (PBS 1X). For further purification, size exclusion chromatography (SEC) was performed using qEV columns (35nm; IZON Science; Christchurch, New Zealand). The isolated EVs suspension was loaded on an SEC column, and fractions enriched in EVs were collected for downstream applications. Purified EV fractions were stored at 4°C until further use. Prior to application in functional assays, EV suspensions (LP-EVs and LPC-EVs) were filtered through 0.22 μ m filters to ensure sterility.

EV concentration and size distribution were determined by NTA (Nanosight, NS300) following a 1:40 dilution in PBS 1X. The morphology and structural integrity of purified EVs were assessed by transmission electron microscopy (TEM). For negative staining, glow discharge was carried out on to carbon-coated collodion 400 mesh nickel grids (Gilder). 15 μ l of each sample were adsorbed on the grid for 2 min, washed in two drops of water and stained with 2% aqueous uranyl acetate (Electron Microscopy Sciences) for 1 min. Grids were visualised in a JEOL 1400 Flash electron microscope (operating at 100 kV). Micrographs were taken with a Gatan OneView digital camera at various magnifications.

3. Evaluation of the immunomodulatory effects of pEVs on activated peripheral blood mononuclear cells (PBMCs)

3.1 PBMCs isolation and activation

PBMCs were isolated from fresh blood obtained from six healthy volunteer donors. Blood was diluted at a 1:1 ratio with PBS 1X and carefully layered over Lympholyte (Cedarlane, Burlington, Ontario, Canada) at a 1:1 ratio. Samples were centrifuged at 1800 rpm for 20 minutes with acceleration 1 and deceleration 0 to allow proper density gradient stratification. After centrifugation, PBMCs were carefully collected. Cells were washed with PBS 1X by centrifugation at 1500 rpm for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in complete Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10% Fetal Bovine Serum (FBS), 0.1% gentamicin, and 1% penicillin/streptomycin (Gibco™, Thermo Fisher scientific, Waltham, Massachusetts, USA) for counting.

PBMCs were activated by adding Phorbol 12-Myristate 13-Acetate (PMA) (1.25 µg/mL) and ionomycin (2.24 µg/mL; Sigma-Aldrich, Merck, Burlington, Massachusetts, United States) to the culture medium and incubated for 24 hours at 37°C. After activation, PBMCs were cultured for an additional 72 hours in RPMI supplemented with IL-2 (100 U/mL; PeproTech, Thermo Fisher Scientific; Waltham, Massachusetts, USA) to sustain cellular activation over time.

3.2 EV treatment

To investigate the immunomodulatory effects of pEVs under inflammatory conditions, a pre-treatment strategy was applied. Activated PBMCs were seeded at 50,000 cells/well in a 96-well plate. For experiments, cells were first stimulated with pEVs (cell:EVs ratio of 1:20,000) for 4 hours, and then further stimulated with LPS (1 µg/mL; Sigma-Aldrich; Burlington, Massachusetts, United States) for 48 hours. Cells treated with LPS, lipoteichoic acid (LTA, 1 µg/mL; Sigma-Aldrich; Burlington, Massachusetts, United States), or medium alone served as controls. After 48 hours, culture supernatants were collected for cytokine analysis by enzyme-linked immunosorbent assay (ELISA), while cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

3.3 Assessment of cell viability by MTT assay

After 48 hours of incubation, plates were centrifuged at 1,500 rpm for 5 min. Culture supernatants were carefully collected and stored at -80°C until cytokine analysis. The remaining cell pellets were used for the MTT assay. Briefly, MTT solution was added to each well to obtain a final concentration of 0.5 mg/mL. Plates were incubated for 3 hours at 37°C in the dark, allowing metabolically active cells to reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. Wells containing culture medium and MTT reagent only were included as blanks to correct for background absorbance. Following incubation, the formazan crystals were dissolved by adding acidified isopropanol (2 mM HCl in isopropanol). Absorbance was measured at 570 nm using a microplate spectrophotometer (Tecan), with blank values subtracted from all readings. Cell viability was expressed as a percentage relative to untreated control cells.

3.4 Quantification of cytokine production by ELISA

The concentrations of TNF- α , a pro-inflammatory cytokine, and IL-10, an anti-inflammatory cytokine, were quantified in culture supernatants using DuoSet kits (R&D Systems, Bio-Techne, Milan, Italy) according to the manufacturer's instructions. Briefly, 96-well plates were coated with 100 μ L of capture antibody and incubated overnight at room temperature. Wells were washed three times with 200 μ L of washing buffer (PBS 1X + 0.05% Tween 20) and blocked with 300 μ L of 1% bovine serum albumin (BSA) for 1 hour at room temperature. Following washing, 100 μ L of supernatants or serially diluted standards were added and incubated for 2 hours at room temperature. After washing, 100 μ L of detection antibody were added for 2 hours. Wells were washed and 100 μ L of Streptavidin-HRP were added for 20 minutes. After washing, 100 μ L of 3,3',5,5'-Tetramethylbenzidine (TMB) were added for 20 minutes in the dark and blue color appeared. The reaction was stopped by adding 50 μ L of stop solution and absorbance was measured at 450 nm using a spectrophotometer. Cytokine concentrations were interpolated from the standard curve.

4. Evaluation of the effects of pEVs on fibroblast-like synoviocytes (FLS) from RA patients in 2D and 3D culture models

4.1 Cell culture

Primary FLS isolated from patients with RA were provided by Queen Mary University of London (QMUL, London, UK). Cells were expanded in complete Dulbecco's Modified Eagle Medium F12 (DMEM-F12) supplemented with 10% FBS, 0.1% gentamicin, and 1% penicillin/streptomycin under standard culture conditions (37°C, 5% CO₂). Upon reaching >80% confluence, cells were detached by trypsinization and used at passage 3-6 for subsequent experiments.

4.2 Experimental design

The effects of LP-EVs and LPC-EVs were evaluated using both 2D monolayer cultures and 3D gelatin methacryloyl (GelMA) hydrogel cultures. Inflammation was induced by stimulation with

a cytokine cocktail containing TNF- α (20 ng/mL), IL-1 β (10 ng/mL), and IL-6 (10 ng/mL) (PeproTech, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

For each culture model, the following experimental groups were established:

1. Inflammatory control (CTR): cytokine stimulation only;
2. Cytokine-stimulated cells treated with LP-EVs;
3. Cytokine-stimulated cells treated with LPC-EVs.

EV treatment was done with a cell:EVs ratio of 1:20,000 for 72 hours.

4.2.1 2D culture

RA-FLS were seeded at a concentration of 2,500 cells/well in a 96-well plate. Each well contained 90 μ L of cell suspension in complete medium supplemented with the inflammatory cytokine cocktail (TNF- α , IL-1 β , and IL-6) and 10 μ L of LPEVs or LPC-EVs or PBS 1X (control). Plates were incubated for 72 hours (37°C, 5% CO₂).

4.2.2 3D GelMA hydrogel culture

Dehydrated GelMA was kindly provided by AO Research Institute (Davos, Switzerland). Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; Sigma-Aldrich; Burlington, Massachusetts, United States) was used as photoinitiation crosslinker for the hydrogel. Powders were weighed and sterilized separately by UV exposure for 20 min in closed microcentrifuge tubes. The powders were dissolved in PBS 1x supplemented with 10% FBS under agitation conditions for 1 hour at 60°C to obtain stock solutions containing 20% (w/v) GelMA and 2% (w/v) LAP.

The GelMA/LAP solution was mixed with the RA-FLS suspension to achieve final concentrations of 5% (w/v) GelMA and 0.3% (w/v) LAP. Cell density was adjusted to 5 x 10⁶ cells/mL. Drops of 5 μ L of the cell-laden GelMA suspension were dispensed onto the bottom surface of wells in a 96-well plate and photocrosslinked by UV exposure for 1 min. Following crosslinking, 90 μ L of complete medium containing the inflammatory cytokine cocktail were added to each well. Treated groups received 10 μ L of pEVs as in the 2D culture.

4.3 Viability assay

In order to confirm the cytocompatibility of pEVs (both in 2D and 3D), FLS viability was assessed using a Live/Dead fluorescence assay. Cells were stained with calcein-AM (2 μ M; BD Pharmingen™, BD Biosciences, USA) that labels metabolically active live cells with a green fluorescent color and propidium iodide (PI, 50 μ g/mL; Thermo Fisher scientific; Waltham, Massachusetts, USA) that labels dead cells with a red fluorescent color. Samples were incubated with staining solution for 20 minutes at room temperature in the dark, washed once with PBS, and imaged by a fluorescence microscope (Thunder, Leica Microsystems). Quantification of live and dead cells was performed using ImageJ (FIJI software).

4.4 Lubricin quantification

To assess intracellular lubricin expression (both in 2D and 3D), protein secretion was first inhibited by incubating samples with 1:1,000 GolgiPlug™ (brefeldin A; BD Biosciences; USA) for 4 hours. Samples were then washed with PBS 1X and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. After fixation, samples were washed with PBS 1X and stored at 4°C until the staining. Cells were permeabilized with 0.2% Triton (Sigma-Aldrich; Burlington, Massachusetts, United States) for 15 minutes, washed with PBS, and blocked with 4% BSA for 30 minutes at room temperature to prevent non-specific antibody binding. Samples were incubated with a rabbit anti-human lubricin primary antibody (Invitrogen™, ThermoFisher Scientific, Waltham, Massachusetts, USA) diluted 1:500 in 4% BSA for 1.5 hours at room temperature. Following PBS washes, samples were incubated with an anti-rabbit secondary antibody (Invitrogen™, ThermoFisher Scientific, Waltham, Massachusetts, USA) diluted 1:500 in 1% BSA for 1 hour at room temperature in the dark.

Nuclei were counterstained with Hoechst solution (1:250 dilution in PBS 1X) for 10 minutes. After washing with PBS, samples were imaged using a fluorescence microscope (Thunder). Quantitative analysis of lubricin expression was performed using ImageJ by measuring the mean fluorescence intensity normalized by the number of cells.

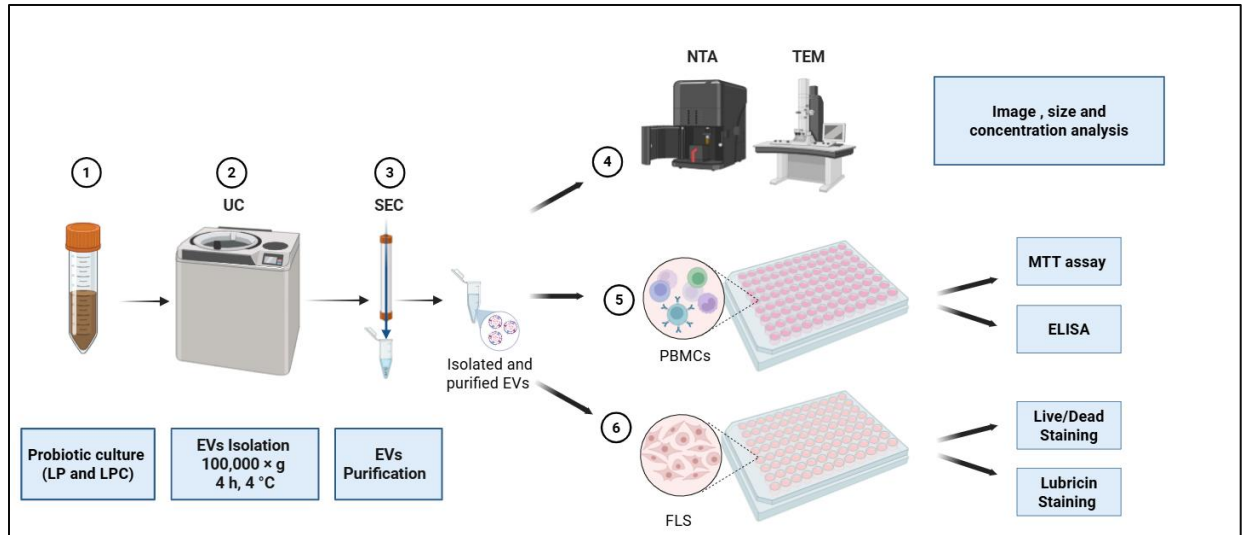


Figure 4) An overview of the experimental workflow. Probiotic-derived EVs (pEVs) were isolated from two different probiotic bacterial strains (*L. plantarum* and *L. paracasei*) using ultracentrifugation and purified via size exclusion chromatography (SEC). Characterization of the isolated pEVs was performed using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) to assess particle size distribution, concentration, and morphology. The immunomodulatory potential of pEVs was evaluated *in vitro* using two cell models: peripheral blood mononuclear cells (PBMCs) and fibroblast-like synoviocytes isolated from rheumatoid arthritis patients (RA-FLS) in both 2D and 3D models. Cell viability was assessed by MTT assay and Live/Dead staining, while functional responses were quantified by ELISA and lubricin quantification. Figure created with BioRender.com.

5. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 9; GraphPad Software, Inc., San Diego, CA, USA). For normally distributed data, comparisons between two groups were performed with the Student's t-test, whereas comparisons among three groups were performed by repeated-measures one-way ANOVA. For non-normally distributed data, comparisons among multiple groups were performed using the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. EV characterization experiments were performed using $n = 3$ independent bacterial cultures, PBMC experiments were performed using cells obtained from $n = 6$ healthy donors, and RA-FLS experiments were performed using cells derived from $n = 4$ different RA patients; all represent biological replicates. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

1. PEVs: isolation and characterization

Concentration and size distribution of pEVs isolated from both the probiotic strains (LP-EVs, LPC-EVs) were characterized by NTA (Figure 5).

To evaluate pEVs size distribution, the concentration was plotted against vesicle size. For LP-EVs (Figure 5a), the unpurified profile showed a broader distribution curve with a peak intensity reaching 6.50×10^9 particle/ml. After SEC purification, the curve shifted downward and showed a decreased peak intensity below 2.60×10^9 particle/ml with a narrower size distribution profile. For LPC-EVs (Figure 5b), a similar downward shift was observed post-purification. The unpurified sample demonstrated a sharp and clear peak maximizing at 4.44×10^{10} particle/ml. After SEC purification, the distribution remained sharp but with a reduced peak height maximized at 1.34×10^{10} particle/ml.

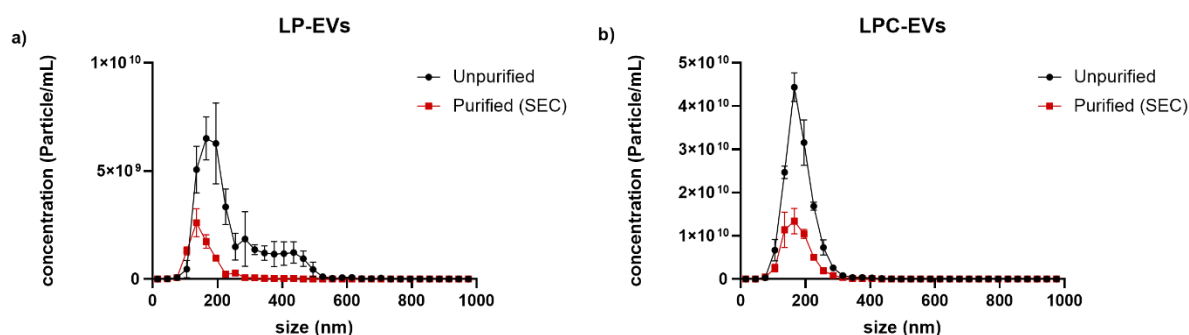


Figure 5) Size distribution profiles of probiotic EVs. Size distribution curves were plotted as concentration (particles/mL) versus vesicle size (nm) for unpurified and SEC-purified (a) LP-EVs and (b) LPC-EVs.

To evaluate the impact of purification, measurements were performed on both unpurified and purified samples across three independent replicates. For LP-EVs (Figure 6a), the mean particle concentration was significantly higher in the unpurified samples, reaching $3.30 \times 10^{10} \pm 8.25 \times 10^9$ particles/ml compared to $7.48 \times 10^9 \pm 1.13 \times 10^9$ particles/ml for purified ones.

A similar trend was observed for LPC-EVs, which had a significantly higher concentration of $1.37 \times 10^{11} \pm 9.90 \times 10^9$ particles/ml in unpurified conditions compared to $4.69 \times 10^{10} \pm 8.21 \times 10^9$ particles/ml after purification, as shown in Figure 6b. Overall, purification significantly reduced particle concentration in both strains.

To compare the production yield of the two probiotic strains, pEV concentrations were compared between LP- and LPC-EVs groups (Figure 6c). LPC strain showed a significantly higher production capacity. Specifically, the mean concentration of LPC-EVs reached $4.69 \times 10^{10} \pm 8.21 \times 10^9$ particle/ml, whereas the LP-EVs had a concentration of $7.48 \times 10^9 \pm 1.13 \times 10^9$ particle/ml.

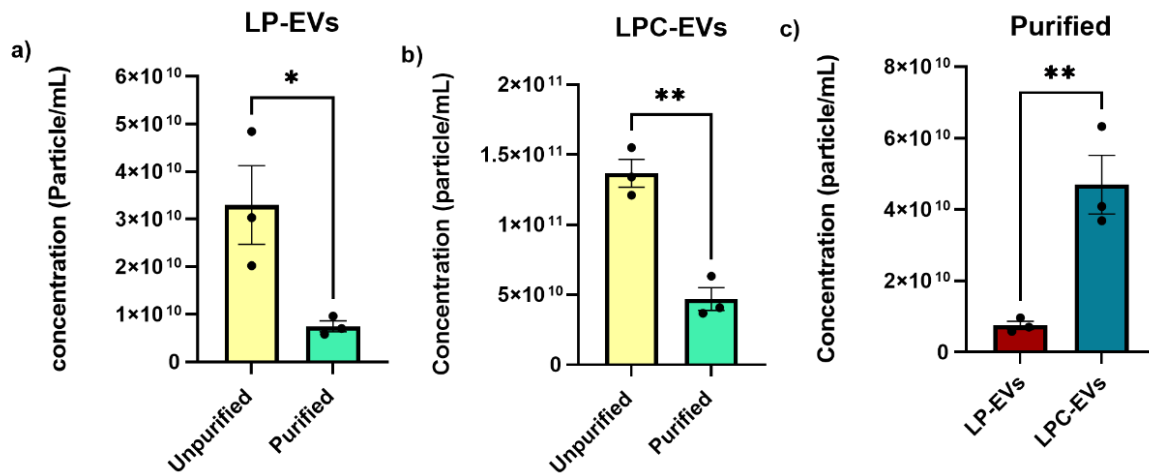


Figure 6) Concentration profiles of probiotic EVs. Comparative evaluation of EV concentrations (particles/mL) between unpurified and purified samples for both (a) LP and (b) LPC strains. (c) Comparison of EV concentrations between purified LP- and LPC-EVs. Data are presented as mean and SEM (n=3). Statistical analysis was performed using t-test (*p<0.05; **p<0.01).

Regarding particle size, the mean dimension of unpurified LP-EVs was significantly larger (232.87 ± 13.10 nm) compared to purified ones (160.77 ± 3.98 nm; $p = 0.006$) (Figure 7a). Conversely, for LPC-EVs, no difference in mean size was observed between the unpurified and purified EVs, averaging 181.00 ± 3.16 nm and 177.77 ± 7.48 nm respectively as shown in Figure 7b.

The mean particle size of the two probiotic strains was further compared after purification (Figure 7c). Purified LPC-EVs demonstrated a mean size of 177.77 ± 7.48 nm, slightly higher than that of purified LP-EVs (160.77 ± 3.89 nm). However, this difference was not statistically significant, indicating that vesicle size was comparable between the two strains after purification.

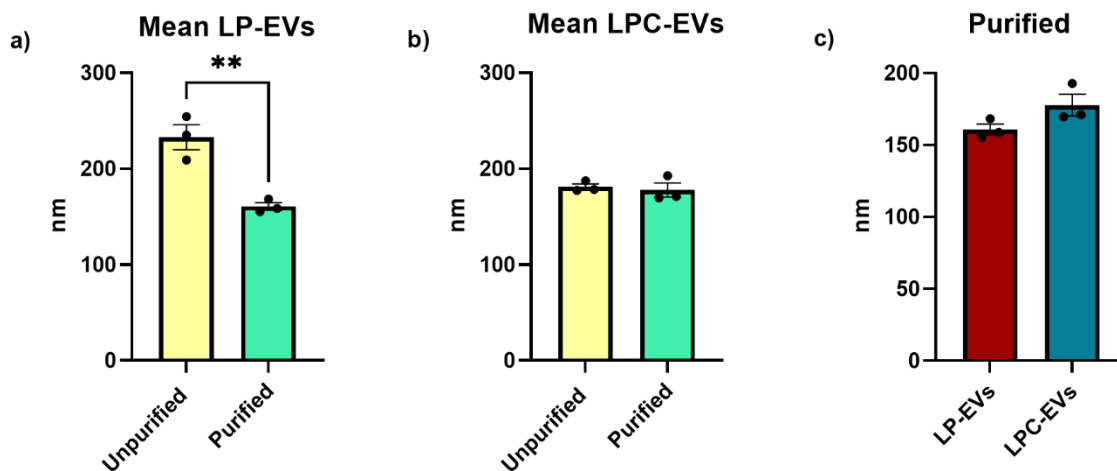


Figure 7) Mean particle size of pEVs before and after purification. Mean particle size (nm) of EVs derived from (a) LP and (b) LPC strains, before and after SEC purification. (c) Comparative analysis of mean particle size between purified LP-EVs and LPC-EVs. Data are presented as mean $\pm$ SEM ($n = 3$). Statistical significance was assessed using t-test (** $p < 0.01$).

For the mode size, unpurified LP-EVs had a higher mode of 175.03 ± 5.36 nm compared to 137.87 ± 4.50 nm for the purified ones showing a statistically significant difference (Figure 8a). For LPC-EVs, the mode values remained closely comparable between the unpurified (169.10 ± 2.15 nm) and purified (165.13 ± 9.88 nm) EVs (Figure 8b).

Further comparison between the mode particle size of the two probiotic strains was performed following purification (Figure 8c). Purified LPC-EVs demonstrated a mode size of 165.13 ± 9.88 nm, slightly higher than that of purified LP-EVs (137.87 ± 4.50 nm). However, this difference was not statistically significant, indicating that mode size of vesicles was comparable between the two strains after purification.

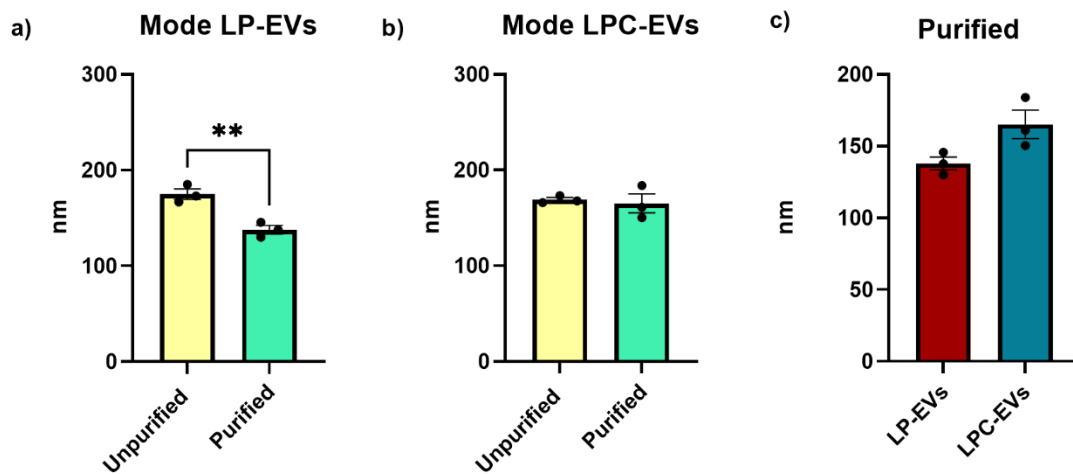


Figure 8) Mode particle size of pEVs with and without purification. Mode particle size (nm) of EVs derived from (a) LP and (b) LPC strains, before and after SEC purification. (c) Comparative analysis of mode particle size between purified LP-EVs and LPC-EVs. Data are presented as mean $\pm$ SEM ($n = 3$). Statistical significance was assessed using t-test.

To visualize the morphological features of the isolated vesicles, LP-EVs and LPC-EVs were examined by TEM (Figure 9). Both LP-EVs and LPC-EVs exhibited the characteristic spherical to cup-shaped morphology typical of EVs, with visible lipid bilayer membranes. No major morphological differences were observed between the two probiotic strains.

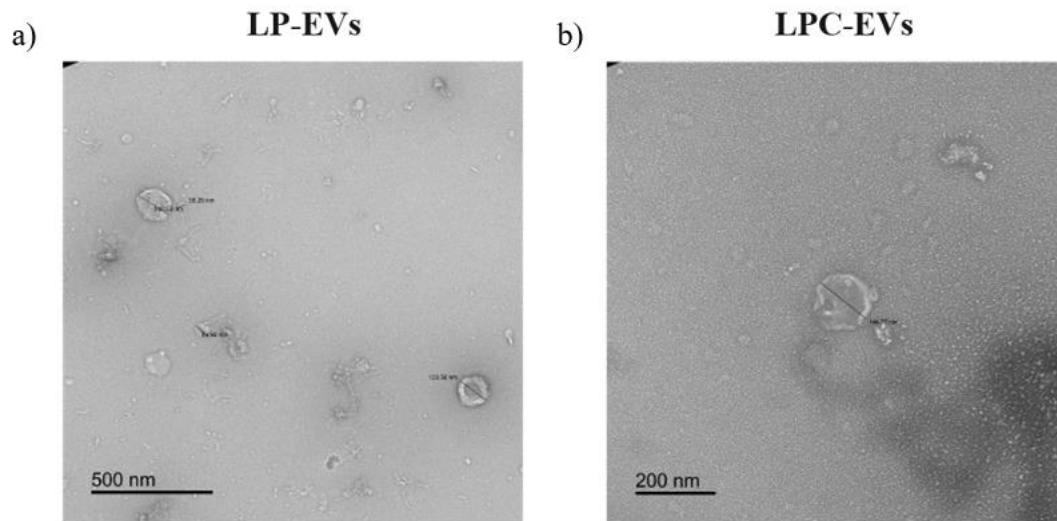


Figure 9) Transmission electron microscopy (TEM) micrographs of purified probiotic EVs. TEM images of (a) LP-EVs and (b) LPC-EVs, showing spherical vesicle morphology with measured diameters (nm). Scale bars: 500 nm (a) and 200 nm (b).

2. Evaluation of pEVs impact on activated PBMCs viability

The metabolic activity and therefore cell viability was assessed by MTT assay after 48 hours. Cell viability percentage consistently remained near CTR level for the majority of treatments. Interestingly, a statistically significant difference was detected between the CTR group and the LPC-EVs + LPS group ($86.87 \pm 2.72\%$) indicating a slight but clear reduction in viability percentage. In contrast, the LP-EVs + LPS group maintained a mean viability slightly above the baseline ($110.6 \pm 10.26\%$), as shown in Figure 10.

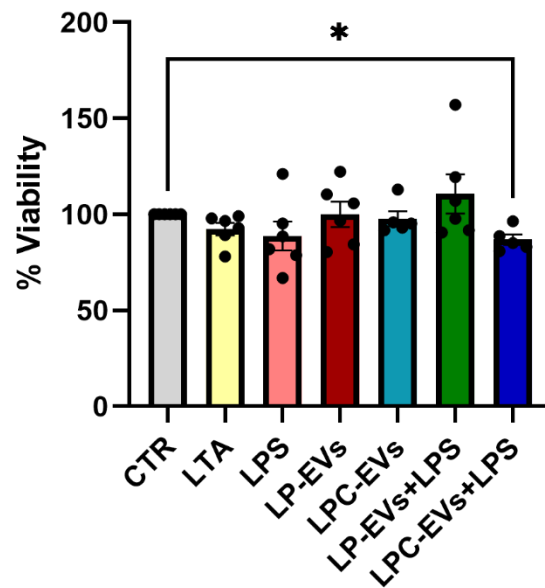


Figure 10) Cell viability of human PBMCs derived from healthy donors (n=6) after 48 hours of incubation across different experimental groups. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using Kruskal-Wallis test (* $p < 0.05$).

3. Evaluation of the immunomodulatory effects of pEVs on activated PBMCs

To investigate the immunomodulatory potential of pEVs treatment, TNF- α and IL-10 secretion were quantified in the supernatants of activated PBMCs after 48 hours (Figure 11). None of the treatments induced a statistically significant alteration in TNF- α levels compared to the CTR group (Figure 11a). Secretion of TNF- α in the CTR group stood at an average of approximately 36.59 ± 15.28 pg/mL.

Regarding IL-10 (Figure 11b), its secretion remained balanced across all evaluated conditions after 48 hours. Treatments with LP-EVs and LPC-EVs, either individually or in combination with LPS, did not induce any statistically significant changes in IL-10 levels relative to the CTR group. The CTR demonstrated a mean secretion of 50.40 ± 15.91 pg/mL.

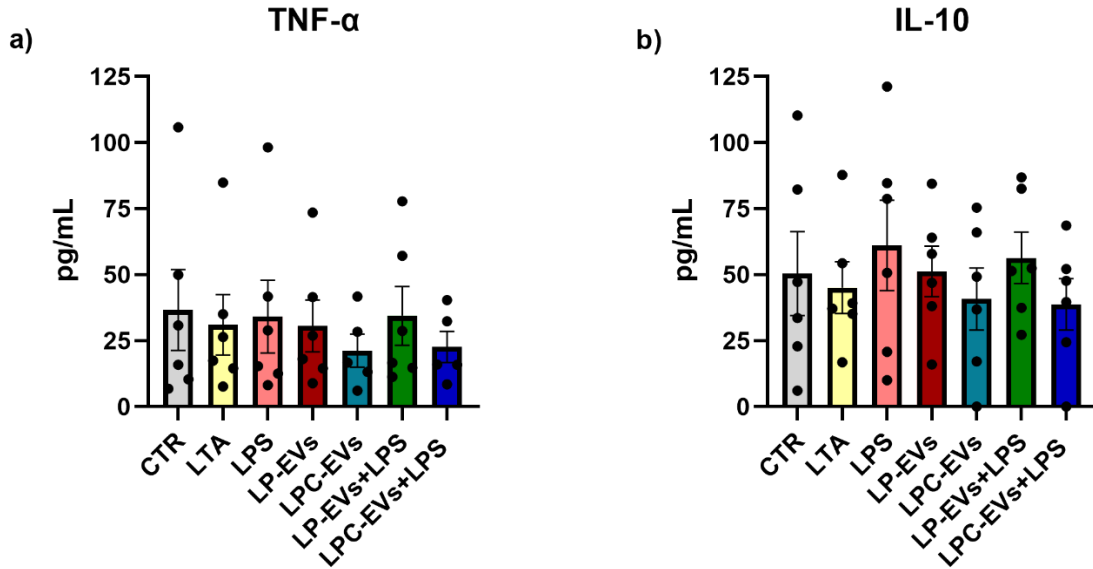


Figure 11) TNF- α and IL-10 secretion profiles in activated human PBMCs. Quantified levels of TNF- α and IL-10 (pg/mL) in the supernatants of PBMCs (n=6) after 48 hours of treatment with different experimental groups. Data are expressed as mean $\pm$ SEM. Statistical significance was assessed using Kruskal-Wallis test.

4. Effects of LP-EVs and LPC-EVs on RA-FLS viability

Cell viability was assessed using a Live/Dead assay to ensure that pEVs did not exert cytotoxic effects on RA-FLS, under both 2D and 3D GelMA culture conditions following 72 h of treatment (Figure 12). RA-FLS were stained with Calcein-AM/PI, which revealed a predominance of viable (green) cells across all treatment groups in both 2D and 3D culture conditions, with minimal dead (red) cells observed (Figure 12a).

In the 2D culture model (Figure 12b), RA-FLS maintained high viability across all conditions, with no statistically significant differences observed between EV-treated and control groups. The

control group exhibited an average viability of $81.07 \pm 11.80\%$, while treatment with LP-EVs and LPC-EVs resulted in viabilities of $92.89 \pm 2.14 \%$ and $88.11 \pm 3.93 \%$, respectively.

Similarly, in the 3D GelMA model (Figure 12c), pEV treatment did not compromise cellular viability, with no significant differences detected among the groups. The control group showed a mean viability of $87.65 \pm 3.29 \%$, whereas cells treated with LP-EVs and LPC-EVs displayed viabilities of $84.88 \pm 5.52 \%$ and $87.50 \pm 3.96 \%$, respectively. Overall, these results demonstrate that both LP-EVs and LPC-EVs are highly cytocompatible with RA-FLS in both 2D and 3D inflammatory culture models.

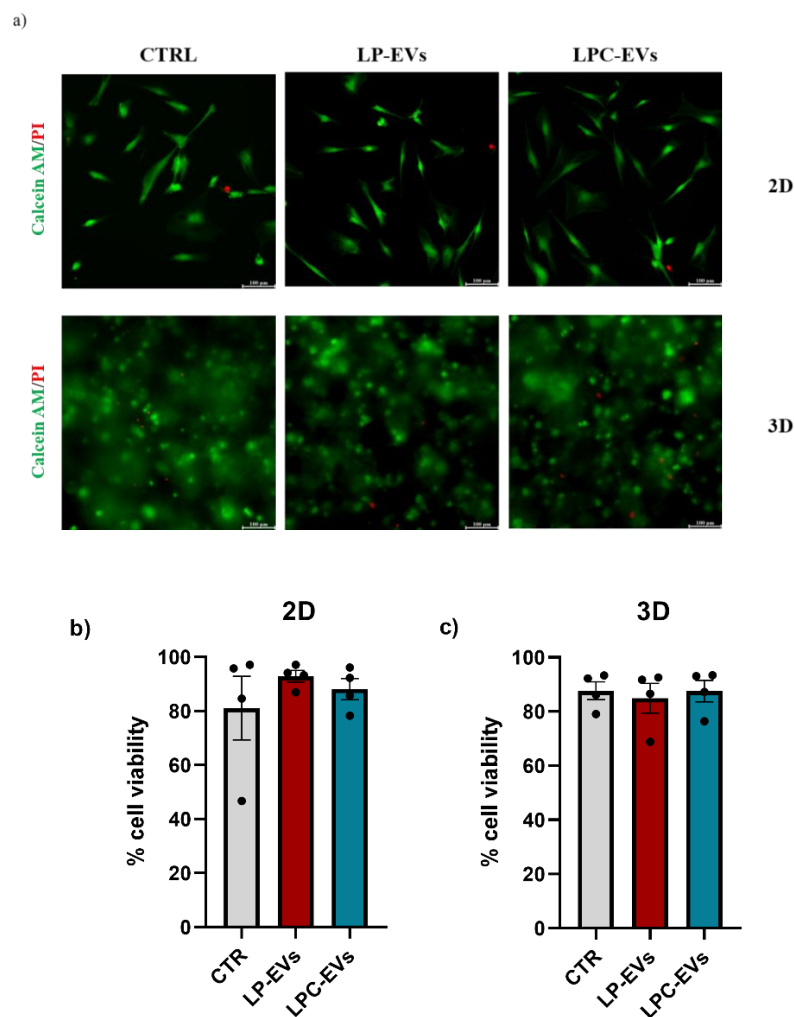


Figure 12) RA-FLS cell viability profiles. (a) Representative fluorescence microscopy images of Calcein-AM/PI-stained RA-FLS following 72 hours of inflammatory stimulation and treatment with pEVs under 2D and 3D culture conditions (scale bar 100 μm). Cell viability percentages of (b) 2D and (c) 3D GelMA model after stimulation and treatment with LP-EVs or LPC-EVs. Data are expressed as mean $\pm$ SEM (n=4). Statistical significance was assessed using RM-one way ANOVA test.

5. Evaluation of pEV effects on RA-FLS lubricin expression

To investigate any therapeutic potential of pEVs on joint inflammation, lubricin expression was assessed in RA-FLS treated with LP-EVs and LPC-EVs in both 2D and 3D culture models (Figure 13). Immunofluorescence staining revealed lubricin expression (green) in RA-FLS across all treatment groups in 2D and 3D culture conditions (Figure 13a).

In the 2D culture model, treatment with either LP-EVs or LPC-EVs did not result in statistically significant changes in lubricin expression compared with the control group. Nevertheless, both pEV-treated groups displayed a modest increase in lubricin levels relative to the control condition, suggesting a limited response under conventional 2D culture conditions (Figure 13b).

In contrast, a more pronounced effect was observed in the 3D GelMA model (Figure 13c). Treatment with LP-EVs induced a clear increase, with lubricin levels of $11,795.22 \pm 1,795.85$ MFI/cell, while LPC-EVs caused a smaller increase in lubricin expression, reaching $10,048.18 \pm 537.81$ MFI/cell. Notably, only the LPEV-treated group showed a statistically significant increase compared with the control condition, whereas the increase observed following LPC-EV treatment showed only a trend toward statistical significance ($p=0.09$). These findings suggest that the stimulatory effect of pEVs on lubricin production is more evident in the 3D microenvironment, with LP-EVs displaying the strongest response under the tested conditions.

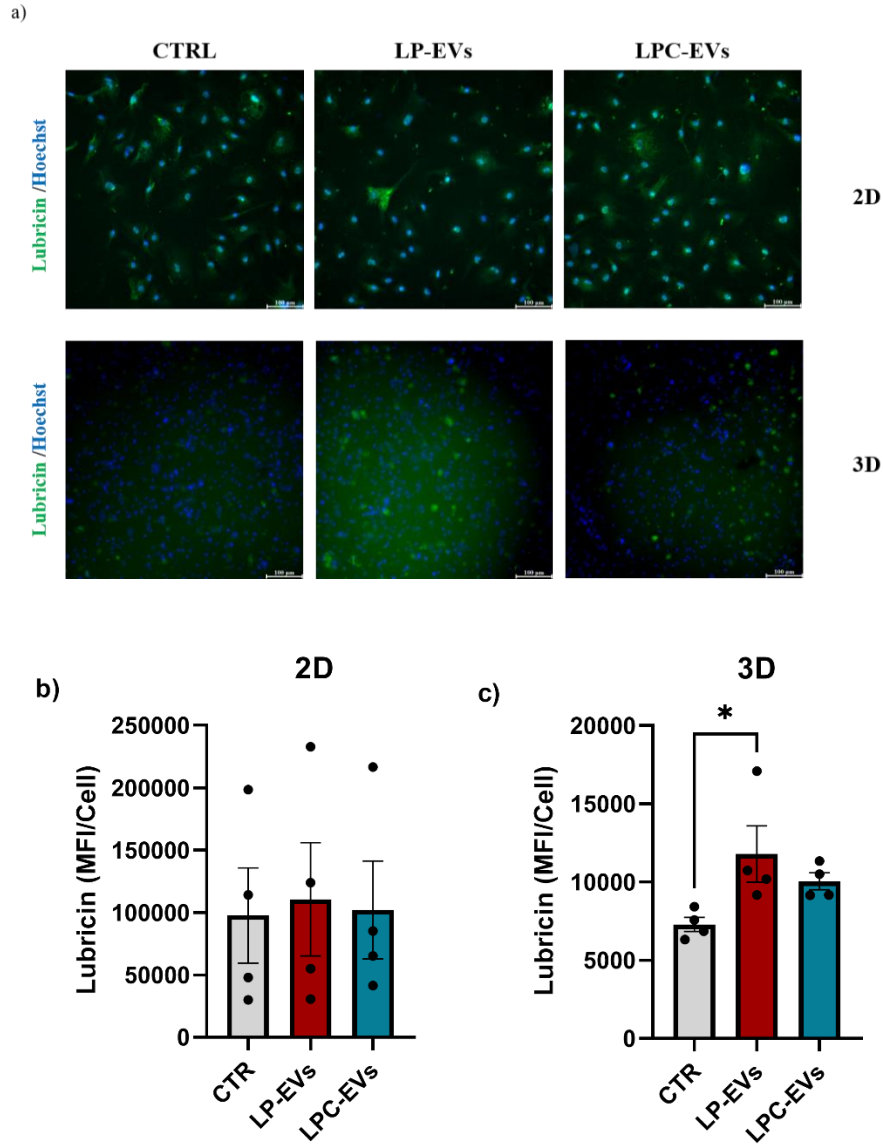


Figure 13) Lubricin expression in RA-FLS. (a) Representative fluorescence microscopy images of lubricin expression (green) following 72 h of inflammatory stimulation and treatment with LP-EVs or LPC-EVs under 2D and 3D culture conditions. Quantified lubricin levels (mean fluorescence intensity (MFI)/cell) of (b) 2D and (c) 3D models after inflammatory stimulation and treatment with LP-EVs (LP) or LPC-EVs (LPC). Data are represented as mean $\pm$ SEM (n=4). Statistical significance was assessed using RM-one way ANOVA test (*p<0.05).

Discussion

PEVs are increasingly recognized as key mediators of host-microbe crosstalk that contribute to intestinal homeostasis. This positions them as promising therapeutic candidates in inflammatory and immune-mediated conditions^{53,54}.

In this study, pEVs derived from *L. plantarum* (LP) and *L. paracasei* (LPC) were isolated and further purified by SEC. The physical, morphological, and functional properties of LP-EVs and LPC-EVs were characterized, with the aim of investigating their immunomodulatory and therapeutic potentials in the context of RA-related inflammation.

NTA analysis showed that SEC purification effectively reduced particle concentration and narrowed the size distribution profile in both strains, consistent with the known ability of this technique to successfully remove contaminants and produce high purity EV preparations⁵⁵. This reduction reflects the presence of contaminants, such as protein aggregates, in the unpurified samples, which were probably counted as particles by the NTA system and thus affected the measured concentration prior to purification. Taken together, these findings confirm the reliability of our purified EV preparations for subsequent functional assays.

Interestingly, particle concentration data also revealed distinct EV production profiles between the two probiotic strains, with LPC-EVs yielding significantly higher EV production than LP, both before and after purification. Supporting this finding, comparative analyses of *Lactobacilli* strains have shown substantial variability in EV production, with *L. paracasei* yielding more particles than *L. plantarum*⁵⁶.

On the other hand, SEC purification also affected vesicle size. NTA revealed that SEC purification decreased both the mean and mode particle sizes of LP-EVs, suggesting that it was able to exclude proteins and other non-EV debris. This finding is consistent with a previous comparative study of EVs derived from *Fasciola hepatica* which similarly demonstrated that SEC-purified vesicles were smaller in size compared to those isolated by differential centrifugation alone⁵⁷. However, after purification, both LP-EVs and LPC-EVs showed a mean size of respectively 160.77 and 177.77 nm, consistent with previously reported sizes of *Lactobacillus*-derived EVs⁵⁸.

The viability assay showed that both LP- and LPC-derived EVs were well tolerated and did not exhibit cytotoxic effects on activated PBMCs under either basal or inflammatory conditions.

Although comprehensive toxicity assessment remains limited, our data are in line with previous studies suggesting pEV biocompatibility when applied to intestinal and immune cells (i.e. macrophages), to explore their immunomodulatory properties^{58,59}. Although LP-EVs and LPC-EVs were safely tolerated by activated PBMCs, their immunomodulatory potential on TNF- α and IL-10 secretion was limited, contrasting with reports of *Lactobacillus*-derived EVs modulating cytokine release in other immune models⁶⁰. This can be explained by cell type-specific responses to bEVs. In fact, a study on *Bacteroides thetaiotaomicron*-derived EVs showed that they are able to differentially target immune cell populations and activate NF- κ B by TLR4/TIRAP pathway depending on both the cell type and healthy or disease states⁶¹.

Since PBMCs are a heterogeneous population (lymphocytes, NK cells, monocytes, dendritic cells) with variable TLRs expression and functional responses⁶², this may have masked an effect that can be more detectable in homogeneous models (e.g., monocytes/macrophages).

Regarding RA-FLS viability, pEVs derived from both strains demonstrated high cytocompatibility under both 2D and 3D inflammatory culture conditions. This consistency across two distinct culture systems reinforces the reliability of these cytocompatibility findings. Notably, the maintenance of viability under inflammatory stimulation is particularly relevant in the context of RA, where synovial fibroblasts are chronically exposed to a pro-inflammatory microenvironment. Importantly, the fact that neither LP-EVs nor LPC-EVs compromised RA-FLS survival under these conditions indicates that any effect exerted by these vesicles, such as the induction of lubricin expression described below, is unlikely to be secondary to cytotoxic stress but rather reflects a modulatory effect on RA-FLS function.

Treatment with LP-EVs and LPC-EVs in the 2D model did not show differences in the lubricin production compared to the control. In contrast, a more pronounced and potentially strain-dependent effect emerged in the 3D model, where LP-EVs significantly increased lubricin expression. This model-dependent variance suggests that the 3D microenvironment may be required to unmask a functional response that remains latent under 2D culture conditions. This is compatible with previous reports demonstrating that 3D culture conditions can better preserve or unmask the functional response of synovial/joint-derived cells, likely due to closer mimicry of the native joint microenvironment compared to standard 2D monolayer conditions⁶³. The induction of lubricin production by LP-EVs is of therapeutic relevance, as their content also exerts direct joint-

protective functions. In fact, it has been reported that the PGN of Gram+ bacteria can interact with TLR2 and TLR4 and play an anti-inflammatory role within the joint⁶⁴.

To our knowledge, this is the first study reporting lubricin induction by pEVs in RA-FLS. Although no direct evidence links *Lactobacillus*-derived EVs to lubricin before, this result offers a plausible biological interpretation: pEVs have been shown to reduce TLR-NF- κ B signaling and decrease inflammatory cytokines, like TNF- α , and TNF- α /IL-1 β are known to suppress lubricin production. This suggests LP-EVs may increase lubricin by reducing local inflammatory signals. The lack of a significant LPC-EV effect may reflect strain-dependent molecular cargo differences between LP-EVs and LPC-EVs, warranting further multiomics characterization of pEVs to identify the specific bioactive components responsible for this effect.

Limitations

Several limitations should be considered when interpreting these findings. Only PBMCs from healthy donors were available for the immunomodulatory assays, and only a single EV dose and exposure time were tested. Moreover, PBMCs are a heterogeneous cell population, which may have masked potential strain-dependent immunomodulatory effects more detectable in homogeneous models such as monocytes/macrophages. Within the RA-FLS 3D model, inflammatory cytokines (e.g., TNF- α , IL-1 β) were not measured, so the mechanism behind increased lubricin expression remains to be better investigated in future studies.

Future Perspectives

Further research could extend and strengthen the findings of this study. First, future work should focus on PBMCs from RA patients rather than healthy donors. Within the RA-FLS 3D model, assessing inflammatory cytokines alongside lubricin induction would help to clarify how they are linked. Moreover, PBMCs were activated using PMA/ionomycin, which induces a broad and non-specific activation of multiple immune cell populations. Therefore, to specifically investigate the effects of the treatment on T-cell responses, future experiments should be performed using PBMCs activated with anti-CD3/CD28 antibodies. Furthermore, proteomic or metabolomic

characterization of LP-EVs and LPC-EVs would help to identify the specific bioactive cargo responsible for the potentially strain-dependent effect.

It should also be noted that the Live/Dead assay and lubricin quantification used in this study do not distinguish between functional modulation and potential increase in cell number resulting from altered proliferation. Since RA-FLS exhibit a hyperproliferative phenotype, future studies assessing pEVs effects on RA-FLS proliferation (e.g., via Ki-67 staining) are needed to fully rule out proliferation as a confounding factor and to confirm that the observed effects reflect true functional modulation rather than changes in cell number. We did not investigate the impact on cytokine secretion; this can be performed by multiplex cytokine analysis or ELISA on the supernatants already collected within the experiments.

Additionally, organ-on-chip technology would provide a more physiologically representative model of the joint microenvironment, closely mimicking the mechanical and cellular dynamics of synovial tissue, thereby leading to more accurate and reliable findings than static culture systems. The incorporation of continuous perfusion in such systems would better simulate synovial fluid flow and shear stress conditions experienced by RA-FLS *in vivo*, factors entirely absent in static 3D culture systems. These platforms can further integrate multiple joint-relevant cell types, such as chondrocytes, immune cells, and endothelial cells, alongside RA-FLS, to better mimic the complex cellular crosstalk within the inflamed joint microenvironment.

Conclusions

In this thesis we demonstrated that LP-EVs and LPC-EVs are biocompatible with both PBMCs and RA-FLS under inflammatory conditions. While neither EV type significantly modulated TNF- α or IL-10 secretion in activated PBMCs, LP-EVs significantly induced lubricin expression in RA-FLS specifically under 3D inflammatory culture conditions, an effect not observed with LPC-EVs or in the 2D model. In conclusion, these findings suggest a potentially strain-dependent and immunomodulatory potential of pEVs, supporting further investigation of their potential role as postbiotic modulators of synovial fibroblast function in RA.

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