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Immunomodulatory potential of probiotic-derived extracellular vesicles in human peripheral blood mononuclear cells (PBMC)

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Summary

The human gut microbiota is essential to maintain immune homeostasis through continuous interactions with the host immune system. An unbalancement in microbial composition, characterized by a reduction in beneficial bacterial populations and an increase in opportunistic microorganisms, lead to a condition known as dysbiosis, which has been associated with enhanced inflammatory responses and immune dysregulation. Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, contribute to immune balance through multiple mechanisms, including the production of antimicrobial compounds, short-chain fatty acids, and other bioactive metabolites, as well as modulation of epithelial barrier integrity and immune cell signaling. Common probiotic strains, such as *Lactobacilli*, have been widely studied for their ability to regulate inflammatory pathways and promote anti-inflammatory responses. In the last years, increasing attention has been directed toward extracellular vesicles (EVs), nanosized particles released also by microorganisms, which are able to transport bioactive molecules including proteins, lipids, nucleic acids, and metabolites. Probiotic-derived EVs might represent a promising cell-free therapeutic strategy, as they can exert immunomodulatory effects while reducing potential safety concerns associated with the administration of live microorganisms.

The aim of this thesis was to isolate and characterize EVs produced by two probiotic strains, *Lactiplantibacillus plantarum* (PBS067) and *Lacticaseibacillus paracasei* (LPC1114), and to evaluate their functional effects on human peripheral blood mononuclear cells (PBMCs).

Bacteria were cultured in particle-depleted MRS broth for 24 hours and EVs were isolated from culture supernatants by ultracentrifugation. Vesicle characterization was performed by Nanoparticle Tracking Analysis (NTA) to determine particle size distribution and concentration, while total protein content was quantified using the micro-BCA assay. PBMCs were isolated from peripheral blood of healthy donors by density gradient centrifugation and subsequently treated with probiotic-derived EVs both in absence and presence of a pro-inflammatory stimulus, lipopolysaccharide (LPS), in order to mimic basal and dysbiotic conditions. Cell viability was assessed by MTT assay, and cytokine levels in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA).

The results demonstrated that EVs derived from both probiotic strains displayed size distributions consistent with bacterial vesicles and contained a similar amount of protein concentration. Treatment of PBMCs with probiotic EVs did not induce significant cytotoxic effects, indicating good biocompatibility. Functional analyses revealed modulation of immune responses, as indicated by changes in the production of the pro-inflammatory cytokine TNF-α

and the anti-inflammatory cytokine IL-10. These findings suggest strain-dependent immunomodulatory properties and highlight the potential of probiotic EVs to contribute to the regulation of inflammatory processes.

Overall, this study supports the potential application of probiotic-derived EVs as novel postbiotic tools capable of modulating immune cell functions. Further investigations are required to elucidate the underlying molecular mechanisms and to explore their possible therapeutic applications in inflammatory and immune-mediated diseases.

Introduction

1. Overview of the immune system

The immune system comprises an interconnected network of defence mechanisms responsible for protection against pathogens, malignant cells, and tissue damage [1]. The primary role of the immune system is to differentiate the body's own harmless components from external or altered elements that might be harmful and to respond to the latter by removing such threats with little collateral damage to the host tissues [2]. The activation of immune responses has to be balanced through regulatory processes to ensure that physiological homeostasis is maintained [1,3].

The protective function of the immune system is mediated by two interconnected parts that work together, namely innate immunity and adaptive immunity. The innate immunity is the first line of defence that provides immediate and non-specific reactions to both invading microorganisms and signals from the body indicating danger, thus representing the frontline of host defence [1,4]. Pattern recognition receptors (PRRs) recognise conserved patterns, which lead to the activation of inflammatory reactions that help defend the body against infection, and at the same time, promote tissue repair [4,5].

Adaptive immunity is mediated by two major cell populations, B and T lymphocytes. Each lineage is uniquely identified by expression of an antigen receptor generated by somatic V(D)J recombination [6,7]. The process creates a diverse set of diverse repertoires of antigen receptors, which enable the system to identify numerous antigenic markers [6]. B lymphocytes use B-cell receptor (BCR) as their antigen receptor, which contain a membrane-bound immunoglobulin molecule that binds to Igα (CD79a) and Igβ (CD79b) signalling subunits through non-covalent interactions [7,8]. The surface of mature naive B cells displays both IgM and IgD isotypes. The BCR identifies unprocessed native antigens, which include soluble proteins, polysaccharides, lipids and conformational epitopes [9,10]. T lymphocytes have T-cell receptors (TCRs), which function as heterodimers composed of α and β chains in most cases or γ and δ chains in less common cases [11]. TCR-mediated antigen recognition is major histocompatibility complex (MHC)-restricted, as T cell recognises short peptide fragments presented by MHC molecules on antigen-presenting cells. CD4+ helper T cells recognise peptides presented by MHC class II molecules, whereas CD8+ cytotoxic T cells recognise MHC class I molecules [12,13].

After antigen exposure in the context of appropriate costimulation, B cells proliferate and differentiate into two major population: antibody-secreting plasma cells that produce soluble antibodies against the stimulating antigen, and long-lived memory B cells that remain available

to mount a rapid aggressive secondary response upon re-encounter with the antigen [14,15]. Concomitantly, antigen-primed T cells undergo clonal expansion and differentiate into specialized effector subsets, including cytotoxic T cells ($CD8^+$), regulatory T cells (Treg), and helper T cells ($CD4^+$), each fulfilling specialized roles in immune defences and regulation [16]. These lymphocyte subsets traffic to secondary lymphoid organs and peripheral tissues according to their effector functions, with T cells predominantly localizing to sites of antigen presentation inflammation, whereas B cells primarily reside within lymphoid tissue follicles[16].

One of the most important features of the immune system is self-tolerance [17]. There are several mechanisms in place to ensure the immune system does not react against its own tissue. These mechanisms include the deletion of lymphocytes clones that recognise antigens as “self”, and the suppressive activity of certain types of immune cells, referred to Tregs [3,17]. The disruption of these mechanisms may result in the inappropriate activation of the immune system and the development of illness [1,17].

2. Overview of gut microbiota

The gastrointestinal tract has the densest microbial community in the human body and it consist of dynamic population of different microorganisms, including bacteria, viruses, fungi, and archaea [18,19]. Among bacteria the phyla Firmicutes (*Faecalibacterium prausnitzii A2-165*, *Roseburia spp., Lactobacillus rhamnosus GG, Lactobacillus plantarum WCFS1*) and Bacteroidetes (*Bacteroides fragilis, Prevotella spp.*) constitute the majority of the bacterial population in healthy adults, with Actinobacteria (*Bifidobacterium longum BB536, B. breve*), Proteobacteria (*Escherichia coli*), and Verrucomicrobia (*Akkermansia muciniphila MucT*) are important commensal genera, many of which are involved in the fermentation of food substrates and the synthesis of bioactive metabolites.

Through its roles in nutrients metabolism, vitamins production, bile acid conversion, and maintenance of the intestinal epithelial integrity, the gut microbiota plays a critical role in preserving physiological homeostasis in the host [20–22]. Additionally, the maturation of the immune system, the establishment of immune tolerance, and the modulation of baseline inflammatory tone depend on continuous interactions between the microorganisms and the host immune cells [23,24].

2.1 Gut dysbiosis

Alterations in the composition and/or function of the gut microbiota, known as gut dysbiosis, may occur as a result of dietary patterns, use of antibiotics, infection, environmental factors, or aging. Dysbiosis has been defined as a condition in which microbial diversity, the relative balance between beneficial and opportunistic microorganisms, or microbial metabolic activity is disrupted [25,26]. For instance, this condition is frequently characterized by the expansion of pathobionts such as Proteobacteria (e.g., *Escherichia coli Nissle 1917*) and the altered relative abundances of dominant commensal taxa, such as Firmicutes (e.g.*, Faecalibacterium prausnitzii A2-165, Roseburia spp.*), Bacteroidetes (e.g., *Bacteroides fragilis BB536, Prevotella spp*.), and beneficial Actinobacteria (*Bifidobacterium longum*) [27–29].

Microbial imbalance has been linked to metabolic disorders, increased susceptibility to infections, low-grade chronic inflammation, and impaired mucosal barrier function, highlighting the gut microbiota as a key modulator of host physiological homeostasis [30–32].

2.2 Gut-immune axis

The gut-immune axis represents the dynamic interface between the microbiota and the host immune system and plays a crucial role in maintaining immune homeostasis [33,34]. The gut microbiota affects immune tolerance and regulates the balance between pro- and anti-inflammatory responses. Dysbiosis can impair immune regulation and contribute to pathological inflammatory conditions [33–35]. This condition is associated with increased intestinal permeability, which facilitates the translocation of microbial components across the epithelial barrier and promotes aberrant activation of the innate immune system. Microbial imbalance also alter the differentiation of Tregs and other T helper cell subsets [34–36].

Alterations in microbial composition have also been linked to excessive pro-inflammatory immune responses. For instance, in inflammatory bowel disease (IBD), dysbiosis has been associated with pro-inflammatory pathways and disruption of mucosal immune regulation [35,36]. Overall, these findings highlight that host-microbiota interactions are essential not only for maintaining immune homeostasis but also represent potential targets for therapeutic modulation of inflammatory disorders.

2.3 Microbial metabolites as mediators of immune modulation

Through the synthesis of bioactive metabolites such as short-chain fatty acids (SCFAs), secondary bile acids, indole derivatives, and polysaccharide antigens, gut bacteria can exert significant immunomodulatory effects in addition to direct cell-to-cell contact [20,23,37–39].

By modulating epithelial barrier function, inhibiting pro-inflammatory signaling pathways, and promoting the development and proliferation of regulatory immune cells, especially Tregs, these metabolites control immune responses [38,40].

The metabolites exist in two different from because they can be both secreted as soluble compounds and delivered through bacterial extracellular vesicles (EVs) which transport bioactive substances that include SCFAs and polysaccharide A (PSA) and indole derivatives to host cells [41,42]. EVs from *Faecalibacterium prausnitzii* and *Bacteroides fragilis* carry metabolites that strengthen epithelial barriers and increase Tregs growth, and this serves as another way that intestinal bacteria may influence body defences during normal and abnormal states [42–44] and acting as carriers of microbial-derived signals [45–47]. This highlights an additional layer of microbiota–host communication linking microbial metabolism to immune regulation under both physiological and dysbiotic conditions [45–48].

3. Extracellular vesicles

EVs are lipid bilayered particles released by both eukaryotic and prokaryotic cells, typically ranging from 30 nm to 5 µm in diameter. As carriers of proteins, lipids, RNA, DNA, and other biomolecules, EVs play a key role in immune modulation, genetic material transfer, intercellular communication, and different physiological and pathological processes [49,50].

Eukaryotic EVs can be classified according to their origin, size and function, with each subtype characterised by distinct biogenesis mechanisms and functional properties that reflect their roles in cell-to-cell communication, immune modulation, and host-microbe interactions [50–53]. Bacterial EVs, instead, are commonly classified according to the structural characteristics of the bacterial cell envelope, which distinguishes vesicles released by Gram-positive (Gram +) and Gram-negative (Gram -) bacteria.

3.1 Eukaryotic EVs

Eukaryotic EVs are released through complex and distinct biogenesis pathways. Exosomes originate from the endosomal sorting system, whereas microvesicles (MVs) are formed through outward budding of the plasma membrane. Apoptotic bodies are released during the process of programmed cell death and are considered fragments of dead cells. In general, EVs are composed of lipid membranes enriched in ceramides and cholesterol and display on their surface membrane proteins such as tetraspanins (e.g., CD9, CD63, CD81) [54,55]. Eukaryotic vesicles are involved in intercellular communication, immune modulation, tissue repair, and disease progression.

Exomeres, typically smaller than 50 nm, lack a clearly defined biogenesis pathway. Their biological roles are still under investigation, but actual evidence suggests a potential involvement in the control of signalling and cellular metabolism [56]. Exosomes, instead, range in diameter from 30 to 150 nm, derive from the endosomal pathway and are released when the multivesicular bodies fuse with the plasma membrane. They transport proteins, lipids, and RNA, and are crucial mediators of immune signaling and cell-to-cell communication [57,58]. Microvesicles (MVs), which range in diameter from 100 to 1000 nm, originate from direct outward budding of the plasma membrane and are enriched in signalling molecules, including integrins and tetraspanins [57]. Apoptotic bodies (500 nm to 5 µm) are produced during apoptosis and contain DNA, cytoplasmic components, and fragments of organelles [58] (table 1).

Table 1. Classification of Eukaryotic EVs based on size. Each subtype is characterized by specific biogenesis pathways and specialised biological functions related to intercellular communication, immune regulation, and tissue homeostasis.

Type	Size	Origin	Key characteristics
Exomeres	< 50 nm	Small nanoparticles; biogenesis is not well defined.	Non-membranous particles; potential role in intercellular communication, metabolism, and signalling.
Exosomes	30 - 150 nm	Endosomal membrane budding and release via multivesicular body fusion.	Enriched in protein, lipids, RNA; regulate cell-to-cell communication and immune responses.
Microvesicles (MVs)	100 - 1000 nm	Outward budding and shedding from plasma membrane.	Enriched in tetraspanins and integrins; involved in cell signalling.
Apoptotic bodies	500 nm - 5 µm	Released during apoptosis as cell fragments.	Contain organelles, DNA fragments and cytoplasmic debris.

3.2 Bacterial EVs

It has also been shown that EVs are secreted by both Gram- and Gram+ bacteria **(Figure 1).** Bacterial EVs not only contribute to microbial survival and adaptation but also shape host–microbe interactions through their structural components and bioactive cargo. These vesicles differ in composition, biogenesis, and biological roles, ranging from pathogenic mechanisms to promising therapeutic applications.

3.2.1. Gram– derived EVs

Gram– bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa,* mainly release outer Membrane Vesicles (OMVs) enriched in periplasmic components, including lipopolysaccharide (LPS) [59,60]. The biogenesis of these OMVs is driven by outer membrane bulging and pinching, often triggered by envelope stress, peptidoglycan remodelling, or accumulation of misfolded protein [49,50]. These OMVs contribute to bacterial proliferation, delivery of virulence factors, and adaptation to environmental conditions. Their cargo includes toxins, periplasmic enzymes, DNA fragments, and signalling molecules that enable bacteria to neutralize antibiotics, modulate the host immune system, and signalling molecules that enable bacteria to neutralise antibiotics, modulate the host immune system, and promote horizontal gene transfer [49,61]. They are involved in nutrient acquisition processes, antibiotic neutralisation, immune system modulation, and biofilm formation. They also facilitate communication with other microbes and competition or cooperation within microbial communities [49,50,61].

3.2.2. Gram+ derived EVs

In contrast, Gram+ bacteria, such as *Lactobacillus spp*. and *Staphylococcus spp.*, lack an outer membrane, but their EVs are enriched in proteins, teichoic acids such as lipoteichoic acid (LTA), and lipoprotein [62,63]. Their biogenesis is thought to occur through localised degradation of the peptidoglycan layer, which allows cytoplasmic membrane protrusions to bud outwards [63,64]. These vesicles play a role in bacterial communication, including both inter-bacterial signalling and host-bacteria interactions. They also contribute to biofilm formation and facilitate immune evasion mechanisms [64]. Notably, EVs derived from *Lactobacillus* or *Bifidobacterium* species show therapeutic potential, acting as modulators of gut immunity and skin health, and are being explored for applications in immunotherapy and anti-ageing treatments [57,65]. Cargo studies showed that Gram+ EVs encapsulate proteins, lipoproteins, LTA, as well as small RNAs that regulate host gene expression and mediate

interspecies communication. Functional studies further highlight their immunomodulatory capacity [64]. Despite the considerable progress, important gaps still concern EV biogenesis, sorting of the cargo, and therapeutic applications, which require further research to enhance their potential in biological and clinical domains [51,57,58].

Gram+ and Gram- bacteria have very different processes for EV biogenesis. In Gram- bacteria, such as *E. coli,* EVs are commonly released either by outer membrane blebbing or through explosive cell lysis, which results in the release of both inner and outer membrane vesicles [66,67]. Conversely, in Gram+ bacteria, enzyme such as autolysins remodel the thick peptidoglycan layer, enabling cytoplasmic membrane-derived vesicle budding through energy-dependent mechanisms [50,68,69]. Several environmental and physiological factors can influence bacterial EVs release. For example, the EVs yield, molecular composition, and activity of *L. rhamnosus* are greatly impacted by culture conditions such as pH 7.5, incubation duration, and changes in culture broth concentration [70]. The biological functions of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* derived EVs depends on their specific cargo with contains membrane and cytosolic proteins together with lipids and metabolites and nucleic acids because different EV compositions produce different anti-inflammatory and neuroprotective responses in specific situations [46,71,72]. Probiotic Gram+ species, including *L. plantarum* and *L. paracasei*, use EV biogenesis through localised peptidoglycan remodeling, with autolysins and cell wall hydrolases generated by these enzymes producing cytoplasmic membrane protrusions that result in vesicle release [66–68]. The molecular regulators which control EV production in *L. plantarum* and *L. paracasei* remain mostly unknown while *Listeria monocytogenes* uses vesicles production to respond to stress through its specific stress-response regulators σ^B and CovRS two-component system [73,74]. Their vesicles contain more membrane-associated proteins and lipoproteins and peptidoglycan-modifying enzymes, lipoteichoic acid, and small RNAs, which indicates that they use selective cargo packaging instead of passive cytoplasmic leakage. The mechanisms which control cargo sorting and the signals which activate vesicle release during various environmental situation have not been fully established yet [49,75,76].

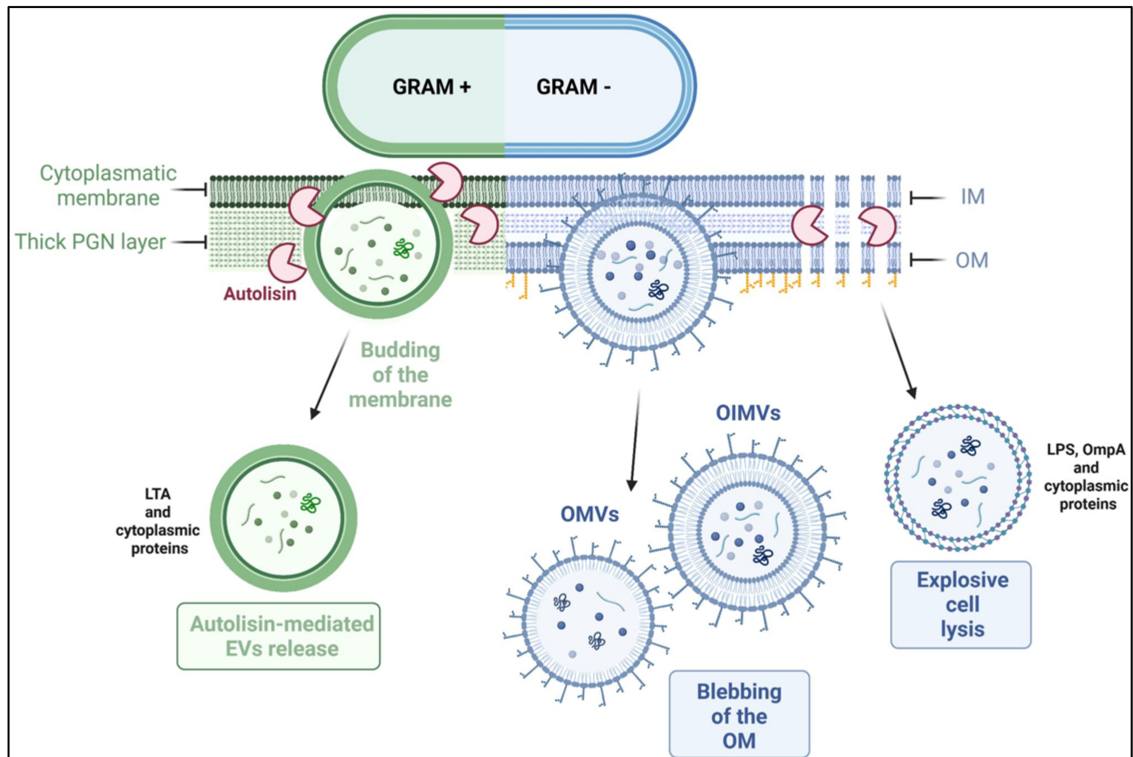

Figure 1: Structural comparison of the biogenesis of EVs between Gram+ and Gram– bacteria [77].

4. Isolation and characterisation of EVs

Isolation of EVs represents the first critical step in EV research and depends on the source material, intended downstream applications, and the required purity. Various techniques are used for EV isolation, each possessing specific strengths and limitations. Ultracentrifugation remains the benchmark method, with density gradient centrifugation used especially to optimise purity. Size-exclusion chromatography (SEC) preserves vesicles integrity, whereas ultrafiltration improves concentration efficiency. Precipitation techniques are simple to implement, but they also co-isolate impurities. More advanced approaches, such as affinity-based capture and a microfluidic platforms, offer improved selectivity and sensitivity and show potential for clinical translation [78–81].

In order to fully understand their biological functions and potential applications, EVs must be precisely characterised. A single method is not sufficient for a detailed analysis, therefore, a combination of complementary techniques is typically employed to provide information on the physical structure, molecular composition, and functional properties of EVs [78,82].

Electron microscopy has been a cornerstone of EV morphological analysis for many decades. Transmission Electron Microscopy (TEM) provides high-resolution images of vesicles, while Cryo-Electron Microscopy (Cryo-EM) preserves vesicles structure, minimising processing artefacts. Scanning Electron Microscopy (SEM) can also be used to observe surface features,

but it has a lower resolution compared to TEM or Cryo-EM and is therefore less suitable for small vesicles [83].

EV size and concentration are commonly determined by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). In more detail, DLS measures particle characteristics based on fluctuations in scattered light caused by Brownian motion, whereas NTA directly tracks individual vesicles and enables particle visualisation [84].

The molecular characterisation of EVs relies on the analysis of their protein and nucleic acid cargo. Surface antigens can be investigated through flow cytometry, with EV markers such as CD9, CD63, and CD81 typically detected in eukaryotic-derived vesicles [79]. In contrast, bacterial EVs display distinct molecular signatures, where common markers include outer membrane proteins (e.g., OmpA), lipoproteins, and LPS Gram– bacteria. Gram+ bacterial EVs are instead enriched in lipoteichoic acid (LTA) and peptidoglycan-associated proteins, which can be employed as markers [80,85].

The gold standard for confirming the presence of specific proteins remains western blotting, but proteomic techniques are necessary for a comprehensive description of EV composition. In parallel, transcriptomic analyses identify small RNAs, while lipidomic and metabolomic studies reveal structural and bioactive components. Together, these omics approaches provide an integrated understanding of the functional potential of EVs [81,86]. Alongside these molecular approaches, emerging platforms such as microfluidics and single-vesicle technologies (e.g., ExoView or nano-flow cytometry) provide high sensitivity, reduced sample requirements, and multiplex detection, making them particularly promising for clinical applications [87,88].

Although the lack of standardisation remains a significant obstacle for reproducibility and translational value, integrating complementary methodologies is necessary to achieve a reliable, multidimensional characterisation of EVs.

5. Therapeutic Potential of EVs

EVs derived from different cell types play major roles in maintaining physiological homeostasis and mediating disease mechanisms. For instance, EVs derived from stem cells contribute to tissue repair and immune tolerance, while macrophage(M)-derived EVs exert context-dependent functions, with M2-derived vesicles exhibiting pronounced immunoregulatory properties [69,70]. In addition, platelet-derived EVs, alongside their role in coagulation, also modulate immune signalling and tumour progression [71]. Endothelial EVs are involved in maintaining vascular function and are implicated in cardiovascular disease,

whereas eosinophil-derived EVs are increasingly recognised for their roles in allergy, especially asthma [72].

From a therapeutic perspective, EVs show significant promise as drug delivery systems due to their biocompatibility, low immunogenicity, and intrinsic targeting capabilities [89,90]. Moreover, EVs contribute to regenerative medicine by promoting wound healing and supporting tissue repair [90].

EV functionality depends on which cells produce the EVs and the surrounding environment and the specific materials they carry [41,82]. The research community has studied mammalian cell-derived EVs for most of its clinical and translation work, but scientists now turn their focus to microbial vesicles and especially those produced by probiotic bacteria [41,91]. Probiotic-derived EVs function as unique therapeutic vesicles because they deliver specific cargo according to different strains and their protein content changes based on environmental factor [41,92]. The combination of their natural stability and their ability to modulate immune responses and their capacity to bind with immune receptors make these substances strong candidates for developing methods to control immune function [41,65,93]. The following section therefore examines the defining biological and physicochemical properties of probiotic EVs that underpin their therapeutic applicability [41,92].

5.1 Probiotic EVs properties

Accumulating evidence indicates that probiotic-derived EVs play a key role in mediating the biological effects that these bacteria have on the host [60,63]. Probiotics can release EVs that serve as stable carriers of bioactive molecules, such as proteins, lipids, nucleic acids, and metabolites typical of the parental strain, rather than only acting through direct contact of viable bacteria with host tissues [49,50,64]. From a structural point of view, EVs derived from Gram+ bacteria contain diverse molecular cargo that supports bacterial survival and host interaction. These vesicles transport metabolites, peptidoglycan hydrolases, lipoproteins, and stress response enzymes involved in bacterial colonisation, adaptation, and biofilm formation [63]. These EVs interact with host cells through different mechanisms, including endocytosis, direct membrane fusion, and receptor-mediated uptake. These processes facilitate the transfer of molecular cargo that regulates cytokine production, gene expression, and cellular signalling pathways [94,95]. In addition, probiotic-derived EVs can cross biological barriers and reach distant tissues, making them promising candidates for therapeutic applications and drug delivery [96,97].

Probiotic EVs have been shown to mimic different immunomodulatory characteristics associated with whole probiotic bacteria, such as tight junction expression reinforcement and regulation of pro- and anti-inflammatory cytokine responses [98–100]. The immunomodulatory activity of probiotic EVs is primarily mediated through their interaction with PRRs, especially toll-like receptors (TLRs, TRL2 and 4) expressed on epithelial cells and immune cells such as macrophages, dendritic cells, and antigen-presenting cells. Upon receptor engagement, EVs activate intracellular signalling cascades including nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) pathways, leading to the regulation of immune cell polarisation, cytokine production, and epithelial barrier integrity [41,57] [62,101,102]. These signalling pathways can stimulate antigen-presenting cell maturation and modulate inflammatory responses through the release of cytokines. These signals induce the production of anti-inflammatory cytokines (IL-10, TGF-β), promote M2 macrophage polarisation, upregulate tight junction proteins (occluding, claudin-1, ZO-1), and foster a balanced gut microbiota, which collectively culminate in immunological homeostasis and barrier integrity [101,102]. Vesicles isolated from different *Lactobacillus* strains, including *L. plantarum* and *L. paracasei*, display anti-inflammatory properties by promoting M2 macrophage polarisation and increasing the expression of IL-10 and arginase 1 (Arg-1), while reducing the expression of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, and inducible nitric oxide synthase (iNOS) [62,102]. Moreover, EVs from *L. plantarum* (LP25) suppress pro-inflammatory cytokines and induce anti-inflammatory markers through the STAT6/PPAR-γ signalling pathway, thereby promoting M2 macrophage polarisation [62].

In contrast, certain other strains would induce a pro-inflammatory response by stimulating IL-1β, IL-6, TNF-α, and iNOS expression, suggesting that EV-mediated immune modulation is strongly strain-dependent and influenced by local microenvironmental cues [103–105].

However, apart from their potential medical uses, probiotic EVs play an important role in microbial communication, immune regulation, and host–microbe interactions. Therefore, further research of their biogenesis mechanisms, cargo selection, and host interactions will lead to the widening of medical applications for creating new treatment strategies [106] (Figure 2).

5.2 Preclinical evidence of immunomodulatory and barrier-protective effects

Preclinical studies provided more evidence supporting the functional relevance of probiotic-derived EVs in immune modulation and epithelial barrier protection. *In vitro* models have demonstrated that EVs derived from *Lactobacillus rhamnosus* GG activate NF-κB signalling

and stimulate IL-8 secretion in human intestinal epithelial Caco-2 cells, highlighting their ability to regulate epithelial immune responses[107]. These findings are supported by *in vivo* studies showing that bacterial EVs contribute to intestinal immune homeostasis and inflammation control. For example, EVs derived from *L. paracasei* attenuate intestinal inflammation in a dextran sulfate sodium (DSS)-induced colitis mouse model by modulating the endoplasmic reticulum stress response in epithelial cells, suggesting a potential therapeutic role in IBD [100]. *Bacteroides fragilis*-derived EVs induce regulatory T cells through the delivery of polysaccharide A in germ-free or antibiotic-treated mouse models, thereby contributing to immune tolerance and protection against inflammatory disorders such as colitis [98,108,109]. Similarly to the protective effects of *L. paracasei* EVs in intestinal inflammation, *L. plantarum* EVs have been shown to have protective effects in allergic disease models, reducing skin inflammation and immune dysregulation in 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis models, supporting their role in the regulation of allergic responses [102]. In addition to immune modulation, bacterial EVs directly influence epithelial barrier integrity. *E. coli Nissle 1917*-derived EVs were shown *in vitro* to enhance intestinal barrier function by the up-regulation of tight junction proteins such as ZO-1 and claudin-14, and the downregulation of leaky proteins such as claudin-2, in polarised epithelial cell monolayers (Caco-2, T-84). These EVs also enter epithelial cells via clathrin-dependent endocytosis, affirming their dual role in barrier protection and immune regulation [107,110].

Beyond intestinal inflammation, EVs derived from *Akkermansia muciniphila* have demonstrated systemic metabolic and anti-inflammatory benefits in high-fat diet (HFD) mouse models. Treatment with *A. muciniphila*-derived EVs improves lipid metabolism, reduces adipose tissue inflammation, enhances intestinal barrier integrity, and improves circulating metabolic parameters [111].

These studies utilized infection models in living organisms which enabled research to extract peritoneal macrophages for their research on bacterial adhesion and internalization and intracellular killing, which linked physiological exposure to antimicrobial assessment [112–115]. The combination of tumor-bearing mouse models with CD8+ T cell transfer enables researchers to track specific cell clones while conducting functional genetic tests, which improves their understanding of both biological processes and practical applications [116–119]. Intravital and *ex vivo* multiphoton microscopy equipment enables researchers to watch CD4+ and CD8+ T cells move and their interaction with antigen-presenting cells inside intact tissue structures [120–122]. The splenocytes co-culture assays evaluated three aspects of cellular behaviour which include their ability to multiply and their activation status and their ability to

produce cytokines without interfering with the natural process of cell-to-cell communication [123–125]. The combination of neutrophil air pouch models and extracorporeal granulocyte prefusion systems allows research to study three biological processes which include chemotaxis and oxidative burst and viability by using clinical simulation methods [126–128]. The research platforms enable scientists to measure immune mechanisms through their systems which deliver biological results that reflect real human body condition [129,130].

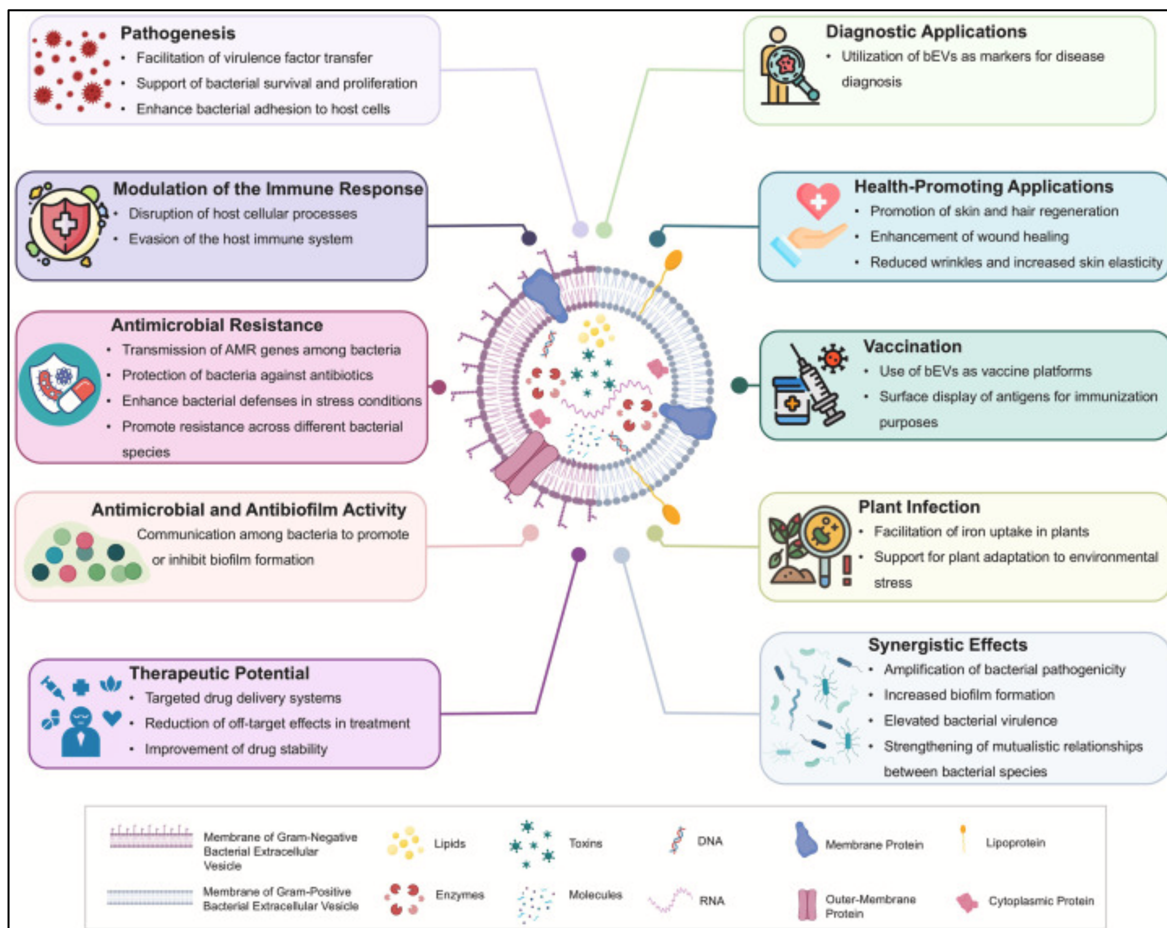

Figure 2: Schematic representation of the dual role of bacterial EVs. On one hand, they contribute to disease pathogenesis by mediating virulence, antimicrobial resistance, and immune modulation. On the other hand, they offer promising therapeutic applications, including drug delivery, vaccination, and health-promoting effects [131].

6. Future Perspective and limits

Despite their promising potential, several challenges must be addressed before probiotic EVs can be translated into clinical applications. The main limits include the standardisation of EVs isolation and purification techniques, ensuring stability and optimal storage conditions, addressing biosafety concerns, particularly those related to strain-specific genetic traits, potential horizontal gene transfer and the use of inadequately characterised bacterial strains,

understanding the immune clearance and biodistribution profiles *in vivo* and overcoming regulatory hurdles and manufacturing scalability issues [132,133].

Probiotic EVs might be a valid alternative to live microbial therapies, offering comparable immunomodulatory and therapeutic effects without the risks associated with administering viable bacteria. EVs can be used for targeted deliveries of bioactive compounds due to their ability to bypass biological barriers, thereby enhancing their efficacy and specificity [41,134]. Additionally, this property defends against immunomodulation of allergies and other immune-mediated illnesses including inflammatory diseases [133,135].

Most probiotic EV biology remains to be understood, including the composition of their cargo and the mechanisms by which they interact with host cells. Additionally, studies should be conducted to investigate their potential as functional foods and nutraceuticals, as well as their immune adjuvant capabilities, thereby further extending preventive and therapeutic strategies [41].

7. Aim of the Thesis

The aim of this thesis was to isolate and characterise EVs derived from LPC and LP probiotic strains namely and to evaluate their immunomodulatory effects on human peripheral blood mononuclear cells (PBMCs). Specifically, this study assessed EV cytocompatibility and quantified the production of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α and the anti-inflammatory cytokine IL-10.

Methods

1. **Preparation of custom-made deMan-Rogosa-Sharpe (MRS) EVs-depleted broth**

The components used to prepare the custom-made MRS broth are listed in Table 2.

COMPONENT	CONCENTRATION
Casein Peptone	10 g/L
K_2HPO_4	2 g/L
Sodium Acetate	5 g/L
Ammonium Citrate	2 g/L
$MnSO_4$	0.05 g/L
$MgSO_4$	0.1 g/L
Glucose	30 g/L

Table 2: Composition of MRS medium used for the cultivation of lactic acid bacteria.

The custom-made MRS broth was prepared by dissolving powders in ddH_2O and sterilised by autoclaving (120°C). After, it was filtered through 0,22 µm filters (Thermo Fisher Scientific™ Nalgene rapid-flow disposable filter unit; Code:566-0020) to remove aggregates. The media was ultracentrifuged for 18 hours at 100000g at 4°C (Thermo Fisher Scientific™ Sorvall WX+ Ultra Series centrifuge, Rotor: SureSpin 632). Lastly, a final filtration step with 0,22 µm filters (Bio-sigma Clearline® Code: 257156) was performed to ensure sterility of the media.

2. **Isolation and quantification of probiotic EVs**

For the isolation of EVs, two probiotic bacteria, *Lactiplantibacillus plantarum* (PBS067; LP) and *Lacticaseibaccilus paracasei* (LPC1114; LPC), gently provided by SynBalance srl, were inoculated in the custom-made MRS broth at 1% and incubated for 24 hours at 37°C. After incubation, the bacterial cultures were centrifuged at 3000 rpm for 15 mins at 4°C (Thermo Fisher Scientific™ Megafuge 16R centrifuge), and the supernatant was filtered with 0,22 µm filters (Bio-sigma Clearline®) to remove any remaining bacterial cells. To isolate EVs, the supernatant of each culture was ultracentrifuged at 100000 g for 4 h at 4°C (Thermo Fisher Scientific™ Sorvall WX+ ultra series centrifuge, Rotor: SureSpin 632). EVs were then resuspended in 1mL of 1X phosphate-buffered saline (1X PBS) and stored at -20°C. In order to measure size and concentration of vesicles, NTA was performed using NanoSight NS300 (Malvern, UK).

3. Micro BCA

To measure protein content, the micro-BCA Protein Assay kit (Thermo Fisher Scientific™, Code: 23235) was used according to the manufacturer's instructions. This colorimetric assay detects protein levels through the reduction of Cu^{2+} to Cu^{+} in an alkaline medium, forming a purple-coloured complex. Briefly, 150µL of each bovine serum albumin (BSA) standard point or samples were added into the wells of a 96-well plate (CytoOne, Code: CC7682-7596). Then, 150 µL of the Working Reagent was added to each well and mixed thoroughly. The plate was covered with sealing tape (Clearline, Code: 760201) and incubated at 37°C for 2 h. After incubation, the plate was allowed to cool at room temperature. Finally, the absorbance was measured at 562 nm using a plate reader Spectrophotometer (Tecan, Switzerland).

4. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from fresh blood obtained from five healthy volunteer donors. Blood was collected and diluted 1:1 with 1X PBS, and cells were isolated by density gradient centrifugation at 1800 rpm for 20 min, using an acceleration setting of 1 and deceleration setting of 0 to minimise gradient disruption. Diluted blood was stratified on Lympholyte (Cedarlane, Burlington, Canada) at a 1:1 ratio. After centrifugation, PBMCs formed a ring at the interface between the plasma/PBS and the Lympholyte. The cells were carefully aspirated, transferred to a new tube, washed with sterile 1X PBS and centrifuged again at 1500 rpm for 5 min. The isolated cells were then resuspended in Roswell Park Memorial Institute (RPMI, Gibco, USA) medium supplemented with 1% of penicillin/streptomycin (PS) (Gibco, USA) and 0,1% of gentamycin (G) (Gibco, USA), and 10% of fetal bovine serum (FBS; Gibco, USA). Cell counting was performed using Trypan blue dye in a disposable Bürker chamber (PAN Biotech, UK).

5. Stimulation of PBMCs with probiotic EVs

To evaluate the cytocompatibility and immune modulation of EVs isolated from LP and LPC, PBMCs were plated at a concentration of 500000 cells/mL and then stimulated with EVs at a concentration of $1x10^{10}$ particles/mL. The volume ratio of EVs to cells was 1:5, with a total of 200 µL per well in a 96-well plate. To mimic inflammatory conditions, PBMCs were stimulated with LPS (from *E. coli* F583, Merck, cod. L6893) at a concentration of 1µg/mL for 4 h before adding probiotic EVs. PBMCs without any stimulation were used as a negative control (NC).
To evaluate the cytocompatibility of LP- and LPC-derived EVs, PBMCs were treated with EVs for 24 and 48 hours, and cellular metabolic activity was measured by (3-(4, 5-

dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay (L6893, Merck). The MTT solution (0,5 µg/ml) was added to each well, and the plate was incubated for 3 h in a humidified incubator (37 °C, 5% CO_2). After the incubation, to dissolve the formazan crystals, MTT solvent (acidified isopropanol with 2 M HCl) was added. Absorbance (A) was measured using a spectrophotometer (Tecan, Switzerland) at 570 nm. To calculate the percentage of viability of PBMCs compared to the NC, the following formula was used:

$$\% \, Viability = \frac{(\text{A} \; - \text{A blank})}{(\text{A NC} - \text{A blank})} \times 100$$

To evaluate cytokine production by PBMCs, TNF-α and IL-10 levels were measured in culture supernatants using ELISA kits (R&D Systems). The samples and reagents were prepared by following the kit instructions. Briefly, 100 µl of the captured antibody was added to a 96-well plate and incubated overnight at room temperature. Then, the plate was washed 3 times with wash buffer (0,05% Tween 20 in PBS) and blocked with 300 µL of reagent diluent (1% BSA) for 1 hour. Following a second washing step, 100 µl of prepared samples and standards were added to each well and incubated for 2 hours. Plates were washed again, and 100 µl of detection antibody was added and incubated for 2 hours. After incubation, plates were washed, and 100 µl of streptavidin-HRP was added and incubated for 20 minutes. After a final washing step, 100 µl substrate (3,3',5,5'-tetramethylbenzidine; TMB) was added and incubated for 20 minutes while avoiding light. Finally, 50 µL of stop solution (2N sulfuric acid) was added to stop the reaction, and absorbance was measured at 450 nm using a spectrophotometer (Tecan, Switzerland). Cytokine concentration was calculated by converting absorbance values to pg/ml, which were fitted with quadratic (second-order polynomial) regression.

6. Statistical Analysis

All experiments were performed in technical duplicates while using PBMCs from 5 healthy donors. Statistical analysis was performed using GraphPad Prism (version 9; GraphPad Software, Inc., USA). For normally distributed data, comparisons between two groups were performed with the Student's one-tailed T-test; multiple comparisons were performed by one-way ANOVA. The level of acceptance was fixed to $p < 0{,}05$. Data are presented as mean ± standard deviation.

Results

1. EVs Concentration and Size

NTA was performed to evaluate the concentration and size of EVs derived from LP and LPC probiotic strains. The mean particle concentration for LP EVs was 1.35 x 10^{10} particles/ml ± 6,76 x 10^{9}, whereas LPC EVs had a significantly higher concentration, averaging 4.78 x 10^{10} particles/ml ± 4.91 x 10^{9} as shown in Figure 4a.

Regarding particle size, LP EVs had a mean diameter of 207.13 ± 21.87 nm, while LPC EVs were slightly smaller, with a mean size of 191.98 ± 3.51 nm (Figure 4b).

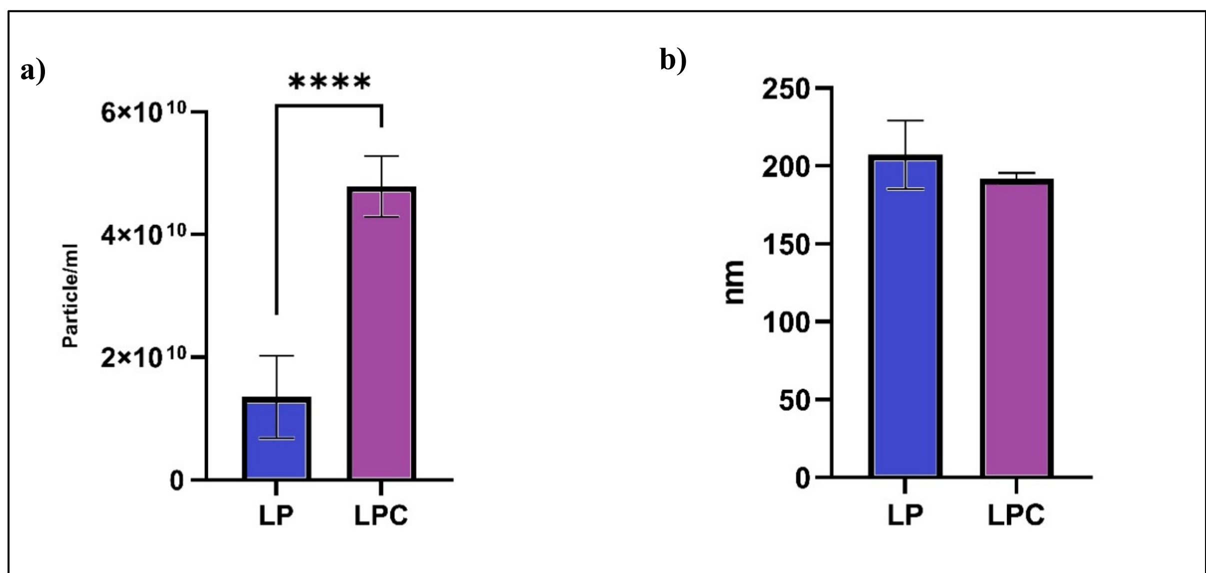

Figure 4: a) Particle concentration, and **b)** mean particle size of EVs derived from LP and LPC. Four asterisks (****) show $p<0.0001$ (Statistical significance by Student t-test).

2. Protein quantification of probiotic EVs

The protein concentration of probiotic EVs was quantified using the microBCA assay. EVs derived from LP showed a mean protein concentration of 645.89 ± 89.26 µg/ml, while the LPC EVs had a higher concentration corresponding to 782.82 ± 132.18 µg/ml (Figure 5), although not statistically significant.

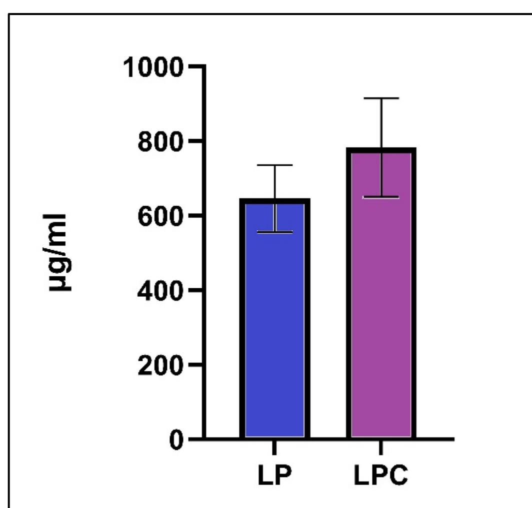

Figure 5: Protein content of EVs derived from LP and LPC, quantified by the microBCA assay.

3. Effects of probiotic EVs on PBMCs viability

The metabolic activity and, consequently, cell viability, were assessed by MTT assay. After 24 hours, PBMCs treated with LPS + LPC EVs (119.19 % ± 12.36) exhibited significantly higher viability than negative control (NC) (100 %; p value of 0.0358). At 48 hours, both LP EVs (119.89% ± 2.46; p value of 0.0418) and LPC EVs (127.26 % ± 17.02; p value of 0.0208) in the presence of LPS groups also showed significantly increased viability relative to NC (100 %), as shown in Figure 6b.

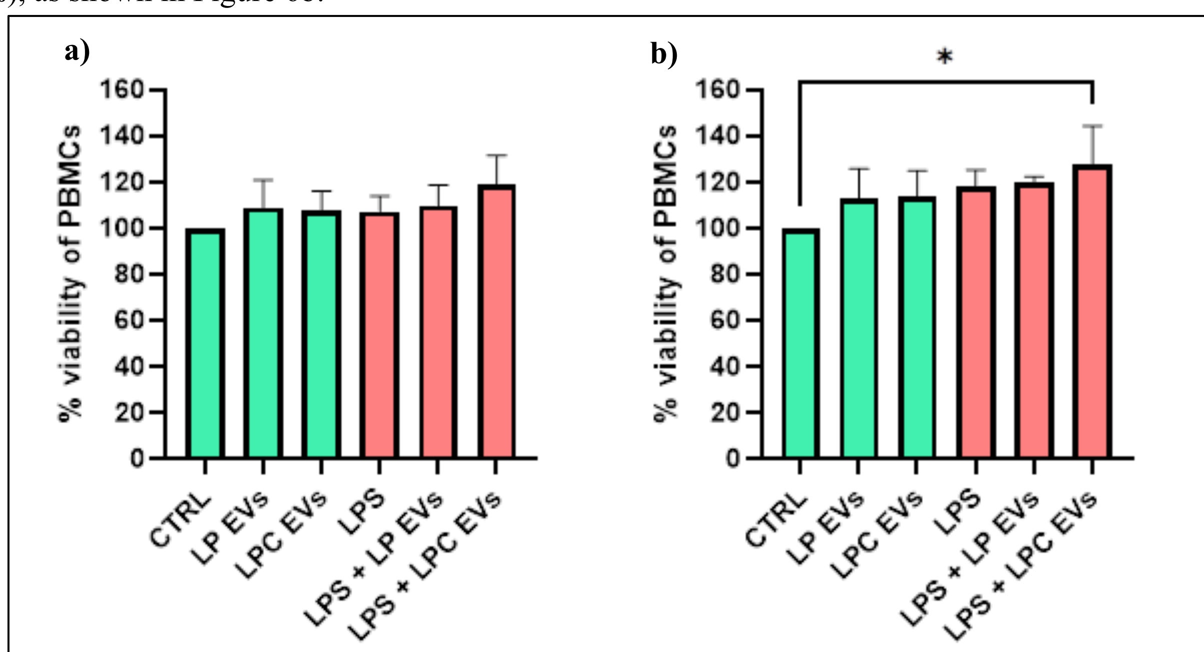

Figure 6: Cell viability was assessed by MTT assay after **(a)** 24 hour and **(b)** 48 hours and expressed relative to the NC, (set at 100%). Both LP EVs and LPC EVs in the presence of LPS significantly increased PBMC viability compared to NC at 48 hours, whereas only LPC EVs showed a significant effect at 24 hours ($p < 0.05$; Anova).

4. Probiotic EVs modulate TNF-α and IL-10 secretion in PBMCs

Since probiotic-derived EVs showed good cytocompatibility, their immunomodulatory effects were further investigated to determine whether these effects are strain-specific. PBMCs were isolated and cultured with probiotic derived-EVs, either alone or in combination with LPS, used as inflammatory stimulus. TNF-α and IL-10 levels were quantified at 24 and 48 hours by ELISA.

After 24 hours, LPC EVs alone induced a marked increase in TNF-α (5399.96 ± 1471.50 pg/ml) compared to the NC (15.04 ± 17.89 pg/ml; $p<0.01$). LP EVs also induced an increase in TNF-α production compared to NC, but to a lower extent than LPC EVs. At 48 hours, TNF-α levels remained elevated in the LPC EV-treated group (4032.77 ± 1460.08 pg/ml) compared to NC (5.88 ± 5.60 pg/ml), despite an overall decrease compared to 24 hours (Figure 7a-b in green).

Regarding IL-10, LPC EVs significantly increased its secretion especially at 48 hours (188.02 ± 46.90 pg/ml) compared to NC (36.98 ± 11.09 pg/ml; $p<0.01$). No significant IL-10 induction was observed at both timepoints for the LP EV-only condition (Figure 8a-b in green).

When combined with LPS, after 24 hours, the LPS + LPC EVs group showed slightly higher TNF-α levels compared to LPS alone; however, this increase did not reach statistical significance. Similarly, at 48 hours, TNF-α levels in the LPS + LPC EVs condition decreased compared to the 24-hour time point but remained comparable to those observed with LPS alone, without statistically significant differences between the two groups (Figure 7a-b in red).

In contrast, IL-10 secretion was enhanced in the LPS + LPC EVs group (211.45 ± 69.66 pg/ml) compared to LPS alone, with this effect becoming more evident at 48 hours (329.75 ± 116.18 pg/ml). These results might suggest a time-dependent induction of an anti-inflammatory response (Figure 8a-b in red).

No significant differences were observed in the LPS + LP EV groups for either TNF-α or IL-10 production at both time points, indicating that LP-derived EVs did not substantially modulate the LPS-induced inflammatory response under these experimental conditions.

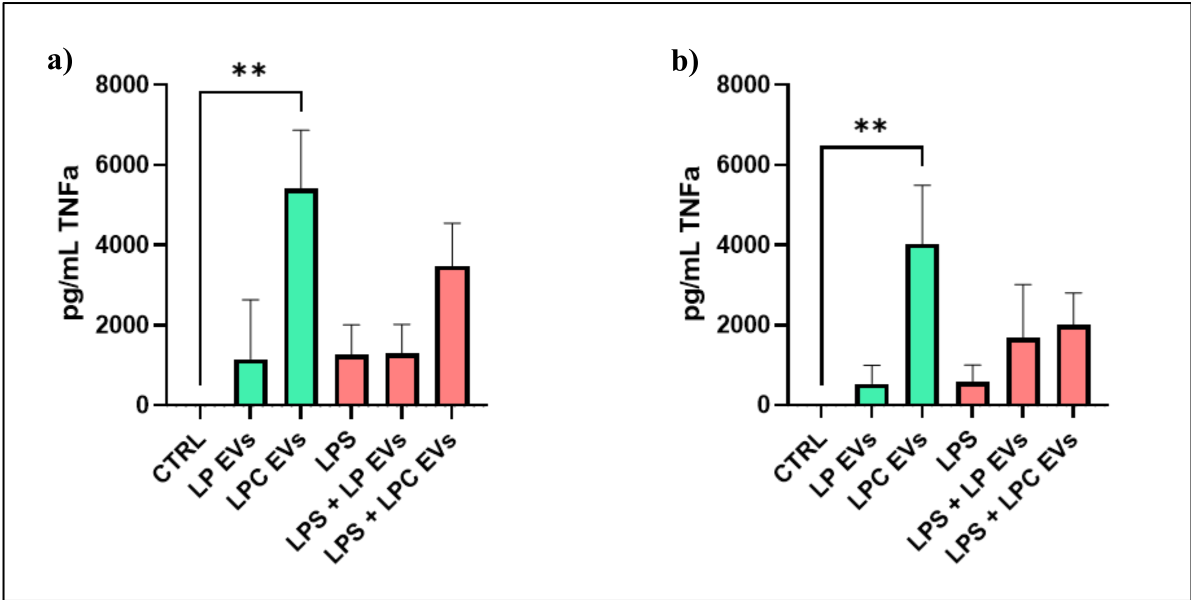

Figure 7: TNF-α secretion in PBMCs treated with EVs at **(a)** 24 hour and **(b)** 48 hours. LPC EVs induced a marked increase in TNF-α compared to NC at both time points (Anova vs NC), while co-treatment with LPS showed no statistically significant difference (Anova vs LPS) (* p<0.05; ** p<0.01).

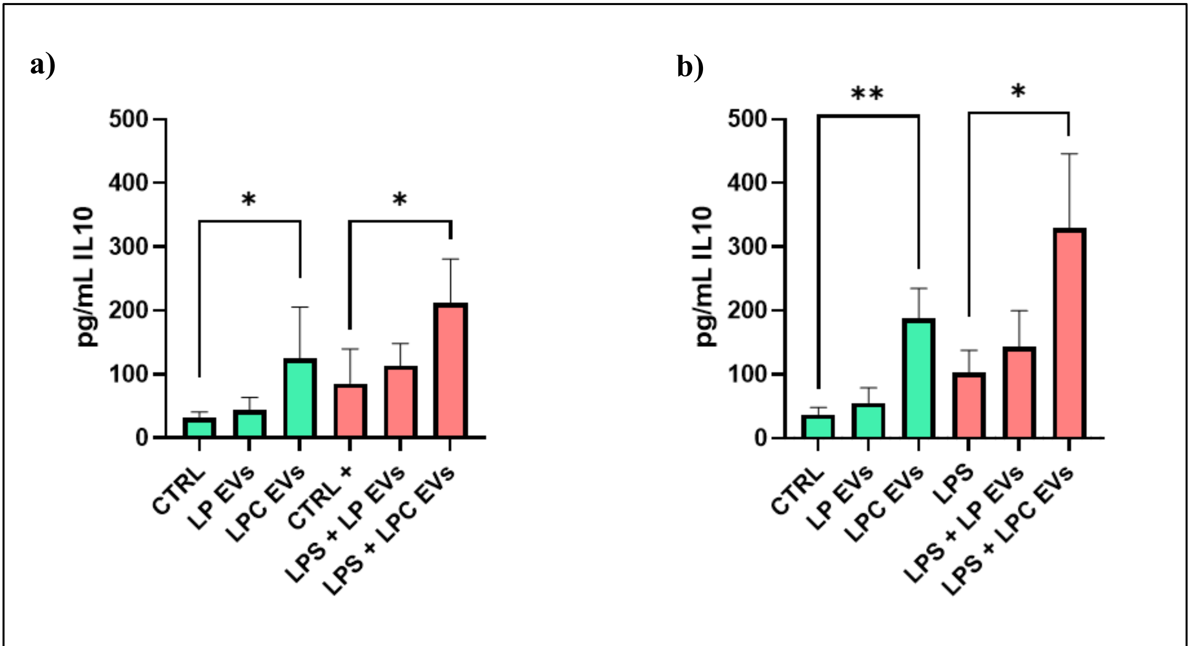

Figure 8: IL-10 secretion in PBMCs treated with EV at **(a)** 24 hours and **(b)** 48 hours. LPC EVs alone and in combination with LPS significantly increased IL-10 levels, respectively compared to NC and LPS (Anova; *p<0.05; **p<0.01), with higher levels observed at 48 hours.

Discussion

EVs have emerged as key mediators of host–microbe communication and have recently gained attention as potential therapeutic tools in immune-mediated disorders [136]. In this thesis, EVs derived from two probiotic strains, (i.e. LP and LPC), were successfully isolated using ultracentrifugation, in accordance with the MISEV2023 guidelines [137].

The main findings were: i) NTA showed that LPC released a significantly higher number of EVs compared to LP; ii) both strains showed no differences in size and a similar amount of protein content ; iii) both LP and LPC-derived EV did not exert cytotoxic effects on PBMCs either under basal or inflammatory conditions (i.e. LPS)and iv) LPC derived EVs induced a significant increase in TNF-α and IL-10 secretions, for the latter also in presence of LPS. The finding that both LP and LPC EVs displayed a mean size of around 200 nm is consistent with Gram+ bacterial EVs size previously reported [60,138]. Gram+ bacteria-derived EVs are composed of a thick bilayer containing membrane and cytosolic proteins, glycolipids and polysaccharides [139,140]. These vesicles tend to carry a wide range of bioactive molecules. Proteomic analysis of *Lactobacillus*-derived EVs has identified cytosolic proteins such as enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and growth operon E, large (GroEL), which are implicated in adhesion processes and immune modulation [140,141]. Other frequently identified proteins include peptidoglycan hydrolases, surface (S-layer) proteins and bacteriocins, all associated with probiotic functionality and host-microbe interactions [139,142]. In addition to proteins, EVs also carry lipids such as phosphatidylglycerol, cardiolipin, and lipoteichoic acids that participate in TLRs signalling and modulation of immune response [73]. Polysaccharides and exopolysaccharide fragments, which are prevalent in *Lactobacillus*-derived EVs, may also contribute to their anti-inflammatory properties of these vesicles through interactions with PRRs of the host, which include TLR2 and C-type lectins, leading to the production of regulatory cytokines such as IL-10, macrophage polarization to M2 macrophages, strengthening of the epithelial barrier, and activation of regulatory immune cell [139]. Significantly, nucleic acids, such as mRNAs and small regulatory RNAs, were also present in probiotic EVs, where they are believed to regulate host gene expression and immune signalling [143].

Functional assays demonstrated that both LP and LPC-derived EV did not exert cytotoxic effects on PBMCs under either basal or inflammatory conditions, such as in the presence of LPS.

Our data are in line with previous studies showing that probiotic-derived EVs are non-toxic to human cells including macrophages, intestinal cells, PBMCs, and placental cell lines and can

even enhance cellular immune functions, as observed with EVs from *L. plantarum WCFS1* (0.3-2.0 μg protein/sample) and *L. crispatus* (10^{10} particles/mL) [60,139,145].

Regarding the cytokine profile, we noted differences among the two probiotics strains. In absence of inflammation, we found that LPC EVs and not LP-EVs induced an increase in TNF-α and IL-10 secretion at both 24 and 48 hours.

Under LPS stimulation, we confirmed these results, though the differences were not statistically significant in TNF-α levels. Overall, these results suggest that LPC have a dual effect by promoting both pro- and anti-inflammatory responses.

Emerging evidence shows that probiotic-derived EVs can replicate or enhance the beneficial effects of the bacteria from which they are released. *L. plantarum WCFS1* EVs restore barrier integrity and promote M2 polarisation in colitis models, while *L. paracasei* EVs suppress TNF-α and IL-1β in activated macrophages and increase IL-10 secretion. Similarly, *L. rhamnosus* GG EVs have protective effects in DSS-induced colitis mice models by decreasing mucosal TNF-α, IL1β, and IL-6 levels [62,100,146].

Mechanistically, Gram+ bacteria-derived EVs carry lipoteichoic acids and lipoproteins that engage TLR2 [64,147], leading to initial NF-κB-mediated TNF-α induction[148], followed by PI3K/Akt-dependent IL-10 upregulation as a compensatory anti-inflammatory response[149,150]. This pattern is consistent with our results, especially for LPC-derived EVs, where the increase of TNF- α secretion is accompanied by a concomitant increase of IL-10. In order to elucidate better the molecules responsible for these immunomodulatory effects, comprehensive metabolomic analysis and RNA or DNA sequencing of LP- and LPC-derived EVs will be needed. Potential effector molecules might include adhesins, enzymes such as enolase and GAPDH, peptidoglycan-modifying proteins, and small regulatory RNAs [140,141]. Identification of these metabolites and their correlation with cytokine expression patterns would provide deeper insight into the mechanisms underlying probiotic EV-mediated immune regulation.

Despite the growing preclinical evidence supporting the therapeutic potential of probiotic-derived EVs, their clinical translation remains limited. This translational gap mainly reflects challenges associated with standardising EV production and purity. Moreover, the absence of unified protocols for bacterial EV isolation, purification, characterisation, and quantification represents one of the major obstacles.

One of the principal technical limitations associated with probiotic-derived EV research concerns vesicle purity. Using only ultracentrifugation as isolation method might lead to co-isolation of protein aggregates, lipoprotein complexes, and other non-vesicular components,

potentially affecting downstream functional analyses. So, as a future perspective, EV purity will be optimized by using other techniques, such as SEC [151]. In our study, we used EV-depleted MRS culture medium. Traditional bacterial culture broths contain protein or lipid particles and vesicle-like structures that may interfere with NTA or other quantification techniques [152]. Although this approach improved sample quality, it did not fully eliminate the possibility of co-isolated contaminants, further emphasising the need for additional purification steps in future investigations. The cytokine analysis performed in this study, restricted to IL-10 and TNF-α, provides only a partial representation of the immunomodulatory potential of probiotic EVs. Expanding the cytokine panel to include mediators such as IL-1β, IL-6, IL-12/23, IFN-γ, IL-17A, TGF-β, and chemokines would allow a more comprehensive evaluation of both pro-inflammatory and regulatory immune responses. Additionally, investigating early intracellular signalling pathways, such as STAT3 activation, NF-κB phosphorylation, and Akt phosphorylation, could help determine whether probiotic EVs preferentially influence innate or adaptive immune pathways.

Conclusion

The results of this study demonstrated that EVs derived from LP and LPC exert a different immunomodulatory effect on PBMCs, whilst preserving cell viability. Further investigation of EV cargo composition, including proteomic, metabolomic, and nucleic acid analyses, will be essential to identify the molecular mediators responsible for these effects and to optimise their therapeutic application. Overcoming current limitations related to standardisation, purification, mechanistic characterisation, and clinical validation will be essential to fully translate their biological properties into safe and effective therapeutic interventions.

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