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Extracellular vesicles from microbiota-derived *Escherichia coli* strains as a postbiotic strategy to Enhance intestinal maturation and modulate gut-liver and gut-brain communication axes

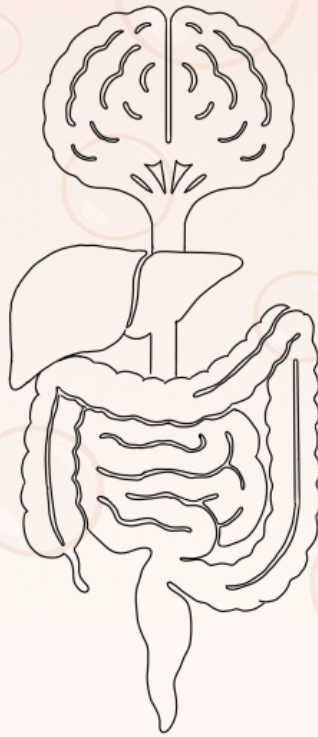
Sergio Martínez Ruiz

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Extracellular vesicles from microbiota-derived
Escherichia coli strains as a postbiotic strategy to
enhance intestinal maturation and modulate gut-
liver and gut-brain communication axis



Sergio Martínez Ruiz

**Barcelona
2025**





UNIVERSITAT DE
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UNIVERSITAT DE BARCELONA
FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Departament de Bioquímica i Fisiologia
Secció de Bioquímica

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UNIVERSITAT DE
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Programa de doctorat

BIOTECNOLOGIA

Extracellular vesicles from microbiota-derived *Escherichia coli* strains as a postbiotic strategy to Enhance intestinal maturation and modulate gut-liver and gut-brain communication axes

Vesículas extracelulares de cepas de *Escherichia coli* derivadas de la microbiota como estrategia postbiótica para favorecer la maduración intestinal y modular los ejes de comunicación intestino-hígado e intestino-cerebro

Vesícules extracel·lulars de soques d'*Escherichia coli* derivades de la microbiota com a estratègia postbiòtica per afavorir la maduració intestinal i modular els eixos de comunicació intestí-fetge i intestí-cervell.

Memoria presentada por **Sergio Martínez Ruiz** para optar al Título de doctor con Menció Internacional por la Universitat de Barcelona

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ABSTRACT

The intestinal microbiota plays a crucial role in maintaining mucosal and systemic homeostasis, particularly during early life when the immune and epithelial systems are still developing. Among microbial-derived components, extracellular vesicles (EVs) produced by beneficial gut bacteria have emerged as promising postbiotic agents capable of modulating host physiology beyond the gut. This thesis investigates the immunomodulatory, antiviral, metabolic, and neurodevelopmental effects of EVs derived from *Escherichia coli* strains EcN (probiotic) and EcoR12 (commensal) in *in vivo* neonatal and *in vitro* models.

In healthy neonatal rats, oral administration of *E. coli* EVs enhanced humoral and cellular immune responses, promoted intestinal epithelial maturation, and accelerated the development of a balanced Th1/Th2 immune profile without inducing inflammation. In a neonatal rotavirus infection model, EV treatment reduced diarrhea severity, preserved mucosal integrity, and stimulated virus-specific immunity. These protective effects were linked to the modulation of serotonin-related pathways and mucin secretion.

Beyond the gut, administered EVs reached the liver and induced gene expression profiles favoring anti-inflammatory, antioxidant, and lipid oxidation pathways, supporting their role in gut–liver axis communication under physiological conditions. In *In vitro* cellular models derived from human inducible pluripotent stem cells (iPSC), EVs upregulated the expression of tight junction proteins in intestinal organoids and enhanced dendritic branching during neuronal differentiation. These findings suggested that microbiota EVs may have a dual action along the gut–brain axis, modulating intestinal epithelial barrier integrity and neurodevelopment.

Collectively, the findings presented in this thesis position EcN- and EcoR12-derived EVs as safe and effective postbiotic candidates capable of supporting early-life immune and epithelial maturation, enhancing antiviral defenses, and modulating organ

functions across diverse gut-organ axis. Moreover, they provide a strong rationale for further investigation of microbiota-derived EVs in disease-relevant models and future translational studies.

Keywords: Extracellular vesicles; Microbiota; Postbiotic; Rotavirus; Gut-liver axis; Gut brain-axis.

RESUMEN

La microbiota intestinal desempeña un papel fundamental en el mantenimiento de la homeóstasis intestinal y sistémica, especialmente durante las primeras etapas de la vida, cuando el sistema inmunitario y el epitelio intestinal aún se están desarrollando. Entre los componentes derivados de los microorganismos, las vesículas extracelulares (EVs) producidas por bacterias intestinales beneficiosas han emergido como agentes postbióticos prometedores, capaces de modular la fisiología del hospedador más allá del intestino. Esta tesis investiga los efectos inmunomoduladores, antivirales, metabólicos y en el desarrollo neuronal de las EVs derivadas de cepas de *Escherichia coli*, EcN (probiótica) y EcoR12 (comensal), en modelos neonatales *in vivo* y modelos celulares *in vitro*.

En ratas lactantes sanas, la administración oral de EVs de *E. coli* potenció las respuestas inmunitarias humorales y celulares, promovió la maduración del epitelio intestinal y aceleró el desarrollo de un perfil inmunológico Th1/Th2 equilibrado, sin inducir inflamación. En un modelo neonatal de infección por rotavirus, el tratamiento con EVs redujo la gravedad de la diarrea, preservó la integridad de la mucosa y estimuló la inmunidad específica frente al virus. Estos efectos protectores se asociaron a la modulación de rutas relacionadas con la serotonina y la secreción de mucina.

Más allá del intestino, las EVs administradas alcanzaron el hígado e indujeron perfiles de expresión génica asociados a rutas antiinflamatorias, antioxidantes y de oxidación lipídica, lo que respalda su papel en la comunicación a lo largo del eje intestino–hígado en condiciones fisiológicas. En modelos celulares *in vitro* derivados de células madre pluripotentes inducidas de origen humano (iPSC), las EVs promovieron la expresión de proteínas de unión estrecha en organoides intestinales y favorecieron el grado de ramificación de dendritas durante la diferenciación neuronal. Estos hallazgos sugieren una acción dual de las EVs de la microbiota intestinal a lo largo del eje intestino–

cerebro, modulando tanto la integridad de la barrera epitelial intestinal como el desarrollo neuronal.

En conjunto, los resultados presentados en esta tesis posicionan a las EVs derivadas de EcN y EcoR12 como candidatos a ser postbióticos seguros y eficaces, capaces de favorecer la maduración inmune y epitelial en etapas tempranas de la vida, potenciar las defensas antivirales y modular la función de órganos distales a través de los distintos ejes de comunicación intestino–órgano. Además, estos hallazgos ofrecen una base sólida para futuras investigaciones en modelos patológicos y estudios translacionales.

Palabras clave: Vesículas extracelulares; Microbiota; Postbiótico; Rotavirus; Eje intestino-hígado; eje intestino-cerebro.

RESUM

La microbiota intestinal juga un paper essencial en el manteniment de la homeòstasi de intestinal i sistèmica, especialment durant les primeres etapes de la vida, quan els sistemes immunitari i epitelial encara estan en desenvolupament. Entre els components derivats de la microbiota, les vesícules extracel·lulars (EVs) produïdes per bacteris intestinals beneficiosos estan emergint com a agents postbiòtics prometedors, capaços de modular la fisiologia de l'hoste més enllà de l'intestí. Aquesta tesi estudia els efectes immunomoduladors, antivírics, metabòlics i sobre el desenvolupament neuronal de les EVs derivades de soques d'*Escherichia coli*, EcN (probiòtica) i EcoR12 (commensal), mitjançant models *in vivo* emprant rates neonatals i models cel·lulars *in vitro*.

En rates lactants sanes, l'administració oral d'EVs d'*E. coli* va millorar les respostes immunitàries humorals i cel·lulars, va promoure la maduració de l'epiteli intestinal i va afavorir el desenvolupament d'un perfil immunitari Th1/Th2 equilibrat, sense provocar inflamació. En un model neonatal d'infecció per rotavirus, el tractament amb EVs va reduir la gravetat de la diarrea, va preservar la integritat de la mucosa i va estimular la resposta immunitària específica contra el virus. Aquests efectes protectors es van relacionar amb la modulació de vies associades a la serotonina i la secreció de mucina.

Més enllà de l'intestí, les EVs administrades van arribar al fetge i van induir perfils d'expressió gènica que afavorien vies antiinflamatòries, antioxidants i d'oxidació lipídica, posant de manifest el seu paper en la comunicació a través de l'eix intestí–fetge en condicions fisiològiques. En models cel·lulars *in vitro* derivats de cèl·lules mare pluripotents induïdes d'origen humà (iPSC), les EVs van augmentar l'expressió de proteïnes de les unions estretes en organoids intestinals i van potenciar el grau de ramificació de dendrites durant la diferenciació neuronal. Aquests resultats suggereixen una acció dual de les EVs derivades de la microbiota al llarg de l'eix intestí–

cervell, modulant tant la integritat de la barrera epitelial intestinal com el desenvolupament neuronal.

En conjunt, els resultats d'aquesta tesi situen les EVs derivades de EcN i EcoR12 com a candidats postbiòtics segurs i efectius, capaços de promoure la maduració del sistema immune i epitelial en les primeres etapes de la vida, potenciar les defenses antivíriques i modular la funció d'òrgans al llarg dels diferents eixos de comunicació intestí-òrgan. A més, aquests resultats ofereixen una base sòlida per a futures investigacions en models de malaltia i estudis translacionals.

Paraules clau: Vesícules extracel·lulars; Microbiota; Postbiòtic; Rotavirus; Eix intestí-fetge; eix intestí-cervell.

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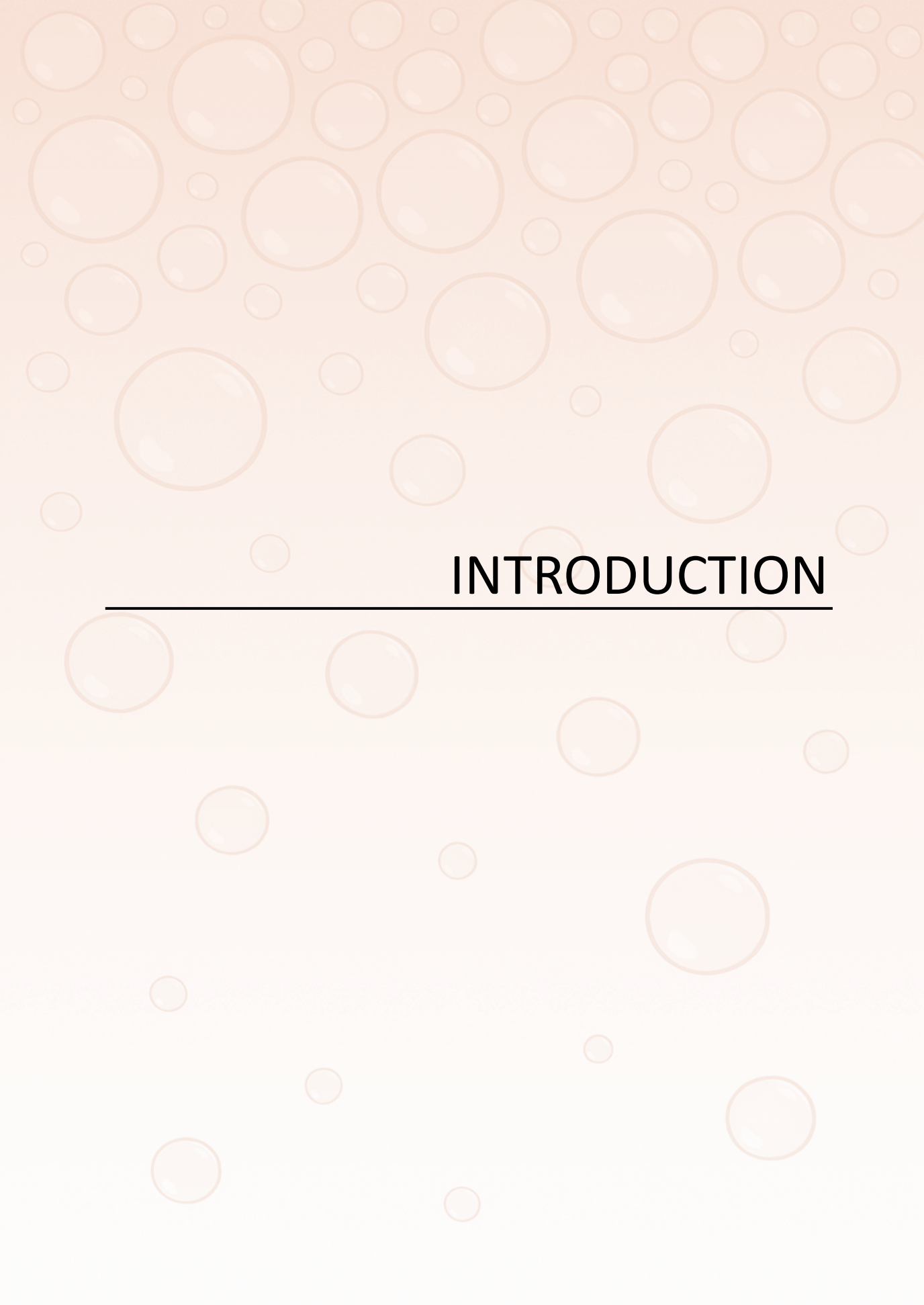
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LIST OF ABBREVIATIONS

5-HPT: 5-hydroxytryptophan	LPS: Lipopolysaccharide
5-HT: 5-hydroxytryptamine (serotonin)	Muc-2: Mucin-2
5-HTR: Serotonin receptor	NAFLD: Non-alcoholic fatty liver disease
AADC: Aromatic L-amino acid decarboxylase	NASH: Non-alcoholic steatohepatitis
ALD: Alcohol-associated liver disease	NK: Natural killer cell
AMPs: Antimicrobial peptides	NKT: Natural killer T cell
BA: Bile acids	NLRs: NOD-like receptors
BBB: Blood brain barrier	OCLN: Occludin
CNS: Central nervous system	OMVs: Outer membrane vesicles
CLRs: C-type lectin receptors	PAMPs: Pathogen-associated molecular patterns
DCs: Dendritic cells	PPAR: Peroxisome proliferator-activated receptor
DLPs: Double-layered particles	PRRs: Pattern recognition receptors
EC: Enterochromaffin cells	RIG: Retinoic acid-inducible gene
ENS: Enteric nervous system	RLRs: (RIG)-I-like receptors
EPEC: Enteropathogenic <i>E. coli</i>	RV: Rotavirus
ER: Endoplasmic reticulum	SCFAs: Short-chain fatty acids
EVs: Extracellular vesicles	SERT: Serotonin transporter
FcRn: Neonatal Fc receptor	slgA2: Secretory immunoglobulin A2
GALT: Gut-associated lymphoid tissues	Tc: Cytotoxic T CD8+ cells
GF mice: Germ-free mice	Th: CD4+ T helper cells
GIT: Gastrointestinal tract	TJs: Tight junctions
GPR: G protein-coupled receptor	TLPs: Triple-layer particles
HCC: Hepatocellular carcinoma	TLRs: Toll-like receptors
IDO: Indoleamine 2,3-dioxygenase	TPH1: Tryptophan hydroxylase 1
IECs: Intestinal epithelial cells	TNF: Tumor Necrosis Factor
Ig: Immunoglobulin	Treg: Regulatory T cells
IFN: Interferon	Trp: Tryptophan
IL: Interleukin	ZO: Zonula Occludens
JAM: Junctional Adhesion Molecules	



INTRODUCTION

1. HUMAN GUT MICROBIOTA

The human microbiome encompasses the collective genomes of the diverse microbial communities residing on and within the human body. They include protozoa, archaea, eukaryotes, viruses, and predominantly bacteria, which coexist in a symbiotic relationship with the host. These microbial communities colonize various anatomical niches such as the oral cavity, genital organs, respiratory tract, skin, and gastrointestinal system [1].

The gastrointestinal tract (GIT) is a long tube-like structure composed of different organs stretching from the mouth to the anus. Notably, essential processes such as food digestion, water/nutrients absorption, and waste elimination occur in the intestine. The intestine, a major component of the GIT, is divided into the small intestine (i) comprising the duodenum, jejunum, and ileum; (ii) the large intestine, which includes the cecum, colon (subdivided into ascending, transverse, descending, and sigmoid regions), rectum, and anus. The intestinal architecture is meticulously adapted for its digestive functions, with a layered organization composed of four layers that from inside to outside are mucosa, submucosa, muscularis, and serosa [2].

The human gastrointestinal tract is colonized by trillions of microbes, with an estimated ratio of at least 10:1 bacterial to human cells. These numbers are derived from the total bacterial cells in colon (3.8×10^{13} bacteria), the organ that harbors the densest number of microbes) [3]. This complex polymicrobial community, known as the gut microbiota, is mainly composed of bacteria from four major phyla, namely Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria [4]. Variations in microbiota composition along the intestine are influenced by chemical gradients, immunological, and nutrient factors that shift throughout the gut, conferring distinct physiological properties to specific intestinal regions (Figure 1) [5].

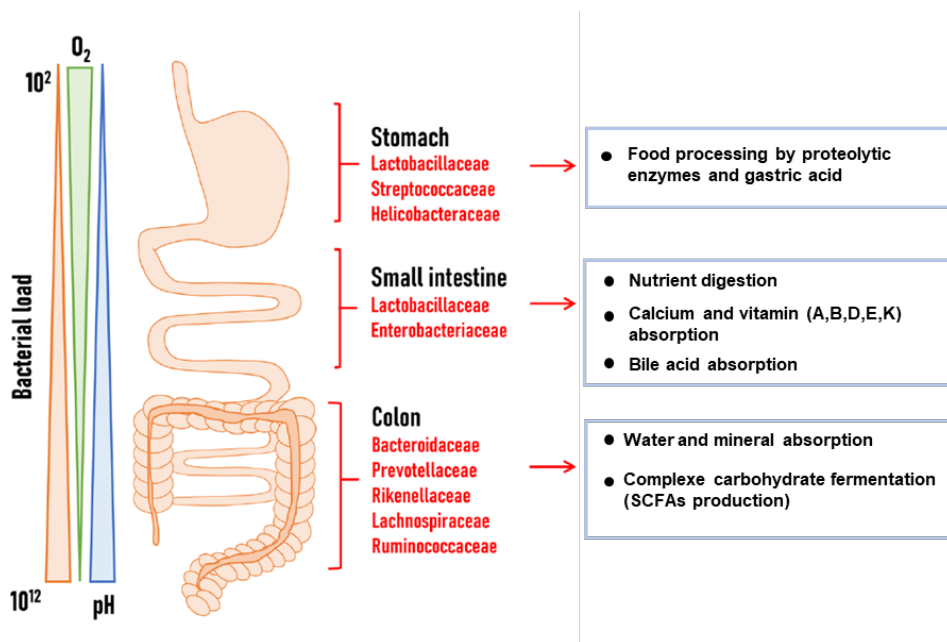


Figure 1: Microbiota distribution in the human gastrointestinal tract. Human microbiota varies along the gastrointestinal tract. The gradient of oxygen, antimicrobial molecules (including bile acids secreted by the bile duct) and pH limits the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. Regional differences are in accordance with the functional heterogeneity of GIT segments.

This diverse and complex microbiome serves as a functional expansion of host genomes, and it is estimated to harbor 50- to 100-fold more genes, compared to the human genome. These extra genes have added various types of enzymatic proteins which were non-encoded by the host, and play a critical role in facilitating metabolism, thus contributing to the regulation of host physiology [6]. However, the microbiota is not only involved in food digestion and nutrient metabolism, it also plays a crucial role in host immune system development and modulation of the gut barrier, immune, and defense responses [7].

Many studies have attempted to describe the normal gastrointestinal microbiota in terms of microbial species that inhabit healthy humans. However, individual differences in the microbiota composition make the definition of this concept very challenging [1,8]. Even though, it is clear that the present view of the microbial

diversity within the human gastrointestinal tract is incomplete, it is widely accepted that alterations in microbiota composition and diversity can trigger ecological imbalances, or “dysbiosis,” predisposing individuals to a broad range of diseases, including cancer, inflammatory, autoimmune, metabolic, and neurological disorders [1,9].

1.1. FUNCTIONS OF THE GUT MICROBIOTA

The gut microbiota is considered the largest endocrine organ in the body due to its capacity to influence and participate in the homeostasis of a wide range of physiological functions. The functions of the intestinal microbiota can be categorized into four types: protective (protection against pathogens), metabolic (nutrient metabolism), immune (immune system modulation), and barrier maintenance (structural integrity of the intestinal barrier) (Figure 2).

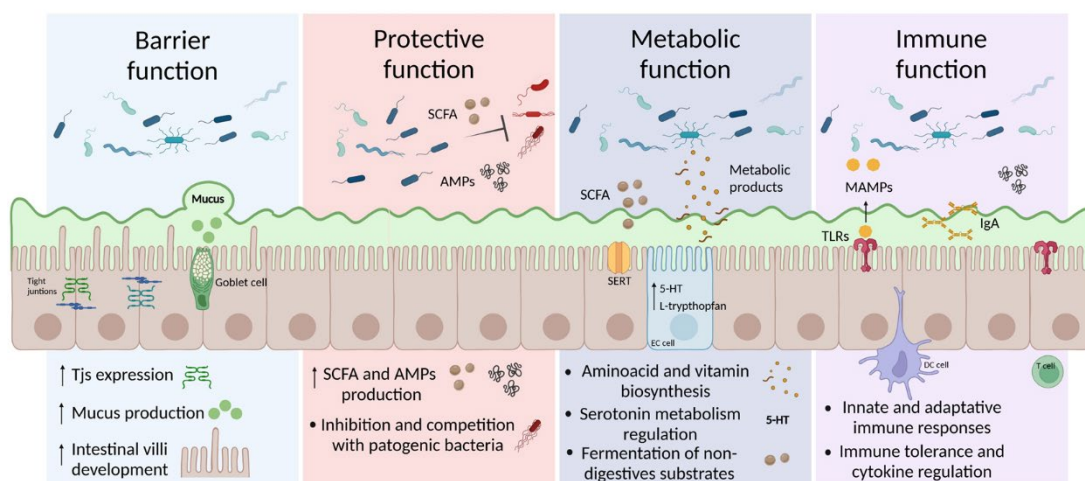


Figure 2: Functions of the gut microbiota. The beneficial roles of gut microbiota can be divided into four major categories: barrier, protective, metabolic, and immune functions.

1.1.1. PROTECTIVE AND ANTIMICROBIAL FUNCTION

The intestinal microbiota and its metabolic products play a fundamental role in protecting the host against the invasion and colonization of pathogenic microorganisms through various mechanisms [10]. Firstly, the intestinal microbiota competes with pathogens for space, essential nutrients, and growth factors within the

gut, effectively limiting their capacity to proliferate and adhere to the intestinal epithelium [11,12]. Furthermore, the gut microbiota secretes a range of bioactive compounds, such as short-chain fatty acids (SCFAs), lactic acid, hydrogen peroxide, and antimicrobial peptides (AMPs), which disrupt the growth of certain microorganisms [13].

Among SCFAs, butyrate exhibits the most significant physiological effects [14] including the ability to inhibit the growth of *Clostridium difficile* [15] and its role in preventing the colonization of opportunistic aerobic pathogens like *Salmonella* [16].

AMPs, also known as bacteriocins, are small, heat-stable peptides naturally produced by many Gram-positive and Gram-negative strains. They exert specific antimicrobial activity through various mechanisms including inhibition of cell wall synthesis, permeabilization of the target cell membrane, pore formation, or cell lysis [17]. Bacteriocins are classified into: (i) microcins and colicins, which are bacteriocins secreted by enterobacteria; and (ii) lantibiotics, which are bacteriocins secreted by Gram-positive bacteria [18].

Another mechanism by which the intestinal microbiota prevents the overgrowth of pathogenic strains is the induction of protein synthesis and the release of antibacterial compounds. It stimulates host cells, such as Paneth cells, to produce antibacterial proteins through the activation of the pattern recognition receptors (PRRs). This leads to the secretion of defensins, cathelicidins, C-type lectins, and lipocalin-1, which are retained in the mucus layer to prevent microbial access to the epithelium [19]. Additionally, secretory immunoglobulin A2 (sIgA2) [20], locally produced through dendritic cell-mediated differentiation of IgA-secreting B cells, coats intestinal microorganisms, restricting their translocation into circulation and preventing systemic immune responses [21,22].

1.1.2. METABOLIC FUNCTION

The microbiota plays a key role in various metabolic processes that directly influence nutrient utilization, body weight regulation, and energy homeostasis. Among these processes, microbial metabolites produced by the microbiota impact both the microbial community itself and the host's overall homeostasis. The intestinal microbiota contributes to this function through two primary mechanisms: the digestion and fermentation of dietary components that the host cannot assimilate and the modulation of lipid, carbohydrate, and protein metabolism [23].

Degradation of indigestible polysaccharides. The gut microbiota ferments host-indigestible carbohydrates, such as fiber, resistant starches, and polyphenols. This fermentation results in the production of SCFAs, vitamins, and other bioactive metabolites which are absorbed and utilized by the host [24]. SCFAs are fatty acids with fewer than six carbon atoms and consist of three major forms: acetate (60%), propionate (20%), and butyrate (20%) [25]. These SCFAs function as key metabolic and immunologic mediators, playing essential functions in maintaining the intestinal barrier's integrity, regulating immune cells development and modulating energy metabolism [26]. Also, they exhibit anti-inflammatory properties, particularly, in immune T cells, neutrophils, and macrophages [27–32]. For instance, butyrate participates in maintaining an anaerobic environment in the intestinal tract and activates peroxisome proliferator-activated receptor gamma (PPAR γ) by promoting mitochondrial β -oxidation in intestinal epithelial cells (IECs) [33,34]. Acetate and propionate induce colonic regulatory T cells (T reg), offering protection against experimentally induced colitis [35]. Extensive evidence suggests that acetate suppresses intestinal inflammation via G protein-coupled receptor (GPR) 43 signaling in neutrophils [36]. SCFAs exert a range of bioactive effects that help maintaining the immune system [37]. Butyrate is an energy source for IECs and has strong anti-inflammatory and immunomodulatory effects [38]. It also enhances intestinal barrier function by stimulating antimicrobial peptide and cytokine production, inhibiting pathogen overgrowth, and strengthening epithelial integrity [37,39,40].

Protein and amino acid metabolism: Recent studies have shown that the human colon harbors a high microbial diversity with a wide range of proteolytic activities responsible for degrading both dietary and endogenous proteins produced by the host [41]. This process results in the formation of peptides, amino acids, and nitrogenous compounds, which can have significant physiological impacts. Proteolytic bacteria are predominantly from the genera *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium*, *Streptococcus*, and *Lactobacillus* [42]. Additionally, the intestinal microbiota is capable of degrading, absorbing, and synthesizing certain amino acids that the body cannot produce or is deficient in, such as alanine, arginine, cysteine, glutamine, glycine, histidine, proline, serine, and tyrosine. Fermentation of amino acids by gut bacteria is associated with increased concentrations of branched-chain fatty acids, phenols, indole derivatives, and ammonia [43].

Serotonin (5-HT) metabolism: Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine primarily recognized for its role as an essential neurotransmitter in the development and function of the central nervous system (CNS). However, only about 5% of the total 5-HT in the human body originates from serotonergic neurons in the CNS. The remaining 95% is produced in the intestine, where it plays a crucial role in regulating the enteric nervous system (ENS), immune responses, and processes such as intestinal motility, secretion, inflammation, and maintenance of epithelial barrier integrity [44].

In the gastrointestinal system, serotonin is synthesized from the precursor amino acid L-tryptophan in enterochromaffin cells (EC) (Figure 3). Only 1% of dietary tryptophan is metabolized and converted into 5-HT, with approximately 4-6% being metabolized by the microbiota [45]. The intestinal microbiota competes with the host for available tryptophan, metabolizing it into indole and skatole, which activate immune responses that promote intestinal homeostasis.

Bacteria contribute to 5-HT synthesis either through direct production of 5-HT or by secreting secondary metabolites that stimulate 5-HT synthesis in EC cells. Indeed,

germ-free mice exhibit elevated levels of tryptophan and 5-HT in serum, which normalize upon colonization with microbiota derived from the feces of wild-type mice [46].

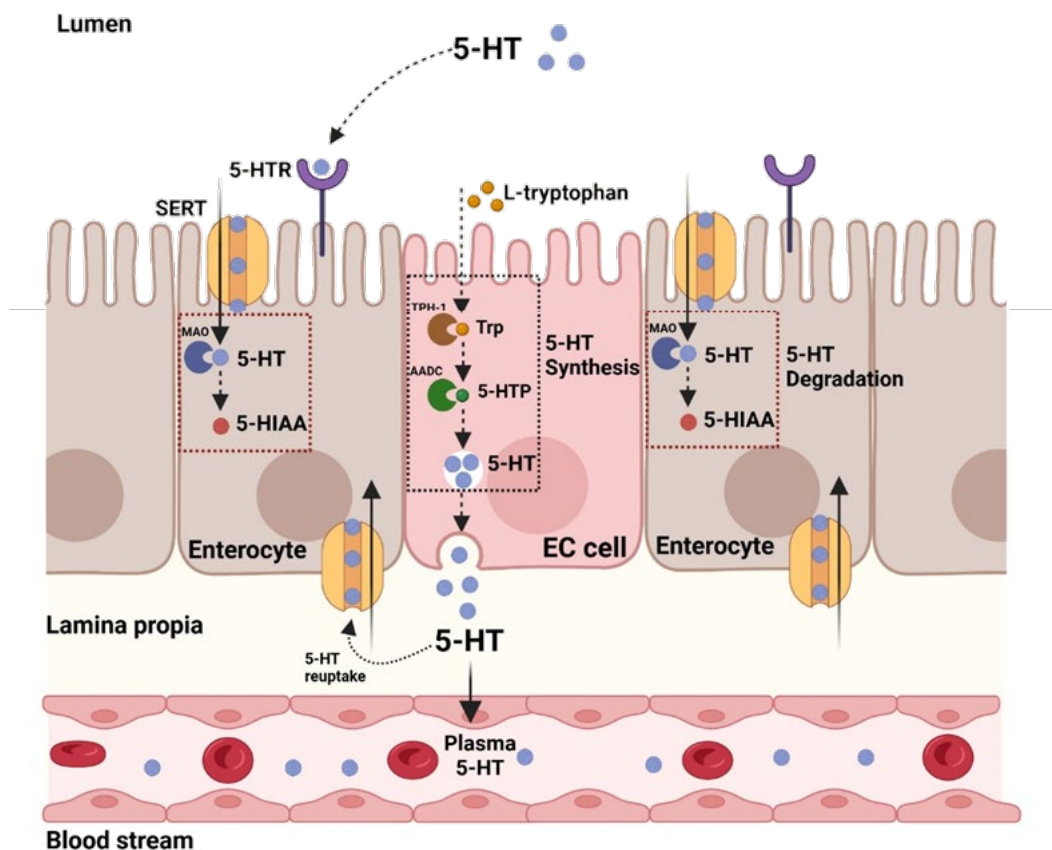


Figure 3. Schematic Representation of the Intestinal Serotonergic System. The intestinal serotonergic system has two main states: "ON" and "OFF." In the "ON" state, enterochromaffin (EC) cells convert tryptophan into serotonin (5-HT) via TPH1 and AADC enzymes, store it in vesicles using VMAT1, and release it into the extracellular space. Serotonin then binds to receptors (5-HTR). In the "OFF" state, 5-HT is reabsorbed by enterocytes or platelets through SERT, ending its action. Binding to platelet receptors like 5-HT_{2A} triggers calcium mobilization and platelet degranulation, releasing more 5-HT. Illustration created using BioRender.com and adapted from Layunta et al. [49].

Bacterial metabolites that promote 5-HT synthesis include SCFAs and bile acids. Acetate, propionate, and butyrate—products of anaerobic fermentation in the gut by bacteria from the Firmicutes and Bacteroidetes phyla—have been shown to activate free fatty acid receptors in EC cells. This activation increases the expression of the

enzyme tryptophan hydroxylase 1 (TpH1), which is responsible for converting L-tryptophan into hydroxytryptophan (5-HPT), thereby stimulating 5-HT production [47]. Recently, metabolites such as α -tocopherol, folate, tyramine, p-aminobenzoate, and lipopolysaccharide (LPS) from *Escherichia coli* have been identified as enhancers of 5-HT secretion in EC cells [48].

The microbiota also exerts an indirect effect on tryptophan metabolism by activating genes involved in its processing. Administration of *Bifidobacterium infantis* in rats has been shown to reduce the activity of the enzyme indoleamine 2,3-dioxygenase (IDO), thereby increasing the availability of tryptophan for 5-HT synthesis [50].

Additionally, other genes involved in serotonin reuptake and receptor function, which directly participate in 5-HT metabolism, are regulated by microbiota action. In germ-free mice, an increase in the expression of the serotonin reuptake transporter (SERT) has been observed. However, *Escherichia coli* AIEC can inhibit both the expression and function of this serotonin transporter [51]. In mouse models infected with *Citrobacter rodentium*, a reduction in the number of EC cells was observed, along with decreased SERT expression ten days post-infection [52].

The ability of 5-HT to influence various physiological responses is attributed to its diverse receptor system. In the intestine, five classes of receptors (5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, and 5-HT₇) are expressed, with 5-HT₃ and 5-HT₄ being the most extensively studied. These receptors mediate the actions of 5-HT released by EC cells. Once activated, they mediate a wide range of intestinal functions, which must be accompanied by regulatory mechanisms; otherwise, disruptions related to increased 5-HT levels can occur [53,54]. Specifically, the 5-HT₃ receptor plays an active role in the regulation of intestinal motility. [55].

Disruptions in serotonin metabolism, and consequently in free serotonin levels, can impact the composition of the microbiota. *In vivo* studies have demonstrated that the inhibition of SERT leads to elevated levels of the *Lactobacillus*, *Streptococcus*,

Enterococcus, and *Listeria*, while significantly decreased abundance of *A. muciniphilia* and *Bifidobacterium* species[56].

Vitamin synthesis: Certain strains of the intestinal microbiota are capable of synthesizing essential vitamins, such as vitamin K [57], which is necessary for blood clotting, and up to eight B vitamins, including B12, which is important for protein metabolism, red blood cell formation and nervous system maintenance [58]. These vitamins are essential for various metabolic reactions. The primary bacteria associated with de novo vitamin biosynthesis are lactic acid bacteria founds in foods [59] and commensal bacteria of the human gut, such as *Bifidobacterium* (e.g., *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis*) [58,60].

Lipid metabolism: The intestinal microbiota plays a key role in modulating the metabolism of lipids consumed or produced by the host by regulating their absorption, storage, and degradation [61]. This function occurs through three primary mechanisms: hydrolysis of triglycerides, synthesis of SCFAs, and regulation of genes and hormones involved in lipid metabolism [23].

The intestinal microbiota produces enzymes, such as lipases and esterases, which hydrolyze triglycerides into free fatty acids and glycerol, releasing free fatty acids and monoglycerides. These products can be absorbed by IECs or utilized by the microbiota as energy substrates [62]. Additionally, it has been shown that the intestinal microbiota can modulate lipid metabolism by influencing the synthesis, absorption, and oxidation of fatty acids, as well as the regulation of cholesterol and triglycerides [63]. Furthermore, the microbiota can affect genes and hormones involved in lipid metabolism, influencing fat absorption, storage, and utilization [62]. This extensive regulatory control has significant implications for energy balance, appetite regulation, insulin sensitivity, and the risk of developing conditions such as obesity, diabetes, and other metabolic disorders [62,64]. Notably, a recent population-based cohort analysis not only confirmed previously established associations between obesity and specific bacterial taxa, such as *Akkermansia*, *Christensenellaceae*, and *Tenericutes* [65], but also

demonstrated that some associations between microbial composition were shared between body mass index and levels of triglycerides and high-density lipoproteins [66].

Secondary bile acids are major bacterial metabolites that influence host physiology. Bile acids are classified into primary and secondary types based on their origin and structure. Primary bile acids, such as cholic acid and chenodeoxycholic acid, are synthesized in the liver [67]. Hepatocytes synthesize primary bile acids from cholesterol. These lipid-soluble bile acids are then conjugated with glycine or taurine to produce water-soluble bile salts, which are stored in the gallbladder and released into the intestine during digestion. Bile acids play a crucial role in the digestion and absorption of lipids, cholesterol, and fat-soluble vitamins [67–69].

The intestinal microbiota can modify the structure and function of bile acids, generating a diverse range of derivatives (30 different bile acids). Secondary bile acids, such as deoxycholic acid and lithocholic acid, are formed through the enzymatic modification of primary bile acids by the intestinal microbiota via processes like deconjugation, dehydroxylation, dehydrogenation, and epimerization processes [67,68]. These modifications alter the physicochemical, biological, and physiological properties of bile acids, affecting their solubility, receptor affinity, antimicrobial activity, and enterohepatic recirculation [69].

Given their antimicrobial properties, the amount and type of bile released into the intestine can influence the composition of the intestinal microbiota, promoting or inhibiting the growth of certain bacterial genera. Low bile salt levels favor the proliferation of Gram-negative bacteria (including pathogenic enteric bacteria), while high bile salt levels promote the growth of Gram-positive bacteria and reduce Gram-negative bacteria, such as those from the genus *Bacteroides* [70].

1.1.3. IMMUNOLOGICAL FUNCTION

The gut microbiota establishes a complex, dynamic, and bidirectional relationship with the host immune system. Throughout human development and into adulthood,

intestinal bacteria contribute to the shaping of the gut immune system, playing a crucial role in both health and disease [1]. This symbiotic relationship is crucial, as the microbiota not only coexists with the host but also serves as an essential component for immune system regulation. Its influence is exerted through several mechanisms: it facilitates the development of immune cells and tissues, modulates innate and adaptive immune responses to maintain a balance between defense against pathogens and tolerance to beneficial microorganisms, and promotes the production of secretory IgA to neutralize pathogens. Additionally, activation of dendritic cells (DCs) in the lamina propria by the gut microbiota help induce immune tolerance and maintain an anti-inflammatory state, thereby preventing autoimmune and chronic diseases.

As previously noted, the intestinal microbiota and the innate immune system are closely interconnected. Together with the intestinal epithelium, they form a physical and biochemical barrier that serves as the body's first line of defense against infections. The innate immune system monitors microbes via PRRs, which are specialized receptors that recognize molecular patterns shared by various microbial groups. PRRs include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins like retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) [71] (Table 1). These PRRs are activated by microbial-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs), including LPS and peptidoglycan, among others. The interaction of PRRs with their specific ligands triggers a signaling cascade that activates transcription factors. These transcription factors stimulate the secretion of cytokines and chemokines, thereby enhancing both innate and adaptive immune responses. They also promote the increased production of mucins and AMPs, which contribute to regulating the intestinal microbial population, modulating apoptosis, and mediating immunoregulatory responses through various generated molecules [72].

Table 1. Classification of PRRs according to their location, ligand and origin.

Receptor	Localization	Ligand	Origin of the ligand
Toll-like receptors (TLR)			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipotechprotein	Bacteria, viruses, parasites
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, parasites
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Dyacyl lipoprotein	Bacteria, viruses
TLR7/8	Endolysosome	ssRNA	Virus
TLR9	Endolysosome	CpG-DNA	Bacteria, viruses
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoo
Retinoic acid-inducible gene (RIG)-I-like receptors (RLR)			
RIG-I	Cytoplasm	Short dsRNA	RNA viruses, DNA virus
MDAS	Cytoplasm	Long dsRNA	RNA viruses
LGP2	Cytoplasm	Unknown	RNA viruses
NOD-like receptors (NLR)			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
C-type lectin receptors (CLR)			
Dectin-1	Plasma membrane	B-Glucan	Fungi
Dectin-2	Plasma membrane	B-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Fungi

Abbreviations: iE-DAP, D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide; SAP130, Histone deacetylase complex subunit.

The commensal microbiota also programs the adaptive immune system. In this context, the microbiome has been shown to influence the differentiation of T cell subpopulations, which play critical roles in infection protection, tissue repair, inflammation, and immune tolerance. The microbiota induces responses from CD4+ T helper (Th) cells, including Th1 (pro-inflammatory immunity and protection against pathogens), Th2 (tissue repair and defense against harmful nematodes), Th22 (tissue protection), Th17 (pro-inflammatory and antimicrobial responses), and Treg (tolerogenic responses) [12,73]. In germ-free mice, a significant reduction in Treg cells and an imbalance in Th1/Th2 cells, favoring a Th2-dominated response, have been

observed [74]. Reconstitution with a healthy microbiota restores immune balance by shifting the immune phenotype toward a Th1-dominated response [75]. Another example of microbiome regulation of T cell responses involves the cytotoxic T CD8+ (Tc) cells, whose effector functions play a pivotal role in targeting and destroying intracellular pathogens and cancer cells. Antigen-activated Tc cells are essential for promoting the transition into memory cells in germ free mice, as microbiota-derived SCFAs are required to support their memory potential [76].

1.1.4. STRUCTURAL AND EPITHELIAL BARRIER FUNCTION

The intestinal mucosa is a dynamic barrier that separates the intestinal lumen from the internal environment of the human body. It consists of: (i) the mucus layer, (ii) the intestinal epithelium, and (iii) the underlying lamina propria, where various immune cells are located in close proximity to the epithelial barrier. The epithelium plays a crucial role in establishing a physical and biochemical barrier between the intestinal microbiota and the host. In turn, the intestinal microbiota influences key proteins involved in epithelial barrier function, including tight junction proteins, mucins, and antimicrobial peptides. Regulating communication between the intestinal lumen and the epithelium is essential for the absorption of nutrients, electrolytes, and water. This regulation also allows the immune system to interact with the microbiota, facilitating the proper detection and elimination of pathogenic microorganisms [77]

Epithelial cells and intestinal barrier. The intestinal epithelium is the largest mucosal surface in the body, covering approximately ~400 m² with a single layer of cells organized into crypts and villi. This epithelial layer has the remarkable ability to renew itself every 4–5 days. This continuous renewal is driven by pluripotent IECs located at the base of the crypts. After undergoing division and differentiation, these stem cells migrate toward the intestinal lumen, where they enter an apoptotic state. This process must be tightly regulated and controlled by paracrine signals to maintain balance and ensure stable cellular renewal [78].

The primary cells comprising the epithelium are enterocytes, which are specialized for metabolic and digestive functions. However, other specialized cell lineages are also present, including M cells, goblet cells, enteroendocrine cells, and Paneth cells. These cell types are distributed differently along the small and large intestines (Table 2) [78].

Table 2. Subtypes of intestinal epithelial cells (IECs) and their roles.

IEC subtype	Localization	Role
Enterocyte	Small intestine (enterocyte) Colon (colonocyte)	Physical barrier Nutrient and water absorption Epithelial shedding Secrete antimicrobial (AMPs)
Goblet cell	Small intestine Colon	Mucin secretion Goblet cell-associated passage Secrete antimicrobial (AMPs)
Paneth cell	Small intestine	Secrete antimicrobial (AMPs) Support the stem cell niche
Tuft cell	Small intestine Colon	Helminth detection ILC2 expansion
Enteroendocrine cell	Small intestine Colon	Secrete hormones
M cell	Small intestine (follicle-associated epithelium)	Antigen uptake

IECs form a continuous physical barrier, connected by tight junctions that link adjacent cells through cytoplasmic networks regulating intestinal permeability [79]. The intestinal epithelium also includes a diverse population of cells with cytotoxic, helper, and regulatory functions, collectively known as intraepithelial lymphocytes. The interactions between IECs and immune cells are critical for maintaining intestinal homeostasis, strengthening the barrier, and protecting the host against external threats [80].

Chemical barrier. The chemical barrier provides passive protection against infections by targeting pathogens and inhibiting bacterial growth through the secretion of antimicrobial molecules including proteins, peptides, and immunomodulatory molecules. Antimicrobial molecules include AMPs, regenerating islet-derived proteins

from the Reg III family of C type lectins, and the enzymes lysozyme and secretory phospholipase A2, among others [81]. AMPs are small peptides released by Paneth cells with antimicrobial properties that contribute to maintaining intestinal homeostasis. Defensins (α and β defensins), in particular, are small cationic peptides secreted by IECs with antimicrobial, antifungal, and antiviral activity [82]. Although AMPs are key components of the innate immune system, certain proteins associated with adaptive immunity, such as secretory immunoglobulin A (sIgA), also contribute to the chemical barrier. SIgA plays a pivotal role in preventing pathogen adhesion to IECs, thereby reinforcing mucosal defense and maintaining intestinal homeostasis. [83].

Physical barrier. Physical barriers primarily consist of the mucus layer covering the intestinal mucosa, the glycocalyx, and intercellular junctions. Their main function is to physically prevent intestinal microorganisms from invading the mucosa.

The mucus layer covering the intestinal epithelium acts as a physical barrier, preventing luminal bacteria from accessing the underlying epithelial cells [84] (Figure 4). In the colon, gel-forming mucin 2 (Muc-2) establishes a well-defined mucus layer stratified into two sections. The inner layer is highly compact and physically excludes luminal bacteria. Additionally, this inner mucus layer contains antimicrobial peptides and immune effectors secreted by epithelial and immune cells, further blocking luminal bacteria and contributing to spatial segregation between the host and microbiota [85]. The outer mucus layer is less compact and serves as an adhesion site for various bacteria colonizing the intestinal tract. This outer layer is degraded by specific gut microbiota, necessitating constant renewal to preserve the integrity of the inner mucus layer. The production of Muc-2 by goblet cells is upregulated through TLR signaling in response to its degradation by commensals or mechanical forces [86].

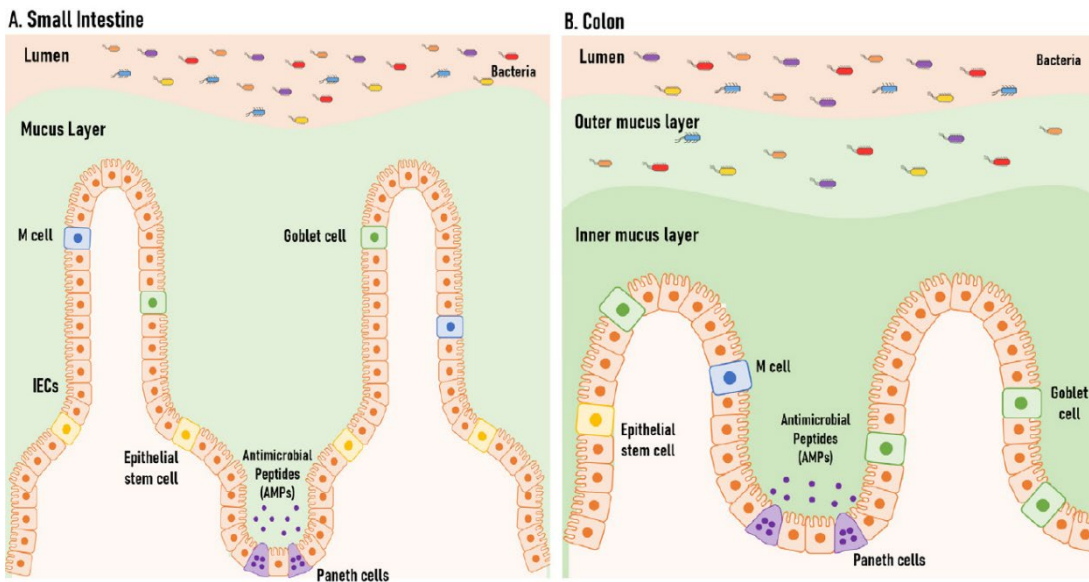


Figure 4: General overview of the mucus layers in small intestine and colon. In the small intestine the mucus is not attached to the epithelium and forms a diffusion barrier containing antibacterial products that limit penetration by bacteria. In the colon, bacteria are compartmentalized to the outer loose mucus layer. The inner mucus layer, which is attached to the epithelium, is almost free of bacteria and protects the barrier.

The *glycocalyx* It is a layer composed of glycoproteins, glycolipids, and transmembrane mucins that coats the surface of intestinal epithelial cells, inhibiting bacterial growth and invasion [79].

The intestinal epithelium functions as a physical barrier that restricts the translocation of luminal bacteria and antigens into host tissues while allowing the passage of ions through the paracellular space. A key mechanism regulating epithelial barrier function is the formation of intercellular junctions between adjacent IECs. While enteric pathogens often target these junctions to compromise barrier integrity as part of their infection strategies, certain beneficial bacterial strains fortify them to support intestinal homeostasis.

Intercellular junctions are categorized into three functional groups: tight junctions, anchoring junctions, and communicating junctions, each contributing uniquely to the maintenance of epithelial integrity and function (Figure 5) [77].

- Tight junctions (TJs): are protein complexes located in the apical zone of the intestinal epithelium, where they play a crucial role in regulating paracellular permeability by sealing the intercellular space and preventing the diffusion of hydrophilic molecules. Tight junctions consist of several transmembrane and cytosolic proteins. The transmembrane proteins include occludin, claudins, junctional adhesion molecules (JAM), and tricellulin, all of which contribute to the selective permeability of the tight junctions [87]. These proteins are anchored to the actomyosin cytoskeleton via cytosolic scaffolding proteins, primarily zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2), which are essential for the assembly, maintenance, and dynamic regulation of tight junctions [88].
- Anchoring junctions are specialized structures that connect the cytoskeleton of a cell to that of adjacent cells or to the extracellular matrix, forming stable and mechanically resilient cellular assemblies [89]. The primary types of anchoring junctions include adherens junctions and desmosomes, both of which establish strong adhesive interactions that preserve cellular cohesion and facilitate intercellular communication [87].
- Communicating junctions: these, also known as GAP junctions, facilitate direct intercellular communication by forming channels that connect the cytoplasm of adjacent cells. These channels enable the exchange of ions, second messengers, and small metabolites, allowing for coordinated cellular responses and tissue homeostasis [90].

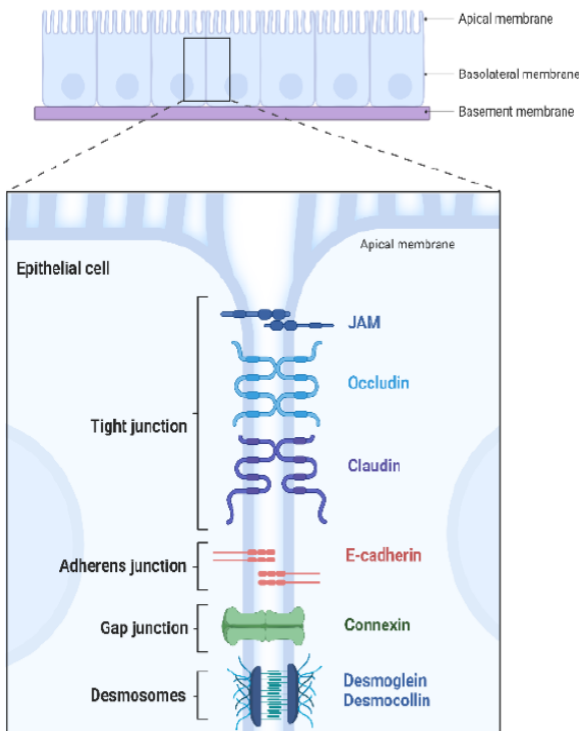


Figure 5: Intercellular Junction Complexes in the Intestinal Epithelium. Each enterocyte is connected to the adjacent one by tight junctions (TJs), adherens junctions, and desmosomes (from the luminal side to the lamina propria). TJs are located in the apical-lateral region of the membrane and consist of three integral transmembrane proteins (claudins, occludin, and JAM). These proteins are linked to the actin cytoskeleton through cytosolic proteins ZO-1, ZO-2, and ZO-3. Adherens junctions and desmosomes anchor epithelial cells to each other, providing mechanical strength to the intestinal epithelial barrier.

Abbreviations: JAM, junction adhesion molecule; ZO, zona occludens. Adapted from Bhat et al. [91].

2. INTESTINAL IMMUNE SYSTEM

The interaction between the gut microbiota and the host immune system is complex, dynamic, and bidirectional. The immune system facilitates a symbiotic relationship with commensal bacteria, maintaining homeostasis by promoting immune tolerance toward the vast microbial population while preserving immune defenses against pathogens and microbial translocation into normally sterile body compartments [92]. Disruptions in gut microbiota composition due to environmental factors (e.g., antibiotic use, diet, or demographic shifts), impairments in host-microbiome interfaces, or immune system dysregulation can lead to systemic dissemination of commensal microbes, increased susceptibility to pathogen invasion, and aberrant immune responses [9].

The gut immune system is highly specialized and functions independently of the systemic immune system, undergoing significant changes after bacterial colonization [93]. Commensal microorganisms are essential for immune maturation, enabling the

system to distinguish between commensals and pathogens through mechanisms involving TLRs in intestinal epithelial and immune cells [93–95]. While some studies suggest microbial colonization may begin in utero, the most substantial colonization occurs postnatally, primarily influenced by maternal microbiota [96,97]. Additionally, maternal antibodies in breast milk provide critical passive immunity to neonates [98]. Proper immune development relies on host-microbiota interactions that regulate immune homeostasis and infection susceptibility.

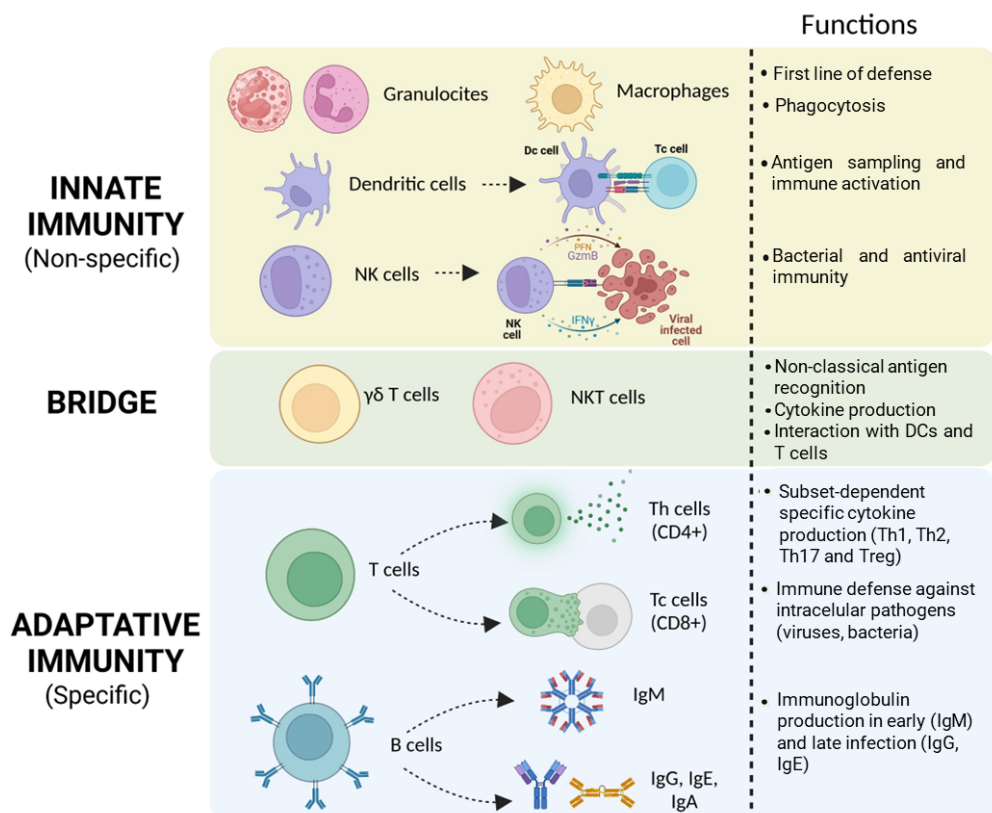


Figure 6. Overview of the main cell types involved in the immune response. Innate immune cells (e.g., macrophages, neutrophils, dendritic cells, NK cells) provide rapid, nonspecific defense. Adaptive immune cells (CD4⁺ and CD8⁺ T cells, B cells) mediate specific, long-term responses. Bridging populations, such as NKT cells and $\gamma\delta$ T cells, share features of both systems and play a crucial role in linking innate and adaptive immunity.

In a healthy state, immune responses to gut microbiota are compartmentalized at the mucosal surface [99]. Intestinal immune cells are distributed throughout gut-associated lymphoid tissues (GALT), including Peyer's patches, crypt patches, and

isolated lymphoid follicles, where antigen-presenting cells facilitate immune tolerance while preserving responsiveness to pathogens [100]. Peyer's patches, located in the small intestine, contain follicular centers covered by M cells, which mediate antigen uptake. These sites harbor diverse immune cells, including B cells, macrophages, dendritic cells, and T cells, which collectively contribute to maintaining intestinal homeostasis through coordinated innate and adaptive immune responses [101] (Figure 6).

2.1. INNATE IMMUNITY

The innate immune system serves as the first line of defense against pathogenic microorganisms and their components. In the gut, innate immunity is mediated by neutrophils, monocytes, macrophages, DCs, innate lymphoid cells, and Natural Killer (NK) cells, which mount rapid, nonspecific responses crucial for early pathogen recognition and elimination.

Among these, DCs in the intestinal lamina propria play a key role in antigen sampling and immune activation. Notably, they express tight junction proteins and extend their dendrites between epithelial-cell tight junctions to directly sample luminal antigens and microorganisms, acting as a gateway for immune responses [102]. Rescigno et al. (2001) demonstrated that intestinal myeloid DCs extend their dendrites into the lumen to acquire bacterial antigens, including pathogens such as *Salmonella typhimurium* [103,104]. Once activated, DCs present antigens to T cells, triggering their activation, clonal expansion, and differentiation, thereby linking innate and adaptive immunity [105].

In addition to DCs, NK cells also interact with gut microorganisms, shaping T cell-mediated adaptive immunity by modulating antigen-presenting cells, particularly DCs [106]. A subset of gut-resident innate lymphoid cells, which share markers with NK cells, contributes to mucosal homeostasis and the formation of gut-associated lymphoid structures [107,108]. NK cells are not only essential for maintaining gut health but also play a role in the pathogenesis of gastrointestinal diseases. In murine

models, NK cells are crucial for the immune response against bacterial infections, including *Listeria monocytogenes*, *Salmonella*, *Citrobacter rodentium*, and *Yersinia enterocolitica* [109–113], with their effectiveness largely dependent on cytokines such as IL-15 and IFN γ .

Beyond bacterial infections, NK cells play a pivotal role in antiviral immunity by directly targeting infected cells and modulating immune responses. In HIV infection, increased mucosal NK cell frequencies suggest their involvement in viral control [114]. Conversely, Human Herpesvirus 6 (HHV-6) can evade NK cell-mediated immunity in the large intestine, weakening immune defenses and promoting persistent infection [115,116]. These findings highlight the crucial role of NK cells in controlling viral infections and the strategies that viruses employ to evade immune surveillance [117].

2.2. BRIDGE BETWEEN INNATE AND ADAPTATIVE IMMUNITY

While the innate and adaptive immune systems have distinct functions, certain immune cells serve as a bridge between these two arms of immunity, facilitating rapid, yet antigen-specific responses. These innate-like lymphocytes, including $\gamma\delta$ T cells and Natural Killer T (NKT) cells, exhibit characteristics of both innate immune responsiveness and adaptive immune specificity. Unlike conventional adaptive lymphocytes that rely on clonal expansion and memory formation, these cells respond swiftly to infections and also influence the development and regulation of adaptive immunity [118,119].

Through their capacity to recognize non-classical antigens, produce cytokines, and interact with DCs and T cells, these populations play a crucial role in orchestrating immune homeostasis, early pathogen clearance, and inflammation regulation. Together, they provide an initial wave of immune defense while shaping the subsequent adaptive immune response, underscoring their crucial role in host defense and immune regulation.

$\gamma\delta$ T cells:

$\gamma\delta$ T cells are a distinct subset of T lymphocytes that bridge innate and adaptive immunity. Unlike conventional $\alpha\beta$ T cells, they recognize non-peptidic antigens in an MHC-independent manner, enabling rapid immune responses [120]. In the intestinal mucosa, $\gamma\delta$ T cells are essential for maintaining epithelial integrity, preventing the entry of microbial pathogens, and limiting their systemic dissemination, making them key players in mucosal immunity [121]. Additionally, these cells produce a wide range of cytokines, chemokines, perforins, and granzymes either constitutively or in response to interactions with surrounding cells such as DCs, epithelial cells, microbial products, and metabolites within the intestinal lumen [122,123].

$\gamma\delta$ T cells are in constant interaction with the commensal gut microbiota, which keeps them activated through their TCR, TLR, and NLR. This continuous crosstalk supports their dynamic role in maintaining intestinal homeostasis and regulating microbial populations [124]. Moreover, their ability to respond rapidly to infection further underscores their essential role in immune defense [118,125,126].

Natural killer T (NKT) cells:

NKT cells are a unique subset of T cells that share characteristics with both T cell and NK cell. Upon activation, they can rapidly secrete cytokines without the need for differentiation into effector cells, making them key players in the early defense against bacterial and viral infections. Many of the cytokines secreted by NKT cells influence $\alpha\beta$ T cell differentiation and function, linking them to adaptive immunity [127].

Unlike conventional T cells, which recognize peptide antigens, NKT cells recognize lipid antigens presented by CD1d molecules[120]. Upon activation, NKT cells produce cytokines such as IFN- γ and IL-4, which impact both innate and adaptive immune responses. They can also synthesize IL-2, IL-10, IL-17, TGF β , TNF, and a broad array of chemokines, allowing them to either promote or suppress immune responses depending on the context [120].

NKT cells interact with a wide range of immune cells, including $\gamma\delta$ T cells, B cells, DCs, macrophages, neutrophils, and eosinophils. For instance, by interacting with CD1d-antigen complexes on DCs cells, NKT cells upregulate CD40L expression, thus activating the DC. This activation enhances the DC's ability to stimulate $\alpha\beta$ T cells and promote IL-12 production, driving Th1 differentiation. Additionally, IL-12 exposure increases IFN- γ secretion from NKT cells, further amplifying Th1 differentiation and supporting the NK cell response. These interactions highlight the pivotal role of NKT cells in immune defense, particularly against viral infections, by regulating immune cell activity and enhancing pathogen clearance [128].

2.3. ADAPTIVE IMMUNITY

The adaptive responses are fundamental in containing the microbiota within its physiological niche and ensuring tissue integrity and regulation. In this regard, antibody- and T lymphocyte-mediated recognition of intestinal bacteria contribute to immune defense and may play a role in the pathogenesis of intestinal and systemic inflammatory diseases.

Following activation of intestinal innate immune responses, lymphocytes enter the mucosa, redistribute into distinct compartments, and trigger an adaptive immune response with the participation of specific cells: (i) B-cells, producing IgA in lamina propria, (ii) Th cells ($CD4^+$), redistributed in the villus-crypt unit, are important in immune-tolerance and (iii) Tc cells ($CD8^+$) that migrate to the epithelium and have cytotoxic activity [129].

B-cells:

B cells play a crucial role in adaptive immunity by producing immunoglobulins that mediate pathogen defense. Gut microbiota induces local immunoglobulin production to control the overgrowth of pathogenic strains. Notably, induction of secreted IgA in response to intestinal bacteria is essential for antimicrobial defense and the maintenance of intestinal microbiota homeostasis [130]. Secreted IgA limits bacterial

association with the intestinal epithelial cell surface and restricts the penetration of symbiotic bacteria across the gut epithelium [131]. The maturation of naïve B cells into mature IgA-secreting plasma cells requires priming by mucosal DCs carrying antigen and live bacteria from the intestinal epithelium [132]. Class switching enables B cells to produce other immunoglobulins, such as IgG for systemic immunity, IgE for allergic responses, and IgM for early infection control [133]. Their interaction with T cells, dendritic cells, and microbial antigens shapes antibody diversity and function. Through these mechanisms, B cells contribute to both protective immunity and immune regulation in various tissues [134].

Th cells:

Th cells play a crucial role in adaptive immunity by proliferating in secondary lymphoid tissues and differentiating into specialized Th cells in response to antigenic stimulation and cytokines. The differentiation and homeostasis of Th cells are influenced by various factors, including the microbiome, which has been shown to modulate their activity [135,136]. Cytokines in the microenvironment influence the activation of Th cells, which then differentiate into highly specialized helper subsets defined by the production of a distinct cytokine signature tailored to combat diverse classes of pathogens with high specificity [137]. In this context, functionally distinct Th effector subsets are differentiated based on their features: T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and T regulatory cells (Treg).

- Th1: The Th1 immune response is primarily activated against intracellular pathogens and viruses, characterized by the production of interferon-gamma (IFN- γ). Probiotic bacteria have been shown to modulate Th1 cell activity, influencing host immunity. Notably, specific *Lactobacillus* strains are closely associated with Th1 responses, and their administration has been linked to the alleviation of viral infection progression by enhancing IFN- γ production and promoting antiviral defense mechanisms [138]

- Th2: The Th2 immune response is primarily involved in defense against extracellular parasites, such as helminths, and plays a key role in allergic reactions. It is characterized by the production of cytokines that promote eosinophil activation, antibody class switching to IgE, and mucosal barrier function. Mazmanian et al. reported that germ-free (GF) mice appeared more biased toward Th2 cell responses than Th1 cell [139].
- Th17: The Th17 immune response is essential for protecting against extracellular pathogenic bacteria and fungi, particularly at mucosal surfaces. Certain probiotics have been reported to influence Th17 differentiation, either enhancing protective immunity or mitigating excessive inflammation [140].
- Treg: Tregs are critical for maintaining immune tolerance and preventing excessive inflammation or autoimmunity. The gut microbiota, including probiotic strains like *Lactobacillus rhamnosus*, has been shown to promote Treg differentiation, contributing to immune regulation and tissue homeostasis. This modulation is essential in preventing inflammatory disorders and ensuring balanced immune responses [128,141].

Tc cells:

Effector Tc cells play a fundamental role in immune defense against intracellular pathogens, including viruses and bacteria [142]. These cells exert their protective function by producing antiviral cytokines, such as interferon-gamma (IFN- γ), and cytotoxic molecules, which contribute to controlling both primary and secondary infections. Tc cells have been shown to be essential for effective immunity against various viral infections in both mice and humans, including influenza virus, poxviruses, coronaviruses, and rotaviruses [142,143]

Recent studies have demonstrated that microbial byproducts mediate Tc cell function. Butyrate directly increases Tc cell activity by upregulating IFN- γ and granzyme B expression [144]. Additionally, butyrate treatment has been shown to enhance the Tc cell response to influenza virus by accelerating their metabolism and effector function

[145]. Induction of IFN- γ ⁺ Tc cells may also be associated with specific bacterial strain-derived metabolites, such as mevalonate and dimethylglycine [146], as well as SCFAs [147], which enter circulation and induce systemic activation of Tc cells. These findings underscore the pivotal role of microbiota-derived metabolites in modulating CD8⁺ Tc cells responses and host immunity.

2.4. GUT IMMUNE SYSTEM DURING EARLY LIFE

Emerging research highlights the crucial role of the gut microbiome in shaping immune system development, regulating intestinal barrier function, modulating host metabolism, and providing colonization resistance against enteric pathogens. These functions are particularly vital during early life, when the immune system is immature, and the gut barrier exhibits increased permeability, increasing neonatal susceptibility to infections [130,148–150]. The neonatal gut is highly receptive to microbial colonization, which plays a fundamental role in immune cell maturation and tolerance to commensal bacteria. However, disruptions to this delicate balance, such as preterm birth, antibiotic exposure or infection, can lead to increased gut inflammation, higher susceptibility to enteric pathogens, and long-term immune dysregulation [151].

2.4.1. ROTAVIRUS AND OTHER EARLY LIFE INFECTIONS

Neonates, particularly those born underweight or premature, are at high risk for infections that can significantly impact both immediate health and long-term disease outcomes [152]. Severe lower respiratory tract infections, such as bronchiolitis and pneumonia, along with acute gastroenteritis caused primarily by rotavirus (RV) and norovirus, remain leading causes of childhood mortality worldwide [153,154]. These infections contribute to high morbidity and mortality rates, particularly in developing countries, where they frequently result in infant hospitalizations [155–157]. Reducing the burden of these diseases remains a critical public health priority.

In regions where RV vaccines have been widely implemented, norovirus has become the primary cause of viral gastroenteritis [157]. Although RV vaccines are effective and safe [158], their widespread adoption faces challenges, particularly in developing

nations, due to cost, storage requirements, and the need for strict adherence to administration timelines for optimal efficacy [159].

Given these limitations, a deeper understanding of the neonatal gut microbiome and the developing immune system offers new opportunities to enhance neonatal health. Microbiome-targeted interventions, such as maternal microbial seeding, probiotics, prebiotics, postbiotics and nutritional immunity approaches, may help strengthen immune function and reduce the risk of infections.

2.4.1.1. ROTAVIRUS STRUCTURE AND PATHOLOGY

RV is a non-enveloped, icosahedral virus belonging to the *Reoviridae* family. It has three concentric capsids and a double-stranded RNA (dsRNA) genome divided into 11 segments. These segments encode six structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6 [160,161] (Figure 7).

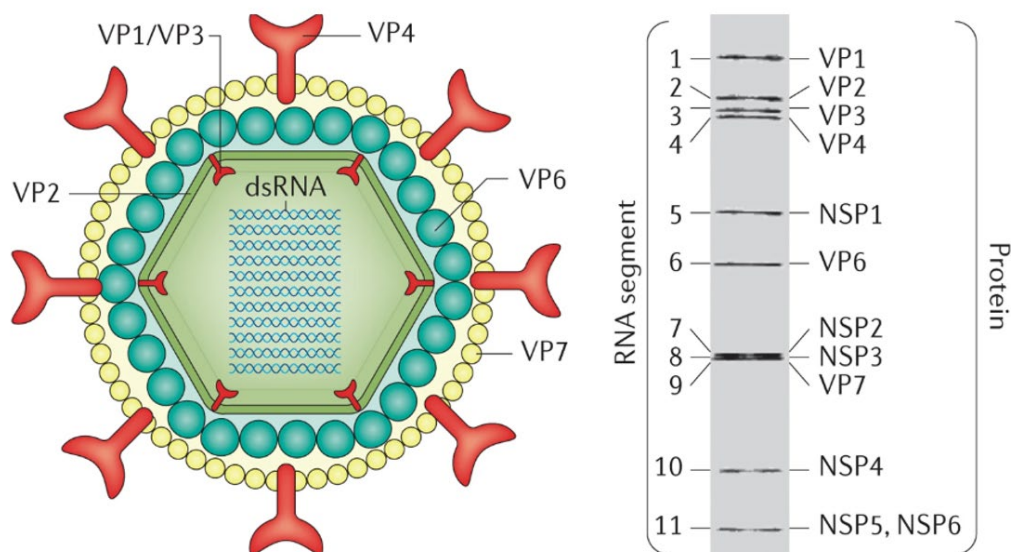


Figure 7. Rotavirus structure and proteins. The virus has a triple-layered capsid with outer proteins VP4 and VP7 involved in cell entry, VP6 forming the middle layer, and VP2 in the inner core. Inside, VP1 and VP3 associate with the segmented RNA genome to support replication and transcription. Figure from [164].

Based on the serological reactivity and genetic variability of the VP6 protein, RV is classified into groups A to H [162]. These groups are distinguished by variations in their surface proteins, with Group A being the primary cause of gastroenteritis in children. While adults can also contract RV, they typically experience milder symptoms [160,163].

Due to its stable triple-layer capsid, RV is highly contagious and primarily transmitted through the oral-fecal route. It spreads via the consumption of contaminated food or water, or by contact with contaminated surfaces. RV infects mature enterocytes and EC cells, in the non-dividing, differentiated cells of the small intestine [160,163] (Figure 8), which is a key factor in its success as a gastrointestinal pathogen. Symptoms of RV infection typically appear two days after exposure and include severe diarrhea, vomiting, nausea, and fever. If fluid loss is not replaced, it can lead to dehydration. Gastroenteritis caused by RV typically lasts between 4 and 10 days.

RV triple-layer particles (TLPs) penetrate the enterocytes at the villus tips by attaching to sialo-glycans on the host cell surface via the viral proteins VP4 and VP7 [157]. Following this, RV interacts with other cellular receptors, including integrins and heat shock cognate protein 70 (Hsc70) [165]. Once inside the endosome, the low calcium concentration triggers the removal of the outer layer, releasing active double-layered particles (DLPs) into the cytoplasm. These DLPs undergo transcription, where the viral RNA genome is replicated and packaged into new DLPs in viroplasm, specialized structures that interact with lipid droplets. The DLPs then interact with NSP4, a transmembrane glycoprotein, which facilitates entry into the endoplasmic reticulum (ER). Within the ER, the TLPs mature and generate progeny virions, which are eventually released from the host cell either through lysis or via a non-classical vesicular transport mechanism [161].

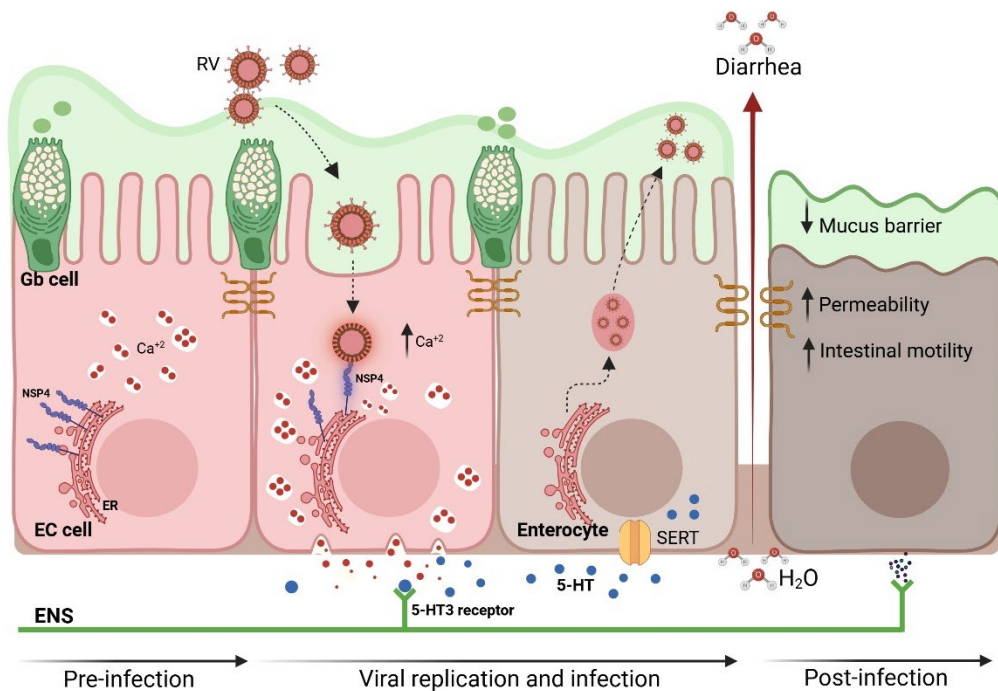


Figure 8. Replication cycle of rotavirus. After cell entry, the virus replicates in cytoplasmic viroplasm, producing structural and non-structural proteins. NSP4 acts as an enterotoxin, disrupting calcium homeostasis and triggering calcium-dependent signaling. This leads to water efflux, diarrhea, and increased intestinal permeability. Additionally, NSP4 and other viral effects alter the serotonergic system, contributing to enhanced gut motility and secretion.

Additionally, the enterotoxin NSP4 acts as a viroporin in the Endoplasmic reticulum (ER), releasing calcium ions (Ca^{2+}) into the cytoplasm [160], which is crucial for RV replication, virion assembly, and maintaining the integrity of the outer capsid layer [163]. By increasing cytoplasmic calcium levels, NSP4 affects the cytoskeleton and tight junction proteins, contributing to malabsorption and causing enterocyte lysis. Additionally, it stimulates chloride channels, causing an overproduction of Cl^- ions in the intestinal lumen, which promotes the movement of water. This, coupled with the diminished absorptive capacity of the epithelial barrier, contributes to the development of secretory diarrhea also disrupts tight junctions, leading to increased water flow into the lumen, contributing to diarrhea [163].

In addition, RV infection has been shown to stimulate serotonin release from human EC cells [166,167]. As mentioned before, serotonin is involved in the regulation of gut motility, intestinal secretion, blood flow, several gastrointestinal disorders [168,169], illness and acute gastroenteritis [170]. Silencing NSP4 significantly reduced serotonin secretion from Rhesus rotavirus-infected EC cells [171]. Moreover, calcium mobilization and diarrhea capacity from virulent and avirulent porcine viruses correlated with release of serotonin from EC cells [171]. Notably, blocking serotonin receptors significantly attenuated RV-induced diarrhea in infant mice, reducing both the severity and duration of symptoms, as well as viral shedding [172]. These findings suggest that serotonin plays a crucial role in RV-induced diarrhea, and targeting serotonin receptors may provide a potential therapeutic approach.

2.4.1.2. ROTAVIRUS AND IMMUNITY

Viral infections trigger immune responses that involve both humoral and cell-mediated immunity [160,161]. Upon infecting host cells, RV introduces its double-stranded RNA, which is recognized by PRRs, such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5). These PRRs activate the transcription factors IRF3 and NF- κ B, which then translocate to the nucleus and promote the production of interferons (IFNs) and IFN-stimulated genes (ISGs) [160,161,173]. This response increases levels of type I IFN, IFN- γ , chemokines, and proinflammatory cytokines (TNF- α , IL-6, IL-8, IL-12, MCP-1), thereby enhancing the antiviral state in IECs [173]. Subsequently, IFNs stimulate the production of antiviral proteins, including Human myxovirus resistance protein 1 (MxA), RNase L, oligoadenylate synthase (OAS), and protein kinase R (PKR) [173]. While other viral sensors, such as endosomal toll-like receptor 3 (TLR-3), may also be involved, RIG-1 and MDA-5 are the primary receptors for RV in IECs [174] and DCs [173].

The induction of an antiviral state in IECs triggers the activation and recruitment of inflammatory cells, which, in turn, leads to the maturation of DCs. This immune response can contribute to epithelial damage and mucosal injury [173]. Studies have

shown that during RV infection, there is a significant reduction in goblet cell numbers in the duodenum and jejunum, along with mucus [175,176]. The immune activation also facilitates the development of antigen-specific immunity, driving the differentiation of Ag-presenting cells into Th1, Th2, Th17, and cytotoxic Tc cells, as well as IgA+ B cells [177].

RV replication is inhibited by certain cytokines, such as IFN- γ and IL-1, produced by RV-specific T cells [178,179]. Studies have shown that intradermal or intranasal immunization with recombinant VP6 (rVP6) induces the production of IFN- γ , IL-4, and IL-17, which promote the differentiation of Th CD4+ lymphocytes into Th1, Th2, Th17 subsets capable of lysing infected cells. However, rVP6 does not appear to stimulate the differentiation of CD8+ Tc lymphocytes [180]. Nevertheless, some research suggests that Tc lymphocytes are essential for virus clearance [181].

IgA and IgG can mediate the lysis of infected cells in mucosal tissues through eosinophils, neutrophils, and NK cells [182]. Secreted IgA plays a critical role in protecting against RV infections and maintaining mucosal homeostasis [177]. However, during intestinal infections, RV can also be detected in systemic circulation, where other immunoglobulins contribute to viral clearance. IgG and IgM provide protection against the virus in the bloodstream, whereas monomeric IgA plays a minimal role in its elimination. Consequently, higher levels of IgG and IgM are typically observed in plasma compared to IgA [200]. Moreover, the level of specific anti-RV antibodies in plasma determine protection against RV infections and the severity of gastroenteritis in children [183].

RV has also developed mechanisms to evade the antiviral immune response, primarily mediated by non-structural protein 1 (NSP1). NSP1 promotes the degradation of key IFN transcription factors such as IRF3, IRF5, and IRF7, as well as RIG-1, TNF receptor-associated factor 2 (TRAF2), mitochondrial antiviral signaling adaptor protein (MAVS), p53, and the Pan3 RNase subunit [161]. Additionally, NSP1 inhibits STAT1

phosphorylation, preventing the STAT1/STAT2 dimer from translocating to the nucleus and establishing an antiviral state [184].

3. PROBIOTICS

As previously discussed, the intestinal microbiota establishes a symbiotic relationship with the host, with certain gut-resident microbes providing additional health benefits. This group includes both commensal and probiotic bacteria, which play key roles in maintaining intestinal homeostasis and overall gut function [185]. According to the Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO, 2002), probiotics are defined as "live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host."

The most extensively studied probiotics are Gram-positive bacteria from the *Lactobacillus* and *Bifidobacterium* genera, commonly found in functional foods and dietary supplements such as fermented vegetables, yogurt, and kefir grains [186,187]. Other probiotic strains available on the market include species from the *Saccharomyces*, *Bacillus*, *Enterococcus*, and *Streptococcus* genera, as well as the Gram-negative *Escherichia coli* Nissle 1917 [188]. To be classified as probiotics, microorganisms must meet specific criteria, including safety for human consumption, the ability to produce antimicrobial metabolites, and the capacity to adhere to the intestinal epithelium and colonize the gut [189].

Due to their well-documented health benefits, probiotics have attracted significant interest within the food industry [186]. They contribute to gut microbiota balance and enhance host defense against pathogens. Clinically validated benefits of probiotics include the prevention and reduction of diarrhea from various causes, modulation of the gut microbiota through antimicrobial activity, mitigation of lactose intolerance symptoms, prevention of food allergies, enhancement of immune function and epithelial barrier, and potential antitumor properties [190,191]. Notably, probiotic properties are strain-specific, which determines their application for particular health

conditions. Additionally, some probiotic strains have been reported to exert systemic effects beyond the gastrointestinal tract [191].

3.1. *ESCHERICHIA COLI* NISSLE 1917

Escherichia coli Nissle 1917 (EcN) (serotype O6:K5:H1; deposited in DSMZ, Germany as DSM6601) is a Gram-negative probiotic strain commercially available in Germany as the active component of Mutaflor (Ardeypharm GmbH, Herdecke, Germany) and in Italy as EcN [192]. This strain was first isolated during World War I by Dr. Alfred Nissle in 1917 from the feces of a soldier who remained unaffected by a shigellosis outbreak that affected his battalion [193].

EcN belongs to the Enterobacteriaceae family and is classified within the phylogenetic subgroup B2 of *Escherichia coli*. It has been designated Generally Recognized as Safe (GRAS) status. Its genome comprises 5,324 genes, of which 108 are strain-specific and contribute to its probiotic characteristics [193]. Genomic analyses indicate that EcN evolved from a common ancestor of uropathogenic *E. coli* (UPEC) strains, undergoing genetic modifications that resulted in the loss of virulence factors while retaining genes encoding fitness-related traits, which enhance its intestinal competitiveness and survival. These fitness factors include microcins, iron acquisition systems (enterobactin, yersiniabactin, aerobactin, salmochelin, EfeU, ChuA), adhesins, and proteases, which facilitate its efficient colonization of the GI, thereby contributing to its probiotic properties [194].

Colonization and Competitive Advantage in the Gut Microbiota. EcN efficiently colonizes the gut epithelial surface within days of administration and can persist in the colonic microbiota for several months [195]. Its ability to express multiple iron uptake systems provides a competitive advantage in the intestinal environment, where iron is a limiting factor essential for bacterial growth. The EcN flagellum is its primary immunogenic antigen, acting both as an immune system activator and as a propulsion mechanism in vivo, enabling it to effectively compete with pathogens for host tissue adhesion sites [196].

Moreover, EcN induces the production of β -defensins in IECs, thereby preventing the adhesion of pathogenic bacteria, such as adherent-invasive *Escherichia coli* (AIEC) strains [197]. Additionally, EcN produces microcins H47 and M, which exhibit antibacterial activity against closely related bacterial species. These microcins also contribute to iron sequestration, further enhancing EcN competitive advantage over other bacteria such as *Salmonella* spp[195]

Immunomodulatory Effects. EcN produces several immunomodulatory molecules, including the H1 flagellin antigen, which is recognized by Toll-like receptor 5 (TLR5), leading to the activation of interleukin-8 (IL-8) production [198]. Flagellin also stimulates the secretion of β -defensins via NF- κ B and AP-1 activation [199]. Notably, β -defensins are dysregulated in ulcerative colitis, and EcN has demonstrated beneficial effects in this condition [197]. Furthermore, the K5 capsule of EcN plays an important role in immune modulation by promoting the induction of MyD88 and TRIF following TLR5 activation [200].

In in vitro studies, the interaction of EcN with immune cells resulted in a reduction of pro-inflammatory cytokines (IL-2, TNF- α , IFN- γ , IL-6, IL-1 β), while inducing an increase in anti-inflammatory cytokines (IL-10, TGF- β) [201]. Additionally, this probiotic strain contributes to the strengthening of tight junction proteins and enhances epithelial barrier integrity by upregulating the expression and function of ZO-1, Claudin-2, and Claudin-14 (Figure 9) [202].

Clinical Applications and Therapeutic Potential. EcN is one of the few probiotic strains approved as a medicinal product for the treatment of gastrointestinal diseases [203]. It is also among the most extensively studied probiotics, primarily due to its immunomodulatory and anti-inflammatory properties [192,204,205]. Preclinical and clinical research has demonstrated that EcN enhances both innate and adaptive immune responses, particularly against viral infections such as rotavirus, while also alleviating associated symptoms, including diarrhea[206–208]

Limitations and Emerging Therapeutic Strategies. Despite its well-documented probiotic properties, the administration of live probiotics presents certain limitations and concerns, particularly in immunocompromised individuals and in early life stages, such as neonates. In such cases, probiotic therapy may exacerbate inflammatory responses, potentially leading to the conversion of non-pathogenic probiotic strains into opportunistic pathogens. To mitigate safety risks, next-generation microbiome-based therapies are increasingly focusing on the use of postbiotics, which consist of inactivated bacterial cells or their bioactive metabolites as safer therapeutic alternatives [209].

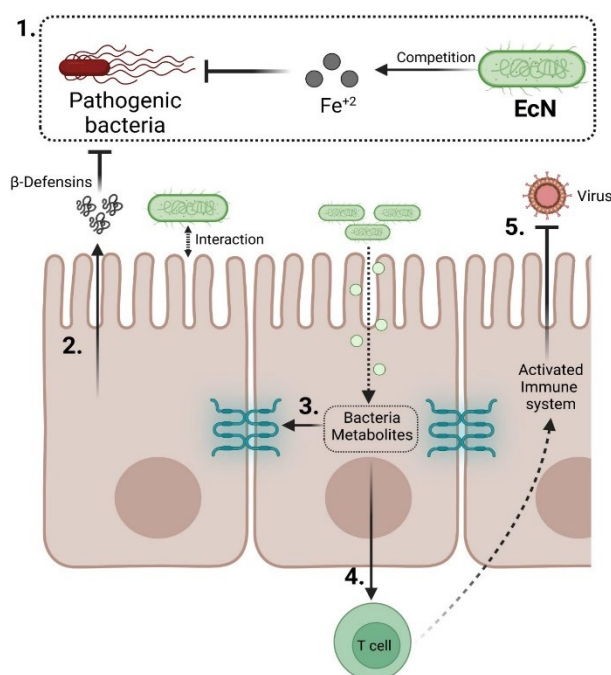


Figure 9. Key mechanisms of the probiotic strain *Escherichia coli* Nissle 1917 (EcN). (1) Competes with pathogenic bacteria for iron, indirectly inhibiting their growth. (2) Stimulates intestinal epithelial cells to produce antimicrobial peptides like β -defensin, which block harmful bacteria and reduce inflammation. (3) Enhances tight junction (TJ) repair and reduces intestinal permeability. (4) Activates the immune system through interaction with antigen-presenting cells. (5) Contributes to the host response against various infections, including viral ones.

4. POSTBIOTICS

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), postbiotics are defined as preparations of inactivated (non-replicative) microorganisms or their components that confer health benefits to the host. This category includes bacterial membrane components, biomolecules, metabolites, and secreted bioactive compounds [210]. Postbiotics exhibit favorable absorption, distribution, and a high

signaling capacity across various organs and tissues, thereby eliciting specific host responses. Their efficacy has been demonstrated for components derived from well-characterized probiotic strains[211,212].

Postbiotics can be classified based on their local or systemic effects, molecular composition, or the bacterial strain from which they originate [213,214]. A key advantage of postbiotics is their improved safety profile, particularly in immunocompromised individuals and neonates, who are more vulnerable due to their immature immune system and increased gut permeability. Unlike live probiotics, postbiotics eliminate risks such as the potential transfer of antibiotic resistance genes and interference with native microbiome reestablishment [215–219].

Additionally, postbiotics offer advantages in production, standardization, and processing. Their high stability enables applications in pharmaceuticals and functional food products. Various techniques are employed in postbiotic production including enzymatic treatment, sonication, heat inactivation, centrifugation, filtration, dialysis, lyophilization, and column purification. Postbiotic characterization, whether qualitative or quantitative, is performed based on the nature of the molecules of interest, employing advanced analytical techniques such as MALDI-TOF MS, HPLC, UPLC, LC-MS, and LC-MS/MS, among others [220].

4.1. PROBIOTIC EXTRACELLULAR VESICLES (EVs)

In this context, extracellular vesicles (EVs) derived from probiotic strains and gut microbiota have emerged as promising candidates for next-generation postbiotics [221,222]. Bacteria universally release EVs as a mechanism of interspecies communication, facilitating molecular exchange within microbial communities and with the host [223,224]. In the intestinal environment, where the mucus layer prevents direct contact between microbiota and host cells, this interaction primarily relies on secreted factors such as EVs [225].

Bacterial EVs are nanoscale, lipid bilayer-enclosed structures (20–300 nm in diameter) that originate from the membranes of both Gram-positive and Gram-negative bacteria. These vesicles encapsulate a diverse array of bioactive molecules derived from the parental strain, including MAMPs, proteins, metabolites, DNA, and RNA. In pathogenic bacteria, they may also contain toxins and virulence factors [226].

Accumulating evidence supports the role of EVs in mediating microbiota-host interactions by transporting effector molecules that modulate host signaling pathways and cellular processes, particularly immune and defense responses. The biological activity of microbiota-derived EVs is highly strain-specific, depending on their bacterial origin and molecular cargo [77,227]. Once released into intestinal lumen, microbiota-derived EVs diffuse through the mucus layer and interact with epithelial and immune cells via PRRs. They are internalized through endocytosis, delivering their molecular components to activate host receptors and signaling pathways. This continuous stimulation maintains controlled basal inflammation, contributing to pathogen defense and intestinal homeostasis [228].

The structural composition of EVs varies between Gram-negative and Gram-positive bacteria. Gram-negative bacteria primarily produce outer membrane vesicles (OMVs), which bud from the outer membrane and encapsulate lipopolysaccharides (LPS), phospholipids, and periplasmic biomolecules [224,229,230]. A second EV type, known as inner-outer membrane vesicles (I-OMVs), arises from explosive cell lysis and contains both inner membrane components and cytoplasmic content [231]. In contrast, Gram-positive bacteria, which lack an outer membrane and possess a thick peptidoglycan layer, produce cytoplasmic membrane vesicles (CMVs). These vesicles lack LPS and periplasmic components but carry molecular cargo similar to that of Gram-negative EVs [232,233].

According to the MISEV 2018 guidelines, the term extracellular vesicle (EV) is recommended as a general designation for lipid bilayer-enclosed particles released by

cells that are incapable of replication (Figure 10) [234]. This standardized terminology ensures consistency in EV research across different biological systems.

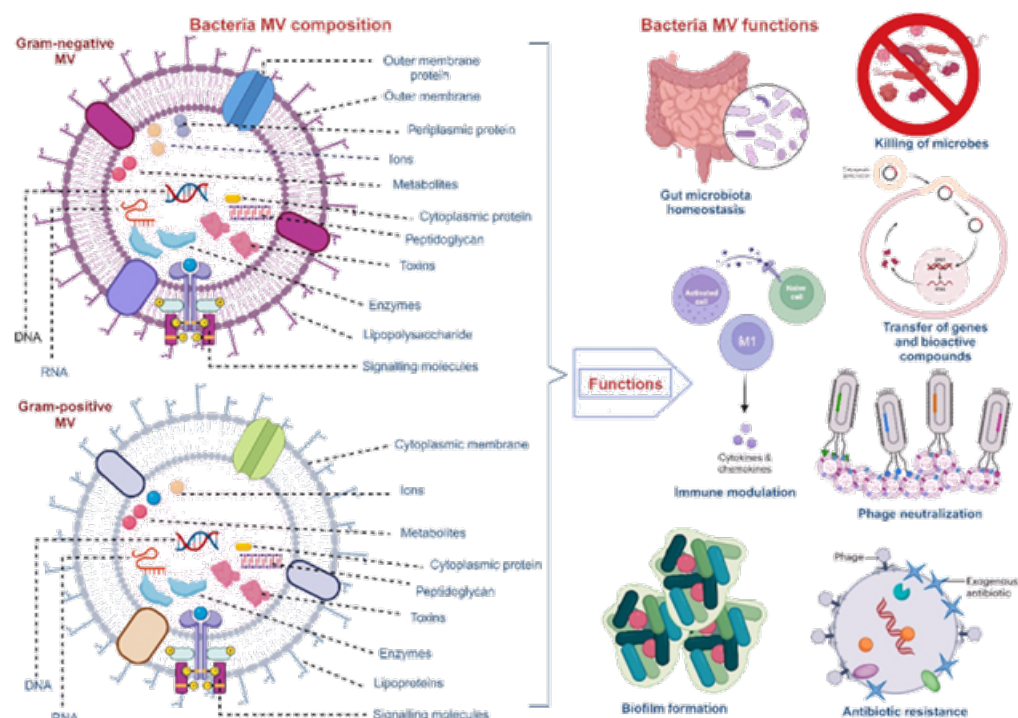


Figure 10. Composition and functions of EVs from Gram-positive and Gram-negative bacteria. The cargo of membrane vesicles (MVs) from Gram-negative bacteria differs slightly from that of Gram-positive bacteria. The illustrated functions include biofilm formation, antibiotic resistance, phage neutralization, immune modulation, gene transfer, microbial elimination, and intestinal microbiota homeostasis. Figure from [235].

In this context, various studies from our group have demonstrated that EVs derived from probiotic and commensal *E. coli* strains exert multiple beneficial effects, particularly in modulating epithelial barrier function, regulating inflammatory responses, and shaping immune responses.

Regulation of Epithelial Barrier Function. EVs from probiotic and commensal *E. coli* strains have been shown to enhance epithelial barrier integrity in different models [202,236–238]. Specifically, EVs derived from EcN counteract epithelial damage induced by enteropathogenic *E. coli* (EPEC) infection by activating compensatory regulatory mechanisms at transcriptional and post-transcriptional levels. These

mechanisms influence tight junction proteins, including occludin, claudin-14, and ZO-1, thereby preserving barrier function [236,239]. Similarly, in human colon explants, EcN EVs induce IL-22 expression, a cytokine that plays a key role in maintaining epithelial integrity [329].

Regulation of inflammation. EVs from intestinal *E. coli* strains activate regulatory pathways to balance anti- and pro-inflammatory responses. In a murine model of DSS-induced colitis, oral administration of EcN EVs alleviated colitis symptoms, improved intestinal permeability, and modulated inflammatory responses similarly to the probiotic itself [240]. In this model, EcN EVs shifted cytokine expression toward an anti-inflammatory profile, reducing proinflammatory cytokines such as IL-1 β , TNF- α , IL-6, IL-12, IL-17, and IFN- γ , while upregulating the anti-inflammatory cytokine IL-10 [236].

Modulation of Immune Responses. EVs from intestinal *E. coli* strains influence immune cell activation through multiple mechanisms. They stimulate dendritic cells to drive specific T-cell responses by activating TLR signaling pathways, regulating miRNA expression, and modulating the differential release of immune mediators via exosomes [77,241]

Moreover, other *in vivo* models have demonstrated the direct involvement of probiotic-derived EV administration in interorgan crosstalk, particularly in the gut-liver and gut-brain axes. This effect is attributed to the ability of these EVs to enhance intestinal barrier function through various mechanisms, including the modulation of inflammatory and immune responses, the strengthening of tight junction proteins, and the regulation of mucin-related proteins [242–244].

Despite their vast potential, the clinical translation of EVs remains challenging, as their precise molecular mechanisms of action require further investigation. However, emerging evidence suggests that EVs hold great promise for developing more effective treatment strategies for gut permeability disorders and metabolic as well as neurological diseases involving the gut-liver and gut-brain axis. Therefore, significant

efforts are anticipated in the coming years to advance EV-based therapeutics and drug delivery systems.

5. GUT-LIVER AXIS

The gut and liver maintain a dynamic interplay through multiple interconnected pathways. The portal vein delivers 75% of the liver's blood supply, carrying signals influenced by diet, genetics, and the environment [245,246]. This communication process is regulated by two main components: gut microbiota and gut barrier [247]. The microbiota impacts the liver directly through metabolites or indirectly by modulating the intestinal barrier, with imbalances in its composition potentially contributing to intestinal dysfunction and liver diseases. In the other hand, the liver exerts regulatory effects on the gut by secreting bile enriched with bile acids, IgA, and antimicrobial molecules into the small intestine. This feedback loop influences the composition and function of the gut microbiota (Figure 11).

A fully intact intestinal barrier is essential for nutrient absorption and for containing the vast microbial populations within the gut lumen, preventing systemic spread [247]. Microbial products and metabolites play a key role in preserving both gut barrier integrity and liver health. However, disruptions in the intestinal microbial communities, pose significant risks for liver diseases such as non-alcoholic fatty liver disease (NAFLD), obesity, and diabetes [245]. Notably, microbiota dysbiosis has been shown to influence liver disease progression. Preclinical studies demonstrate that fecal microbiota from individuals with liver disease can transmit the condition to healthy subjects [246,248]. These findings underscore the critical role of the intestinal microbiota in liver function.

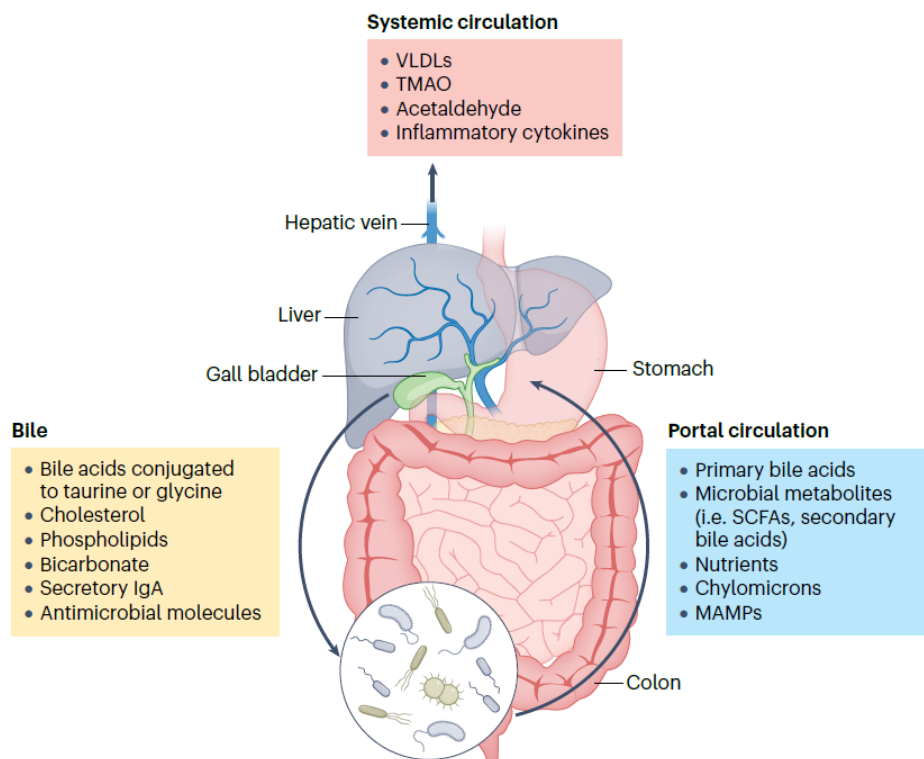


Figure 11. Gut-liver axis. The liver produces bile, composed of cholesterol, phospholipids, proteins, bicarbonate, and bile acids conjugated to taurine or glycine (yellow box). Bile reaches the small intestine to support fat digestion, and in the colon, microbiota convert primary bile acids into secondary ones. Both types are reabsorbed into the liver via the portal circulation (blue box), which also transports nutrients, lipids, microbial products (e.g., LPS), and metabolites (e.g., SCFAs). The liver releases toxic metabolites and inflammatory cytokines into systemic circulation (red box), illustrating the bidirectional communication of the gut-liver axis. **Abbreviations:** MAMP, microorganism-associated molecular pattern; TMAO, trimethylamine N-oxide; VLDL, very low-density lipoprotein.

EVs are gaining attention for their role in gut-liver interactions. These nanoparticles, found in sources like milk, plants, and probiotics, carry bioactive molecules and are resistant to the digestive environment [249,250]. When administered orally, EVs are absorbed by gut epithelial and immune cells, modulating gut and liver health [251–254]. Emerging evidence suggests that EVs protect the liver from injury and improve metabolic conditions, offering potential for treating liver diseases and metabolic disorders [254–256]. However, the mechanisms underlying their effects remain unclear, highlighting the need for further research into their therapeutic potential, particularly in relation to the gut-liver axis.

5.1. GUT MICROBIOTA IN THE GUT-LIVER AXIS IN HEALTH

The gut microbiota is a key component of the gut-liver axis (Figure 12). During physiological activities, the intestine and liver secrete bioactive molecules that either interact with the gut microbiota or are transported between the two organs. Intestinal microbes produce metabolites that can influence intestinal barrier permeability and be absorbed by intestinal tissues, subsequently traveling through the portal vein to impact hepatic function [245,257]. Additionally, systemic circulation extends the gut-liver axis by transporting liver-derived metabolites, such as free fatty acids (FFAs) and ethanol metabolites, back to the intestine, affecting overall axis functionality [245].

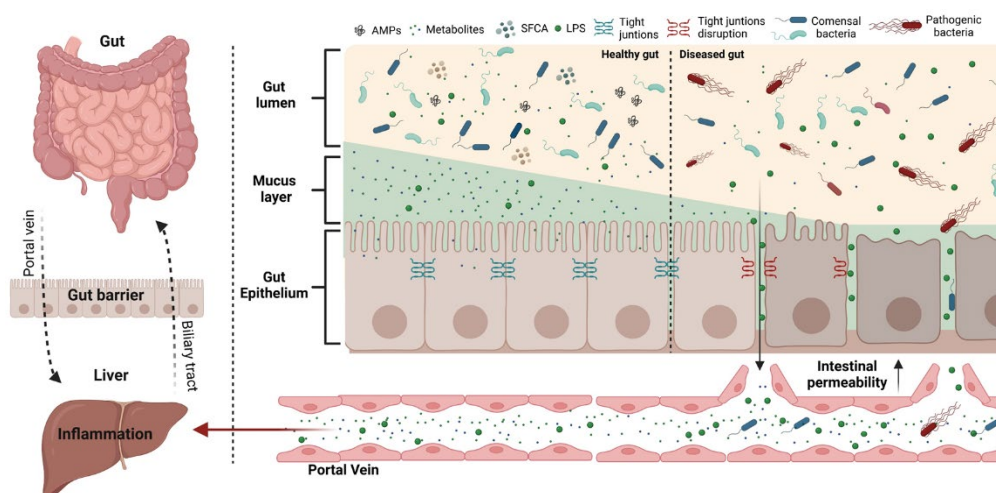


Figure 12. Impact of the gut barrier integrity and liver function gut microbiota. Gut microbiota alterations influence the production of metabolites, including SCFAs, bile acids, and aromatic amino acids. Disruption of gut barrier integrity increases intestinal permeability, allowing harmful compounds to enter circulation, which can trigger liver inflammation and exacerbate liver diseases (e.g., NAFLD, NASH, HCC) and metabolic disorders, such as diabetes, obesity, and cardiovascular disease. **Abbreviations:** NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease. Adapted from [243].

Gut microbes and their metabolites can also migrate directly from the gut to the liver [258]. Gut eubiosis, the balance of microbial communities, is maintained through mechanisms such as limiting bacterial overgrowth, facilitated by bile salts and endogenous antimicrobial molecules secreted by the liver via the biliary tract and bloodstream [257]. The gut microbiota modulates plasma levels of key metabolites,

including SCFAs, bile acids (BAs), and aromatic amino acid (AAA) derivatives, which mediate gut-liver interactions. SCFAs like acetate, propionate, and butyrate are primary fermentation products of carbohydrates by gut bacteria [259,260]. Meanwhile, AAAs such as tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr), which are broken down by the gut microbiome, yield metabolites critical for immune, metabolic, and neuronal responses at both local and systemic levels [261].

In addition, the gut microbiota produces EVs containing diverse bioactive cargos, enabling them to regulate the gut microbiome and its metabolites. The specific composition of these cargos determines their ability to modulate microbial communities by promoting cooperation or competition between different bacterial species. For instance, bacterial EVs can carry polysaccharide-degrading enzymes, which not only support the nutritional needs of the producing strain but also benefit other bacteria with similar dietary requirements [262].

5.2. GUT BARRIER IN THE GUT-LIVER AXIS IN HEALTH

The intestinal barrier comprises three distinct, structured layers that work in concert to maintain gut integrity. The outermost layer is the mucus layer, rich in mucin secreted by intestinal goblet cells. This layer not only provides a habitat for microbes but also acts as a protective barrier, preventing direct contact between bacteria and epithelial cells [263]. Beneath the mucus lies the epithelial barrier, formed by specialized epithelial cells joined by intercellular junctions, which seal the GI space and block the paracellular passage of bacteria [264]. Beyond the epithelial layer is the immune barrier, composed of immune cells in the lamina propria (LP), which mount defensive responses against pathogens that cross the intestinal epithelium.

Together, these three layers synergize to regulate the gut-liver axis, controlling the transport of molecules and compounds from the gut to the liver and systemic circulation. Under healthy conditions, the epithelial tight junctions remain intact, allowing the absorption of water and nutrients while effectively blocking the penetration of harmful intestinal contents into the bloodstream and internal organs

[265,266]. However, dysregulation of the gut microbiota can compromise the gut barrier, leading to a leaky gut characterized by increased intestinal permeability (Figure 13) [267]. This issue can be exacerbated by gut-vascular barrier damage [268].

In summary, the disruption of the gut barrier facilitates the translocation of inflammatory microbial products to the liver, triggering hepatic inflammation and exacerbating liver disorders through the gut-liver axis.

5.3. GUT-LIVER AXIS IN DISEASE

Gut microbiota dysbiosis is implicated in the progression of multiple liver diseases, including alcohol-associated liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) [269–272]. Unhealthy lifestyles, such as chronic alcohol consumption, excessive caloric intake, and insufficient physical activity, impose stress on the gut microbiota, triggering dysbiosis [247].

Dysbiosis is frequently associated with intestinal barrier permeability, endotoxin leakage, alterations in BA composition, and decreased choline levels [273]. These changes facilitate the translocation of gut microbiota, their metabolites, and bacterial EVs across the compromised intestinal barrier, ultimately entering the portal system and exacerbating liver disease progression [274,275]. For instance, sustained exposure of the liver to microbial metabolites activates hepatic immune responses and exacerbates hepatic steatosis [276–278].

In ALD, beyond the direct toxic effects of ethanol on liver parenchymal cells, gut microbiota dysbiosis and intestinal barrier disruption also play critical roles in pathogenesis [279]. Dysbiosis, which often precedes liver fibrosis in ALD, leads to elevated gut bacterial endotoxins in the bloodstream following excessive alcohol intake, thereby stimulating hepatic immune cell activation and contributing to liver injury [280–282].

In contrast, NAFLD is predominantly driven by sedentary lifestyles and poor dietary habits. Importantly, therapeutic interventions such as gut microbiota transplantation, as well as the use of probiotics, prebiotics, and postbiotics, have demonstrated efficacy in mitigating liver disorders in both preclinical models and clinical studies [283–285]. These findings underscore the pivotal role of gut microbiota in NAFLD/NASH. While dysbiosis and increased intestinal permeability contribute to hepatic inflammation in both ALD and NAFLD [286], the two diseases differ in specific aspects, including gut microbial composition, gut permeability, bile acid metabolism, ethanol byproducts, and choline metabolites [276,287]. Notably, disruptions in microbial BA metabolism are strongly associated with hepatic steatosis and insulin resistance [259]. In cirrhosis, a leaky gut is a key driver of major complications. Pathological bacterial translocation promotes liver inflammation, which further impairs bile acid synthesis [288].

The gut-liver axis is also involved in metabolic diseases such as obesity, type 2 diabetes, and cardiovascular diseases. Gut microbiota influences obesity through pathways like energy absorption, fat storage, inflammation, and circadian rhythms, with butyrate enhancing fatty acid oxidation and potentially limiting weight gain [289,290]. In type 2 diabetes, reduced butyrate-producing bacteria and increased opportunistic pathogens contribute to chronic inflammation, insulin resistance, and metabolic dysfunction [291,292]. Dysregulation of the gut-liver axis further disrupts hepatic lipid metabolism, exacerbating insulin resistance [293].

This intricate interplay highlights the central role of gut barrier integrity and microbiota in the pathogenesis and progression of liver diseases.

5.4. THERAPEUTIC POTENTIAL IN THE GUT-LIVER AXIS

The gut-liver axis consists of two interconnected organs, and dysfunction in either leads to reciprocal impacts on both intestinal and liver-related pathologies.

Therapeutic strategies targeting the gut primarily aim to regulate dysbiosis and restore gut barrier integrity. Preclinical studies with the probiotic EcN have demonstrated its

ability to enhance gut barrier function and modulate inflammatory responses in fecal transplantation models [294]. *In vitro* studies further support the anti-inflammatory potential of EcN, highlighting its role in strengthening the gut barrier by upregulating tight junction protein expression [201,202].

Clinical interventions using probiotics, particularly from the *Lactobacillus* and *Bifidobacterium* genera, as well as fecal microbiota transplantation have shown significant efficacy in improving liver conditions such as NAFLD, ALD, and hepatic encephalopathy [295].

More recently, postbiotics, including bacterial EVs, have emerged as promising therapeutic agents for gut-liver axis disorders. EVs derived from probiotics such as *Lactobacillus rhamnosus*, *Lactobacillus plantarum* Q7, and *Clostridium butyricum* have been shown to alleviate colon shortening, reduce colonic tissue damage, and reinforce gut barrier integrity [296–298]. In this context, studies conducted by our group have demonstrated that EVs derived from probiotic and commensal *Escherichia coli* strains modulate epithelial barrier function [202,299] and activate regulatory mechanisms to maintain a balance between anti-inflammatory and pro-inflammatory responses [300,301].

Given the role of EVs in modulating gut microbiota and maintaining gut barrier integrity, their therapeutic potential in liver diseases linked to gut disorders is unsurprising (Figure 13). Two studies on chronic liver injury demonstrated that oral gavage of EVs derived from *Akkermansia muciniphila* reduced serum inflammatory cytokines and alleviated liver and colon damage in a mouse model of high-fat diet and CCl₄-induced liver injury [302,303]. EVs from *Lactobacillus rhamnosus* showed protective properties on the intestinal barrier, mitigating alcohol-induced barrier dysfunction and reducing the translocation of gut bacteria and endotoxins to the liver, thereby protecting the liver from both steatosis and injury in an animal model of alcohol-associated liver disease [255]. EVs from *Akkermansia muciniphila* were also tested in high fat diet-induced diabetic mice. Oral gavage of those probiotic EVs for 5

weeks, tissue inflammation and insulin resistance were reduced in the mice, likely due to enhancement of intestinal tight junction function [304].

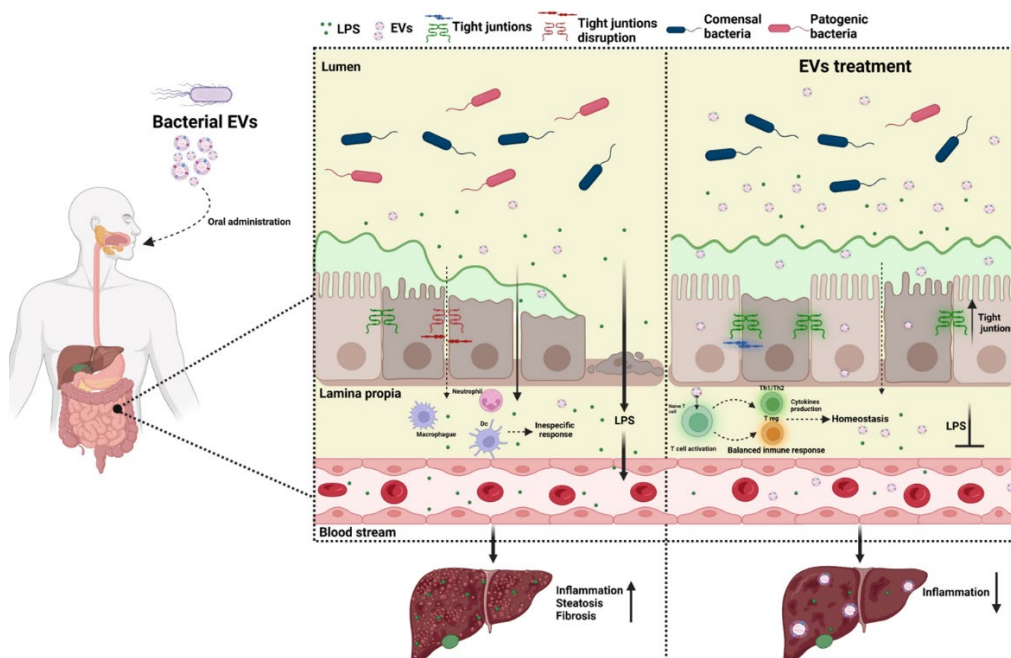


Figure 13. Therapeutic potential of EVs in liver disease prevention. EVs contribute to gut-liver axis protection by enhancing intestinal barrier integrity through stimulation of tight junctions, thereby limiting the translocation of LPS to the liver. Additionally, EVs help modulate dysbiosis, supporting microbial balance and reducing inflammation associated with liver disease progression.

Although research is still limited, the therapeutic potential of EVs has been demonstrated in both gut disorders and various metabolic diseases. Mechanistically, EVs may exert their effects either directly at the site of disease or indirectly through pathways such as the gut-liver axis, gut-brain axis, or others. Despite being in the early stages, these promising preclinical findings have already laid the groundwork for future clinical applications.

6. GUT-BRAIN AXIS

The gut-brain axis is a complex, bidirectional communication network that links the GIT with the central nervous system (CNS), regulating a wide range of physiological and cognitive functions. This intricate system is mediated by neural, endocrine, immune,

and metabolic pathways that facilitate signal exchange between the gut and the brain, thereby influencing both neurological and gastrointestinal homeostasis [305].

Growing evidence underscores the pivotal role of the gut microbiota in modulating gut-brain connection, with its influence extending to behavior, cognition, and emotional regulation. As stated above, under normal physiological conditions, commensal microbiota contribute to immune homeostasis and confer protection against various diseases [306,307]. Conversely, disruptions in gut microbial composition, known as dysbiosis, can impair host immunity and have been implicated in the pathogenesis of chronic metabolic disorders [307]. Furthermore, dysbiosis has been also linked to neurological and psychiatric conditions, including Alzheimer's disease, Parkinson's disease, and mood disorders, suggesting a mechanistic link between gut microbiota alterations and neurodegeneration [308–310].

Recent advances have highlighted the critical role of EVs in mediating gut microbiota-brain communication. These nano-sized vesicles act as carriers of molecular cargo, enabling the transfer of microbial signals across both intestinal and blood-brain barriers. Upon reaching the CNS, bacterial EVs engage innate immune receptors such as TLRs and NLRs in glial cells, initiating immune signaling cascades that drive neuroinflammatory responses. This immune activation has been implicated in the onset and progression of neurodegenerative and neuropsychiatric disorders, revealing a novel mechanistic pathway through which gut dysbiosis may contribute to brain dysfunction.

The recognition of EVs as key mediators in gut-brain signaling underscores their potential as therapeutic targets for modulating neuroinflammation and restoring homeostasis within the gut-brain axis [244]. By elucidating the role of microbial-derived EVs in brain pathology, future research may pave the way for innovative therapeutic strategies aimed at mitigating the impact of dysbiosis on neurological and psychiatric health.

6.1. GUT-BRAIN AXIS IN HEALTH

The gut-brain axis is a fundamental communication network essential for maintaining homeostasis and regulating physiological processes across multiple organ systems. The gut microbiota plays a pivotal role in this axis by producing bioactive metabolites such as SCFAs, which significantly influence brain function and mental well-being. Notably, the integrity of the blood-brain barrier (BBB) in critical brain regions, including the frontal cortex, hippocampus, and striatum, is modulated by commensal gut microbiota. Studies using GF mice have demonstrated increased BBB permeability, a phenomenon evident even during embryonic development due to reduced tight junction integrity, as indicated by decreased expression of claudin-5 and occludin [311]. Remarkably, recolonization of GF mice with a complex gut microbiota or administration of the SCFA butyrate restored BBB integrity, underscoring the microbiota's role in maintaining neurovascular function.

Beyond BBB integrity, the gut microbiota also governs microglial maturation and innate immune responses in the brain. GF mice exhibit immature and functionally impaired microglia compared to specific-pathogen-free mice, highlighting the necessity of microbial-derived factors for microglial development [312]. SCFAs, which can cross the BBB, play a critical role in modulating neuroinflammation, immune signaling, and neurotransmission [313].

Gut-brain communication is also facilitated by bacterial EVs, which transport a diverse range of bioactive molecules produced by the gut microbiota to the brain. These vesicles contribute to neuroimmune regulation, influencing neural circuits and modulating inflammatory responses. Notably, EVs from *Lactobacillus plantarum* have exhibited neuroprotective effects, demonstrating antidepressant-like properties and mitigating amyloid-beta-induced neuropathology in experimental models [314,315].

Collectively, these findings underscore the gut-brain axis as a crucial determinant of both physical and mental health. Through metabolites such as SCFAs and intercellular communication via EVs, the gut microbiota exerts a profound influence on

neurodevelopment, neuroprotection, and emotional well-being, providing novel avenues for therapeutic interventions targeting neuropsychiatric and neurodegenerative disorders.

6.2. GUT BRAIN AXIS IN DISEASE

Dysregulation of the gut-brain axis is increasingly recognized as a fundamental factor in the pathogenesis of neuropsychiatric and neurodegenerative disorders. Conditions such as depression, anxiety, Parkinson's disease, and Alzheimer's disease have all been linked to gut microbiota dysbiosis, underscoring the role of gut-derived signals in maintaining brain function. Imbalances in the gut microbiome can disrupt this communication network within the gut-brain axis, promoting the release of pro-inflammatory cytokines and neurotoxic metabolites that exacerbate neuroinflammation and neuronal damage [316].

In neurodegenerative diseases, alterations in gut permeability and microbial composition contribute to chronic neuroinflammation and oxidative stress, both of which are central drivers of disease progression. Evidence from fecal microbiota transplantation experiments further support the gut's influence on the brain health. For example, GF rodents that received fecal microbiota transplants from patients with major depressive disorder exhibited depressive-like behaviors, whereas transplants from healthy individuals did not induce similar effects [317,318]. This finding suggests that gut microbiota composition plays a causative role in mood disorders.

EVs produced by gut bacteria have emerged as critical mediators of microbiota-brain communication in disease states. Studies in murine models have demonstrated that EVs derived from *Lactobacillus* strains can regulate hippocampal brain-derived neurotrophic factor (BDNF) expression, producing antidepressant-like effects in stress-induced depression models. Moreover, EVs derived from the intestinal microbiota have been shown to modulate the expression of inflammatory markers, regulate serotonin metabolism, and influence neural signaling [319]. In neurodegenerative diseases, both bacterial and host-derived EVs have been implicated in increasing BBB permeability.

This alteration facilitates the entry of microbial-derived pro-inflammatory molecules into the CNS, thereby exacerbating neuroinflammation in conditions such as Parkinson's disease and bacterial meningitis [320,321].

The expanding understanding of the role of EVs in the pathophysiology of these diseases presents novel therapeutic opportunities. Targeting the molecular cargo carried by these vesicles, modifying gut microbiota composition, or manipulating EV-mediated signaling pathways may offer promising strategies for restoring gut-brain axis homeostasis. Such interventions hold potential for alleviating symptoms and potentially slowing the progression of neuropsychiatric and neurodegenerative disorders [316].

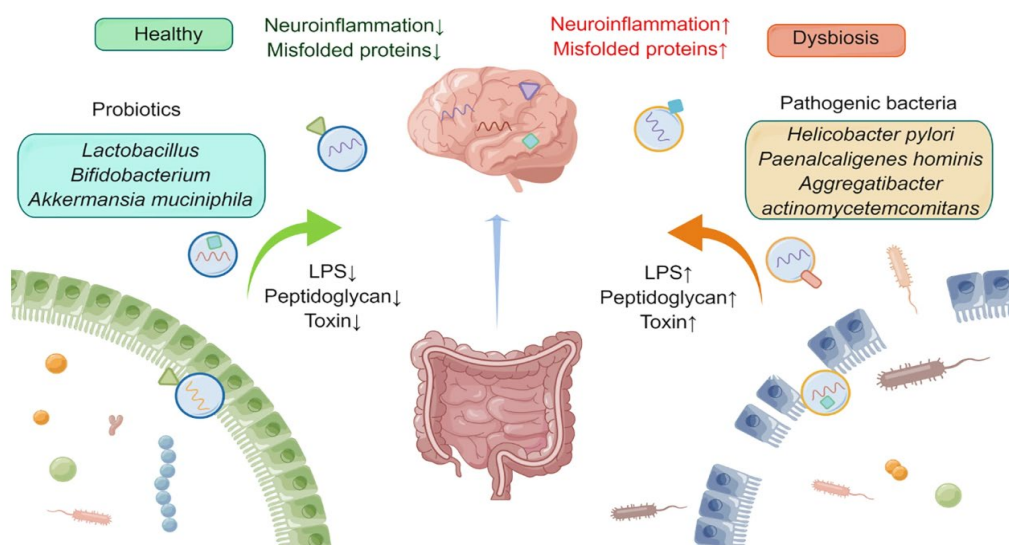


Figure 14. Gut–brain axis: microbiota-derived extracellular vesicles as modulators of neural integrity.

This schematic illustrates the dual role of EVs in the regulation of neural health. EVs released by probiotic and beneficial commensal bacteria exhibit neuroprotective properties and may contribute to the mitigation of neurodevelopmental disorder pathologies. In contrast, disruption of gut homeostasis enables the release of EVs from pathogenic bacteria, which can cross both the intestinal barrier and the BBB. These vesicles may carry pro-inflammatory components into the CN), potentially triggering immune responses, neuroinflammation, and the accumulation of neurotoxic protein aggregates. Figure from [322].



OBJECTIVES

Early life represents a critical window for the development of both the intestinal barrier and the immune system. During this stage, the establishment of an effective mucosal barrier and appropriate immune responses is essential for protecting the host from pathogens, particularly enteric viruses such as RV. The gut microbiota plays a central role in these processes by releasing microbial signals that influence host development and function.

Among these microbial components, EVs produced by probiotic and commensal bacteria have emerged as key modulators of host physiology. EVs are nanosized structures capable of delivering effector molecules to host cells, modulating inflammation, permeability, gene expression, and cellular signaling. Due to their stability and ability to cross biological barriers, EVs have gained attention as promising postbiotic candidates with potential effects beyond the gut, including communication through the gut-liver and gut-brain axes.

To this end, we used EVs derived from two well-characterized bacterial strains: the probiotic *E. coli* EcN and the commensal *E. coli* EcoR12. Oral administration of EVs was assessed in suckling rats to evaluate their ability to mitigate rotavirus infection and stimulate neonatal immune development, as well as their impact on the gut–liver axis. Additionally, to explore gut-brain interactions, we employed intestinal organoids derived from induced pluripotent stem cells (iPSCs) and neurons derived from iPSCs to assess epithelial barrier regulation and neuronal development.

GENERAL OBJECTIVE

Based on this background, the **main objective** of this thesis “to investigate the role of EVs derived from probiotic and commensal *E. coli* strains in the modulation of intestinal barrier function, antiviral immunity, and systemic communication through the gut-liver and gut-brain axes, using *in vivo* and *in vitro* models relevant to early life”. To achieve this general goal, the following specific objectives were established:

OBJECTIVE 1.

1.1. To evaluate the impact of EcN- and EcoR12-derived EVs on **intestinal development, barrier maturation, and systemic immune responses** in **healthy** suckling rats.

1.2. To assess their protective and **immunomodulatory** effects in a **neonatal** model of **rotavirus infection**.

The results obtained from the first objective are included in the following works:
Article 1 and **Article 2**.

OBJECTIVE 2.

2.1. To determine whether EVs from EcN and EcoR12 **reach the liver** following oral administration in suckling rats.

2.2. To assess EVs effect on intestinal **permeability** by evaluating **zonulin** expression as a marker of epithelial barrier regulation.

2.3. To explore the role of EVs in gut-liver communication by assessing their impact on **hepatic gene expression** related to **fatty acid synthesis and oxidation, inflammation, and oxidative stress**, using both *in vitro* and *in vivo* models.

The results obtained from the second objective are included in the following works:
Article 3.

OBJECTIVE 3.

3.1. To investigate the effect of EcN- and EcoR12-derived EVs on epithelial **tight junction** expression in **intestinal organoids** derived from human iPSCs.

3.2. To evaluate their influence on **neuronal development** by assessing morphological features in **iPSCs-derived neurons** model, as part of the exploration of gut-brain axis signaling.

The results obtained from the third objective are included in the following works:
Article 4.



RESULTS

ARTICLE 1

“Microbiota-Derived Extracellular Vesicles Promote Immunity and Intestinal Maturation in Suckling Rats”

Martínez-Ruiz, Sergio; Sáez-Fuertes, Laura; Casanova-Crespo, Sergi; Rodríguez-Lagunas, María José; Pérez-Cano, Francisco José; Badia, Josefa; Baldoma, Laura.

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The results showed in this paper have been presented in the following congresses:

- **XIV Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Pamplona (March 2023). **Martínez-Ruiz S;** Sáez-Fuertes L; Olivo-Martínez Y; Cordero C; Rodríguez-Lagunas MJ; Pérez-Cano FJ; Badia J; Baldomà L. “Efecto de vesículas de *E.coli* probióticas y comensales en un modelo de infección por rotavirus”
- **I Jornada de recerca i divulgació de doctorat de la Universitat de Barcelona.** Sevilla (March 2023). **Martínez-Ruiz S;** Sáez-Fuertes L; Olivo-Martínez Y; Cordero C; Rodríguez-Lagunas MJ; Pérez-Cano FJ; Badia J; Baldomà L. “Efecto de vesículas de *E.coli* probióticas y comensales en un modelo de infección por rotavirus”.

ABSTRACT

Aim: This study aimed to investigate the effects of daily administration of EVs from probiotic and commensal *E. coli* strains in healthy suckling rats during the first 16 days of life.

Methods: Suckling rats received EVs derived from probiotic and commensal *E. coli* strains daily from postnatal day 2 to 16. On days 8 and 16, samples were collected and a range of intestinal and systemic parameters were evaluated, including growth, humoral and cellular immune responses, epithelial barrier maturation, and intestinal morphology.

Results: By day 16, animals treated with probiotic or microbiota-derived EVs showed elevated plasma levels of IgG, IgA, and IgM, along with an increased proportion of Tc, NK, and NKT cells in the spleen. In the small intestine, EV administration enhanced villus area and modulated the expression of genes involved in immune function, inflammation, and intestinal permeability, promoting an anti-inflammatory and barrier protective profile from as early as day 8.

Conclusion: Interventions involving EVs from probiotic and commensal *E. coli* strains may represent a safe and effective postbiotic strategy to support immune development and promote intestinal maturation during early life. The observed systemic and intestinal effects highlight their potential to modulate key processes involved in neonatal health.



Article

Microbiota-Derived Extracellular Vesicles Promote Immunity and Intestinal Maturation in Suckling Rats

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Abstract: Microbiota–host communication is primarily achieved by secreted factors that can penetrate the mucosal surface, such as extracellular membrane vesicles (EVs). The EVs released by the gut microbiota have been extensively studied in cellular and experimental models of human diseases. However, little is known about their in vivo effects in early life, specifically regarding immune and intestinal maturation. This study aimed to investigate the effects of daily administration of EVs from probiotic and commensal *E. coli* strains in healthy suckling rats during the first 16 days of life. On days 8 and 16, we assessed various intestinal and systemic variables in relation to animal growth, humoral and cellular immunity, epithelial barrier maturation, and intestinal architecture. On day 16, animals given probiotic/microbiota EVs exhibited higher levels of plasma IgG, IgA, and IgM and a greater proportion of Tc, NK, and NKT cells in the spleen. In the small intestine, EVs increased the villi area and modulated the expression of genes related to immune function, inflammation, and intestinal permeability, shifting towards an anti-inflammatory and barrier protective profile from day 8. In conclusion, interventions involving probiotic/microbiota EVs may represent a safe postbiotic strategy to stimulate immunity and intestinal maturation in early life.

Keywords: early life; extracellular vesicles; microbiota–host crosstalk; probiotic; *E. coli* Nissle 1917; immunity; intestinal permeability

1. Introduction

The intestinal microbiota plays a key role in the development and function of the intestinal immune system, reinforces the intestinal epithelial barrier, and contributes to host defense responses against pathogens [1]. The gut microbiota is considered an essential organ that establishes multidirectional communication with other host organs through several routes including endocrine, immunological, metabolic, and neural pathways [2]. Thus, alterations in the gut microbiota composition and diversity not only cause gut-related disorders but also contribute to extraintestinal diseases [3,4].

The gut barrier is essential for maintaining the segregation between the luminal microbial community and the host [5,6]. The barrier function is accomplished by a combination of several mechanisms, which include the mucus layer, host-secreted immunoglobulin (Ig) A and antimicrobial peptides, the intestinal epithelium, and the mucosal immune system. The intestinal epithelium is formed by a single layer of polarized cells sealed by tight junctions (TJs) that restrict the paracellular passage of large molecules [7,8]. In adulthood, the passage of molecules through the intestinal barrier is selectively controlled by specific endocytic pathways and regulation of TJs. In contrast, the intestine is highly permeable during early life, especially in rodents. This is due to the characteristics of immature intestinal

epithelial cells, which have high endocytic capacity (transcytosis) and altered expression of TJ proteins that leads to enhanced paracellular passage. Reduced expression of strengthening TJ proteins (i.e., occludin, ZO-1) and overexpression of pore-forming proteins such as claudin-2 have been observed in the intestinal tissue of infant mice [9]. In the neonatal period, increased intestinal permeability contributes to the maturation of the immune system by transferring microbial components and antigens to the underlying immune cells [10]. Moreover, intestinal transfer of maternal IgG antibodies through endocytosis mediated by the neonatal Fc receptor for IgG (FcRn) provides passive immune protection to the offspring [11]. The leaky barrier properties gradually disappear as functional intestinal maturation progresses. The timing and extent of the enhanced endocytic and paracellular transfer activity before gut closure depend on the animal species [10].

The structure of the mature intestinal mucosal barrier prevents direct contact of the gut bacteria with intestinal epithelial and immune cells. Therefore, communication between commensal microbiota and host cells is mainly achieved by secreted bacterial components that can penetrate the mucosal surface. In addition to secreted proteins, metabolites, and signaling molecules [12–14], the gut microbiota releases extracellular vesicles (EVs). These EVs can cross the mucus layer, pass through the epithelial monolayer, and disseminate in the body to perform their actions [15,16]. There is growing evidence that interkingdom crosstalk in the gut is mainly mediated by EVs [17].

Bacterial EVs are nano-size spherical structures (20–300 nm) derived from bacterial membranes. They serve as a secretion system for molecules that are essential for bacterial survival and interspecies communication. During the biogenesis process, EVs selectively enclose a wide variety of bacterial molecules that include characteristic microbe-associated molecular patterns (MAMPs), proteins, metabolites, DNA, and regulatory RNAs. The vesicle cargo depends on the producer bacterial strain and its physiological state. This fact determines functions that are strain specific [18]. In the context of the gut microbiota, cumulative evidence shows that bacterial EVs mediate microbiota functions by transferring effector molecules into host cells (reviewed in [17]). MAMPs on the vesicle surface such as lipopolysaccharide (LPS) interact with extracellular immune receptors, known as pattern-recognition receptors (PRRs) of the host cells to activate signaling cascades to control immune and defense responses [19,20]. Moreover, EVs are taken up by epithelial and immune cells through endocytic pathways [21]. This process allows for the intracellular delivery of MAMPs and other active bacterial molecules that trigger specific host responses. In this scenario, continuous activation of immune receptors by EVs released by gut microbiota results in controlled basal inflammation that is essential to maintain balanced immune responses to preserve intestinal homeostasis [22]. Thus, we hypothesized that in early life, when the intestine is more permeable, the internalization rate of microbiota EVs could increase, and therefore, it could have a greater impact on the developing host.

The study of gut microbiota EVs and their actions on host physiology is an emerging research area. Many reports on EVs from probiotics (*Lactobacillus* sp., *Bifidobacterium* sp.) and gut beneficial bacteria (*Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, *Escherichia coli*) prove their immunomodulatory and barrier-strengthening effects in both in vitro and in vivo models through different mechanisms. Depending on the producer bacterial strain, EVs can regulate the expression of TJ proteins, antimicrobial peptides, and mucus production and induce innate and/or adaptive responses [17,23,24]. In this context, our group has contributed to this knowledge using *E. coli* strains as a model of microbiota-derived vesicles, specifically the probiotic *E. coli* Nissle 1917 (EcN) and commensal *E. coli* strains isolated from the stools of healthy human individuals. We have shown that EVs from these strains activate immune responses and modulate the barrier integrity in intestinal epithelial cells and colonic explants [22,25]. Moreover, EVs from these microbiota strains modulate human dendritic cells and the subsequent T-helper (Th) responses through mechanisms that are strain specific [26].

While microbiota EVs have been extensively studied in cellular and experimental models of human diseases such as inflammatory bowel disease, obesity, diabetes, or neuro-

EcoR12 (EcoR12 EVs). Each experimental group was formed by 3 litters of 8 pups each ($n = 24/\text{group}$). Neonatal rats were orally administered from the second (day 2) to the sixteenth day of life (day 16). Since the pups gained weight through the experimental period, during the first eight days, the EcN EVs and EcoR12 EVs groups were given a concentration of EVs of $2 \mu\text{g protein/animal/day}$ (body weight between 5.5 and 12 g). From the ninth day of life, the administered dose was increased to $5 \mu\text{g protein/animal/day}$ until the last day of the procedure (body weight from 12 to 29 g). The administered volume was adjusted to $100 \mu\text{L}$. The control group received a matched volume of the vehicle (PBS). The selected dose of EVs was based on previous studies in mice with an average body weight of 20–22 g [33]. To confirm that equal amounts of EcN EVs and EcoR12 EVs were administered, the stock EV samples ($40 \mu\text{g/mL}$) were diluted and quantified with the lipophilic fluorescent dye FM4-64. Fluorescence intensity values did not differ between samples (at $8 \mu\text{g/mL}$ protein concentration the fluorescence values were 0.96 ± 0.025 for EcN EVs and 0.94 ± 0.03 for EcoR12 EVs). Oral administration was performed as described previously [33,37], using low-capacity syringes (Hamilton Bonaduz, Bonaduz, Switzerland). Half of the animals of each litter were euthanized on day 8 and the other half on day 16. For this purpose, animals were randomly selected, maintaining similar proportions of males and females at both intervention times.

Body weight was measured daily. To prevent adverse intestinal effects of EcN or EcoR12 EVs in neonatal rats, fecal samples were collected during the first two weeks of life (days 4–7 and days 8–11) by gentle abdominal massage. Fecal specimens were weighed and scored in a blinded manner (from 1–4) based on their color, texture, and amount. The fecal score (FS) values were: (1) normal feces, (2) soft yellow-green feces, (3) totally loose yellow-green feces, and (4) high amount of watery feces, as in previous studies [37].

2.4. Sample Collection

At the end of the experimental procedure (day 8 or day 16 of life) neonatal rats were anesthetized with intramuscular injection of ketamine/xylazine (Imalgene 100 mg/mL , Merial Laboratorios, Barcelona, Spain/Rompun 20 mg/mL , Bayer Hispania, Sant Joan Despí, Spain). At this point, the naso-anal and tail lengths were measured to obtain the body/tail ratio. The body mass index (BMI) was calculated as $\text{body weight}/\text{length}^2$ (g/cm^2) and the Lee index as $3\sqrt{\text{weight}/\text{length}} \times 1000$ ($3\sqrt{\text{g}/\text{cm}}$). Once the animals were fully unconscious, euthanasia was performed by exsanguination. Blood was collected by cardiac puncture to obtain plasma samples, which were stored at -20°C . The spleen, thymus, liver, stomach, small intestine, cecum, kidneys, and heart were obtained and weighed to obtain morphogenic variables. Also, the length of the small intestine was measured. A central portion (1 cm) of the small intestine was placed in liquid nitrogen directly and then stored at -80°C for gene expression analysis. A fragment of the distal jejunum was collected and processed for histological analysis. The gut wash was obtained from the remaining parts of the intestine as described previously [37]. The spleens were processed for lymphocyte isolation as described below.

2.5. Quantification of Immunoglobulins

Plasma concentrations of IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c), IgA, and IgM were quantified using ProcartaPlex™ Multiplex immunoassay (Thermo Fisher Scientific, Barcelona, Spain), as previously described [37]. Briefly, specific color-coded capture beads were bound to the Ig of interest. After adding the different detection antibodies conjugated to phycoerythrin (PE), the concentration of each analyte was obtained using the MAG-PIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB).

2.6. Gene Expression Analysis by Reverse Transcription Quantitative PCR (RT-qPCR)

Small intestine tissue collected as described above (Section 2.4) was homogenized in lysing matrix tubes (MP Biomedicals, Illrich, France) using Fast-Prep-24 equipment (MP

logical disorders [27–33], little is known about their *in vivo* effects in early life, specifically regarding immune and intestinal maturation during the neonatal period. The present study aimed to investigate the effects of daily administration of EVs from probiotic and commensal *E. coli* strains in healthy suckling rats during the first 16 days of life. We evaluated intestinal and systemic variables in relation to animal growth, humoral and cellular immunity, epithelial barrier maturation and function, and intestinal architecture.

2. Materials and Methods

2.1. Bacterial Strains and Isolation of EVs

The probiotic *E. coli* Nissle 1917 (EcN) was provided by Ardeypharm (GmbH, Herdecke, Germany). The *E. coli* strain EcoR12, included in the ECOR reference collection, was isolated from a healthy human stool sample [34].

Bacterial EVs were obtained as described previously [26]. Briefly, EcN and EcoR12 strains were grown in Luria–Bertani broth (LB) for 16 h. Bacterial cells were pelleted by centrifugation and the culture supernatants were filtered through 0.22 µm filters (Merck, Millipore, MA, USA) to remove residual bacteria. The filtrated supernatants were concentrated using centrifugal Centricon Plus-70 100 kDa filters (Millipore). After an additional filtration step using 0.22 µm pore size filters, the vesicles were collected by ultracentrifugation at 150,000× *g* for 1 h at 4 °C. The resulting pellets were washed twice in phosphate-buffered saline (PBS). Finally, pelleted EVs were resuspended in PBS, aliquoted, and stored at −20 °C until use. Sterility of the samples was confirmed on LB plates. Quantification of samples was assessed in terms of the protein and lipid content of EVs using, respectively, the Pierce BCA protein assay and the lipophilic fluorescent dye FM4-64 (Thermo Fisher Scientific, Barcelona, Spain), which intercalates into the vesicle membrane. For the FM4-64 assay, EV samples were incubated with FM4-64 (5 µg/mL in PBS) for 10 min at room temperature. Reactions containing only EVs or the FM4-64 probe were used as negative controls. After excitation at 515 nm, emission at 640 nm was measured with the Varioskan Lux multiplate reader (Thermo Fisher Scientific) [35]. Fluorescence was normalized by protein concentration.

2.2. Animals

A total of nine pregnant Lewis rats (G14), purchased from Janvier (Le Genest-St-Isle, France), were individually housed in cages (2184 L Eurostandard Type II L, Tecniplast, West Chester, PA, USA). All cages contained tissue paper and fibrous particle bedding. Rats were monitored daily until natural delivery. Litters were randomly distributed into three experimental groups (with 3 lactating dams each) and the number of pups was unified to 8 per dam, keeping a similar proportion (40–60%) of males and females per litter. The rats were housed in controlled conditions of temperature, humidity, and light: dark cycles of 12 h, in an isolated room, designed and authorized for working under biosecurity level 2 conditions, at the Animal Experimentation Unit (UEA) of the Diagonal Campus at the University of Barcelona (UB). Rats were fed with a commercial diet corresponding to the American Institute of Nutrition (AIN) 93 G formulation (Teklad Global Diet 2014, INOTIV, Indianapolis, IN, USA) [36] and water *ad libitum*. Neonatal rats had free access to maternal milk. For animal handling, the mother was placed in a new cage and the pups were kept in the home cage. Oral administration to each experimental group was performed randomly, and then the dams were brought together with the corresponding litters. Animal procedures were approved by the Ethical Committees for Animal Experimentation (CEEa) of the UB and the Catalan Government (CEEa-UB Ref.169/20 and Ref.11461, respectively) in compliance with the EU Directive 2010/63/EU, which establishes the rules for the protection of animals used in experimental work.

2.3. Experimental Design

Litters were randomly distributed into three experimental groups: the control group, and animals receiving EVs from the probiotic EcN (EcN EVs) or EVs from the commensal

Biomedicals) [37]. RNA was isolated with the RNeasy®Mini Kit (Qiagen, Madrid, Spain). RNA purity and concentration were assessed using a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA). Then, cDNA was synthesized using the TaqMan®Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany) following the manufacturer's protocol.

Quantitative PCR was carried out using the ABI Prism 7900 HT equipment. The specific PCR TaqMan®primers (AB) were: *TLR2* (Rn02133647_s1), *TLR7* (Rn01771083_s1), *IGA* (Rn01511082_m1), *CD68* (Rn00566655_m1), *FcRn* (Rn00583712_m1), *MUC2* (Rn01498206_m1), *IL12* (Rn00584538_m1), *COX-2* (Rn01483828_m1), *OCN* (Rn00580064_m1), and *ZO-1* (Rn02116071_s1). The relative gene expression was normalized with the endogenous housekeeping gene *Gusb* (Rn00566655_m1) by means of the $2^{-\Delta\Delta Ct}$ method. The results were expressed as the percentage of expression in each experimental group, normalized to the mean value obtained for the control group, which was set at 100%.

2.7. Lymphocyte Isolation from Spleen

Spleens were passed through a sterile mesh cell strainer (40 µm, Thermo Fisher Scientific, S.L.U, Barcelona, Spain) [38]. Then, erythrocytes were removed by osmotic lysis and lymphocytes were suspended in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine. Dead cells were stained with Trypan Blue. Cell numbers and viability were measured using a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, S.L.U, Barcelona, Spain).

2.8. Lymphocyte Phenotypic Analysis by Immunofluorescence Staining and Flow Cytometry

The phenotype of spleen lymphocytes was analyzed by using fluorescent monoclonal antibodies (Ab), as previously described [38]. The Ab used were conjugated to the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), brilliant violet 421 (BV421), brilliant violet 605 (BV605), or brilliant violet 786 (BV786). Ab used in this study were specific against rat and fluorochrome conjugated as follows: FITC-TCRαβ, Bv786-CD8β, FITC-CD25, PE-CD161a, Bv605-TCRγδ, PE-CD4, PerCP-CD8α, APC-CD4, and BV421-CD45RA (BD Biosciences) and APC-FoxP3 (eBioscience, Frankfurt, Germany). Briefly, lymphocytes were incubated with saturating concentrations of fluorochrome-conjugated Ab, fixed with 0.5% p-formaldehyde and stored (4 °C, in darkness) until flow cytometry analysis. For Treg analysis, lymphocytes were incubated (20 min, 4 °C, in darkness) with fluorochrome-conjugated anti-CD4 (PE) and anti-CD25 (FITC) Ab, treated (30 min, 4 °C, in darkness) with a fixation-permeabilization solution (eBioscience) and then incubated (30 min, 4 °C, in darkness) with APC-conjugated anti-Foxp3 Ab (eBioscience). A negative control staining with an isotype-matched monoclonal Ab was included in each cell sample.

Analyses were performed with an AURORA™ Cytometer (Beckman Coulter, Miami, FL, United States) in the Flow Cytometry Unit of the Scientific and Technological Centers of the UB (CCiT-UB), and data were analyzed by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA). The results were expressed as percentages of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FSCs) and side-scatter characteristics (SSCs) or in a particular lymphocyte population.

2.9. Histomorphometry Analysis

Distal jejunum fragments obtained from neonatal rats were processed for hematoxylin-eosin staining as previously described [37]. Intestinal morphology was analyzed by bright-field microscopy (Olympus BX41, Olympus Corporation, Shinjuku, Tokyo, Japan) at 100× magnification. The morphometric measurements were performed with ImageJ v1.53t. Ten villi from each animal were randomly selected to measure their height, width, and area. The villi width was assessed at the crypt-villus junction. The villi area was calculated by delineating the region of interest (excluding the crypts). Crypt depths were

also determined. Statistical analysis was carried out with the mean value of the parameters calculated for each animal.

2.10. Statistical Analysis

For statistical analysis, the Statistical Package for Social Sciences (SPSS v22.0) (IBM, Chicago, IL, USA) was used. Data were tested for normal distribution (by Shapiro–Wilk) and variance equality (by Levene's test). A conventional one-way ANOVA test was carried out followed by the post hoc Bonferroni test when data were homogeneous and had a normal distribution, considering the experimental group as the independent variable. The non-parametric Kruskal–Wallis test followed by the post hoc Dunn's multiple comparison test was performed when data displayed non-normal distribution or dissimilar variances and for non-parametric variables such as the feces scoring. Differences were considered significant at $p < 0.05$. The results were expressed as mean $\pm$ SEM (n animals).

3. Results

3.1. Growth and Morphometry Variables

The body weight was monitored daily until the last day of the intervention (Figure 1A). Pups receiving EcN or EcoR12 EVs have similar weights compared to the control animals. Concerning the impact of the intervention on stool features, administration of EcN or EcoR12 EVs during the first two weeks of life had no significant impact on either fecal weight or the stool consistency. In this sense, the FS value (scale 1–4) was close to 1 for all groups (Figure 1B,C).

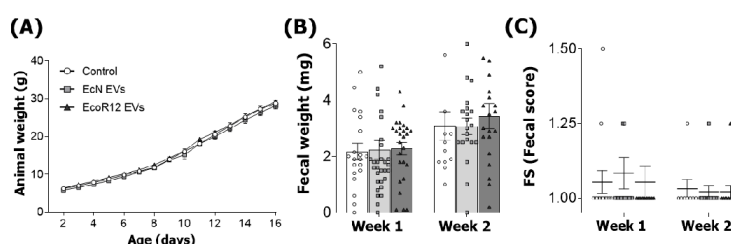


Figure 1. Interventions with EcN EVs or EcoR12 EVs do not affect body weight and feces features. The experimental groups were: Control (white circles), EcN EVs (gray squares), and EcoR12 EVs (black triangles). (A) Body weight (g) for control, EcN EVs, and EcoR12 EVs groups was measured daily over the 16-day experimental period. (B) The weight (mg) and (C) consistency of stool specimens were recorded during the first two weeks for the three experimental groups. Stool consistency was scored as described in the Materials and Methods to obtain the FS. Results are expressed as mean $\pm$ SEM ($n = 12$ –24 animals/group).

In addition, other growth-related parameters and the relative weight of organs were measured on day 8 and day 16 of life, without great changes due to EcN or EcoR12 EVs interventions (Table 1).

On day 8, only minor differences ($p < 0.05$) were observed in the EcoR12 EVs intervention group affecting the body mass, which were no longer evident on day 16. Concerning the relative organ weight, some small differences were observed between the two interventions. In this sense, the spleen weight was higher in animals given EcN EVs, both on day 8 and day 16. Moreover, oral administration of EcN EVs also resulted in a small significant increase in heart weight only on day 8. However, by day 16, the relative weight of this organ in the EcN EVs group was comparable to that of the control animals. In contrast, the intervention with EcoR12 EVs only affected the empty cecum relative weight, which had a slightly higher increase on day 8 (0.05% vs. control) and day 16 (0.04% vs. control).

Table 1. Growth-associated measurements and relative weight of organs.

Day 8	Control	EcN EVs	EcoR12 EVs
Animal length (cm)	9.83 ± 0.15	9.64 ± 0.12	9.96 ± 0.07
Body (cm)	6.87 ± 0.11	6.86 ± 0.12	6.74 ± 0.11
Tail (cm)	3.05 ± 0.06	2.91 ± 0.05	3.15 ± 0.1
Body mass index (g/cm ²)	0.24 ± 0.01	0.25 ± 0.01	0.27 ± 0.01 *
Lee index (g ^{0.33} /cm × 1000)	331.15 ± 2.96	331.23 ± 2.47	343.78 ± 4.41
Intestine weight (%)	3.19 ± 0.06	3.38 ± 0.11	3.16 ± 0.05
Intestine length (%)	231.57 ± 7.39	226.00 ± 6.17	212.31 ± 4.49
Cecum weight (%)	0.19 ± 0.04	0.19 ± 0.03	0.24 ± 0.04 *
Spleen weight (%)	0.48 ± 0.02	0.59 ± 0.03 *	0.50 ± 0.03
Liver weight (%)	2.85 ± 0.02	3.03 ± 0.05	2.86 ± 0.06
Thymus weight (%)	0.29 ± 0.01	0.29 ± 0.01	0.30 ± 0.01
Kidney weight (%)	0.71 ± 0.01	0.69 ± 0.01	0.70 ± 0.01
Heart weight (%)	0.69 ± 0.02	0.77 ± 0.02 *	0.72 ± 0.01
Day 16	Control	EcN EVs	EcoR12 EVs
Animal length (cm)	14.42 ± 0.22	14.36 ± 0.17	14.61 ± 0.04
Body (cm)	9.32 ± 0.12	9.32 ± 0.08	9.51 ± 0.06
Tail (cm)	5.1 ± 0.09	5.03 ± 0.09	5.1 ± 0.05
Body mass index (g/cm ²)	0.33 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
Lee index (g ^{0.33} /cm × 1000)	328.6 ± 3.15	326.98 ± 2.26	321.77 ± 2.9
Intestine weight (%)	2.94 ± 0.04	2.97 ± 0.04	2.95 ± 0.04
Intestine length (%)	126.08 ± 5.51	132.18 ± 3.67	129.27 ± 3.53
Cecum weight (%)	0.21 ± 0.01	0.24 ± 0.01	0.25 ± 0.01 *
Spleen weight (%)	0.44 ± 0.02	0.50 ± 0.01 *	0.41 ± 0.01
Liver weight (%)	3.08 ± 0.03	3.24 ± 0.1	3.20 ± 0.07
Thymus weight (%)	0.52 ± 0.01	0.47 ± 0.01	0.51 ± 0.01
Kidney weight (%)	0.61 ± 0.01	0.63 ± 0.02	0.60 ± 0.01
Heart weight (%)	0.62 ± 0.02	0.65 ± 0.02	0.62 ± 0.01

Data corresponding to relative organ weight are expressed as percentage of animal body weight. Results are expressed as mean ± SEM ($n = 12$ animals/group). * $p < 0.05$ compared to control (by post hoc Dunn's multiple comparison test).

The hematological variables were also assessed in blood samples at the two final time points (days 8 and 16) (Supplementary Table S1). No significant differences were apparent between the three experimental groups.

3.2. EVs from EcN and EcoR12 Stimulate Immunoglobulin Production in Neonatal Rats

Concentrations of Ig types and IgG subtypes were measured in plasma samples of suckling rats on days 8 and 16 (Figure 2). On day 8, no significant differences were observed in total IgGs or IgM or IgA levels between the intervention groups (EcN-EVs and EVs-EcoR12) and the control group (Figure 2A). The interventions did not significantly modify IgG subtypes, nor the Th1/Th2 Ig ratio (IgG2b + IgG2c/IgG1 + IgG2a) (Figure 2A). In contrast, highly significant differences were observed on day 16 in animals given EcN or EcoR12 EVs (Figure 2B). Animals in the EcN EVs and EcoR12 EVs groups showed more elevated plasma IgG ($p < 0.003$), IgM ($p < 0.001$), and IgA ($p < 0.001$) concentrations compared to the control group.

The increase in total IgG plasma levels on day 16 was due to an increase in all the IgG subtypes, without significant differences between the two interventions. Notably, both EcN and EcoR12 EVs raised plasma IgG2b levels by 7-fold ($p < 0.002$), IgG2c by 5-fold ($p < 0.001$), IgG1 by 4-fold ($p < 0.002$), and IgG2a levels by 2-fold ($p < 0.001$). Accordingly, both interventions promoted on day 16 a shift to the Th1 response as shown by the Th1/Th2 IgG ratio (Figure 3B).

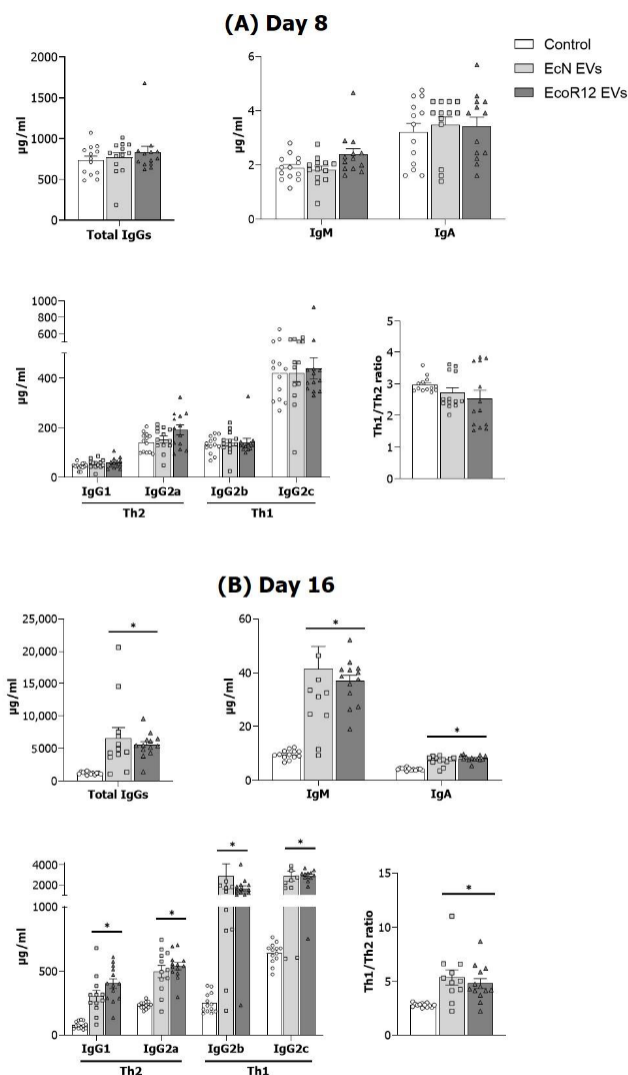


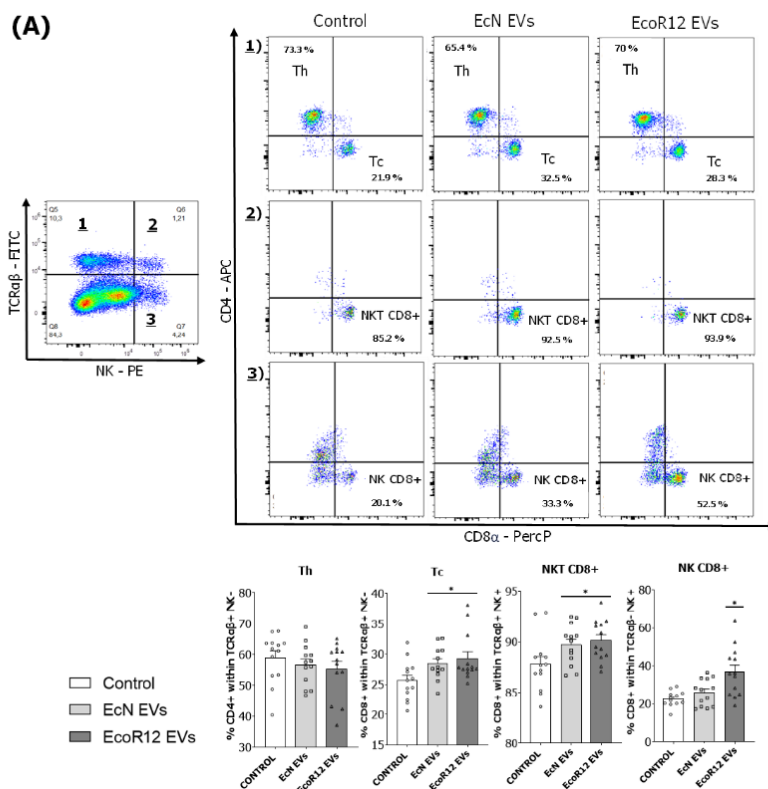
Figure 2. Effect of the interventions with EcN and EcoR12 EVs on the Ig levels in plasma on days 8 (A) and 16 (B). The three experimental groups were: Control (white bars), EcN EVs (light gray bars), and EcoR12 EVs (dark gray bars). The Th1/Th2 Ig ratio applies to the relation $(\text{IgG2b} + \text{IgG2c})/(\text{IgG1} + \text{IgG2a})$. Results are expressed as $\pm$ SEM ($n = 12$ animals/group at both days 8 and 16). Statistical differences: * $p < 0.05$ compared to control (by post hoc Dunn's multiple comparison test).

3.3. Analysis of the Spleen Lymphoid Subsets

The relative percentages of splenic lymphocyte subsets were assessed on day 16 (Figure 3). No significant differences in the proportion of total B and T lymphocytes were apparent among the three experimental groups. However, the suckling rats administered daily with EcN EVs or EcoR12 EVs showed higher percentages of cytotoxic T cells (TCR $\alpha\beta$ + CD8+ NK $^-$) ($p < 0.05$) and NKT cells (TCR $\alpha\beta$ + CD8+ NK $^+$) ($p < 0.05$) in comparison to the control group (Figure 3A). Also, the percentage of NK cells (NK+ TCR $\alpha\beta$ $^-$) expressing CD8 was specifically elevated by the intervention with EcoR12 EVs (Figure 3A).

Interestingly, both interventions raised the proportion of the TCR $\gamma\delta$ T cell subset ($p < 0.05$), principally the CD8+ subpopulation ($p < 0.05$ for the EcoR12 EVs group, while there was a tendency for the EcN EVs group) (Figure 3B). These results showed that the administration of EVs isolated from probiotic and commensal *E. coli* strains to neonatal rats stimulates the innate and adaptive immune systems by increasing the proportion of cytotoxic lymphocytes, which are important to fight against intracellular pathogens.

Moreover, both interventions (EcN EVs and EcoR12 EVs) tend to increase the proportion of Treg cells (CD4+, CD25+ FoxP3+) compared to the control group. Particularly, differences were statistically significant for the EcoR12 EVs group (Figure 3C).



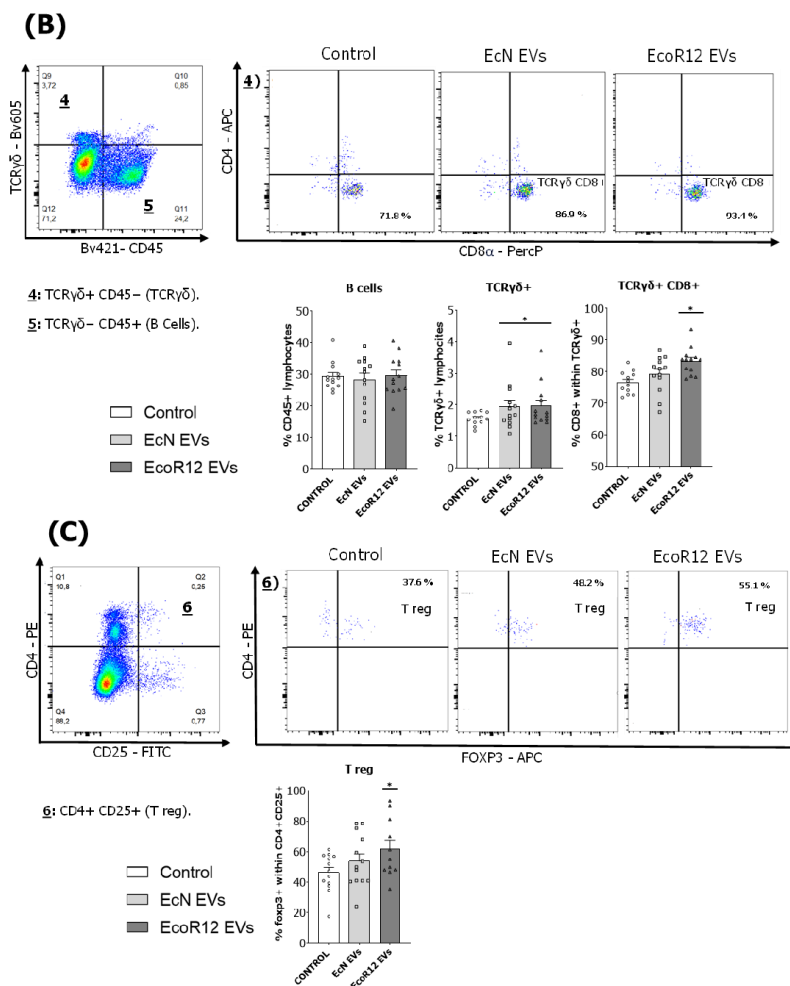


Figure 3. Effect of the interventions with EcN and EcoR12 EVs on the main spleen lymphocyte subsets. Flow cytometric analysis of lymphocytes isolated from spleen samples on the last day of the experimental period (day 16). The analyzed populations were: (A) Th cells (TCRαβ⁺ CD4⁺ NK⁻), Tc cells (TCRαβ⁺ CD8⁺ NK⁻), NKT cells (TCRαβ⁺ NK⁺, classified in CD8⁺ and CD8⁻ subsets), and NK cells (TCRαβ⁻ NK⁺, classified in CD8⁺ and CD8⁻ subsets). (B) B cells (CD45RA⁺) and the T subset population expressing the receptor TCR-1 (TCRγδ⁺, classified in CD8⁺ and CD8⁻ subsets). (C) Treg cells (CD4⁺ CD25⁺ FoxP3⁺). Abbreviations: Th, T-helper; Tc, T-cytotoxic; NK, natural killer; NKT, natural killer T; Treg, regulatory T cells. Representative dot plots for the three experimental groups (control, EcN EVs, and EcoR12 EVs) are shown for the analysis of each lymphocyte subtype gate. The percentage of positive cells is indicated in each quadrant or box. Results, presented in bar diagrams, are expressed as mean ± SEM (*n* = 12/group). Statistical differences: * *p* < 0.05 compared to the control (by post hoc Dunn's multiple comparison test).

3.4. Intestinal Gene Expression Analysis

To further explore the impact of microbiota/probiotic EVs on the intestinal function, the expression levels of genes related to the immune system (*TLR2*, *TLR7*, *IGA*, *CD68*), intestinal maturation (*FcRn*), inflammation (*IL12*, *COX-2*), and gut barrier integrity/permeability (*MUC2*, *ZO-1*, and *OCN*) were measured in the small intestine on days 8 and 16 (Figure 4).

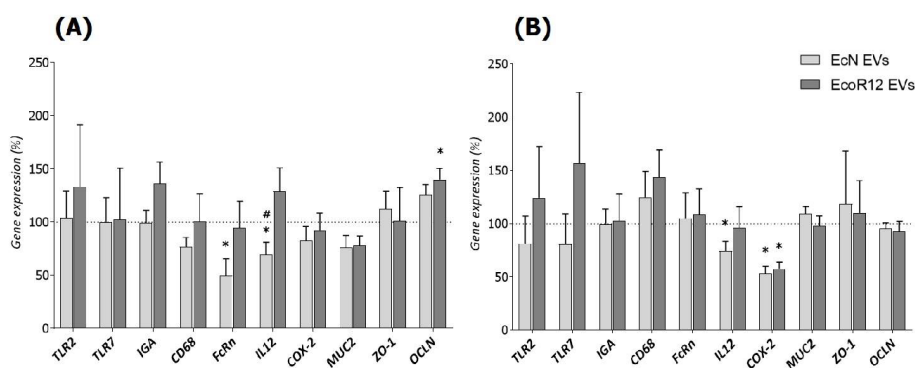


Figure 4. Modulation of gene expression by EcN and EcoR12 EVs in the small intestine of neonatal rats. The expression of genes related with intestinal function including markers of the immune system, maturation, inflammation, and barrier integrity was assessed by RT-qPCR at two points of the experimental period: (A) day 8 and (B) day 16. The relative expression in the interventional groups (EcN EVs, light gray bars; EcoR12 EVs, dark gray bars) was calculated with respect to the control group, which was assigned 100% transcription (indicated by a dotted line). Results are expressed as mean $\pm$ SEM ($n = 12$ /group). Statistical differences: * $p < 0.05$ compared to control, # $p < 0.05$ between interventions (by post hoc Dunn's multiple comparison test). Abbreviations: TLR, Toll-like receptor; IGA, immunoglobulin A; CD68, cluster of differentiation 68; FcRn, neonatal constant fragment receptor; IL12, interleukin-12; COX-2, cyclooxygenase-2; MUC2, mucin 2; OCLN, occludin; ZO-1, zonula occludens-1.

Concerning immune-related genes, the administration of EcN or EcoR12 EVs did not significantly modify their expression. However, there was a tendency toward higher expression of *TLR2* and *TLR7* in the EcoR12 EVs group on day 16. Another gene related to the adaptative immunity that reflects the maturation state of the neonatal intestine is *FcRn*. The encoded protein acts as a receptor that allows the uptake of maternal IgG by the immature neonatal intestinal epithelium [11]. Notably, neonatal rats in the EcN EVs group showed lower *FcRn* expression levels ($p < 0.05$) on day 8 of life but levels comparable to the control group on day 16 of life. In line with the anti-inflammatory effects of the probiotic EcN, the administration of EcN EVs significantly reduced *IL12* ($p < 0.05$) expression at both times. Moreover, both interventions (EcN EVs and EcoR12 EVs) also significantly downregulated the proinflammatory enzyme COX-2 on day 16. Finally, considering the vesicle effects on genes involved in intestinal barrier integrity, both interventions increased occludin expression on day 8, with statistically significant differences observed for EcoR12 EVs ($p < 0.05$). On day 16, *OCN* mRNA levels were comparable to those of the control group. At both intervention times, no significant differences in *ZO-1* or *MUC2* mRNA levels were observed between control and EV-treated groups.

3.5. Intestinal Histomorphometry

The effects of microbiota EVs on the intestinal morphology of neonatal rats were assessed on days 8 and 16 (Figure 5).

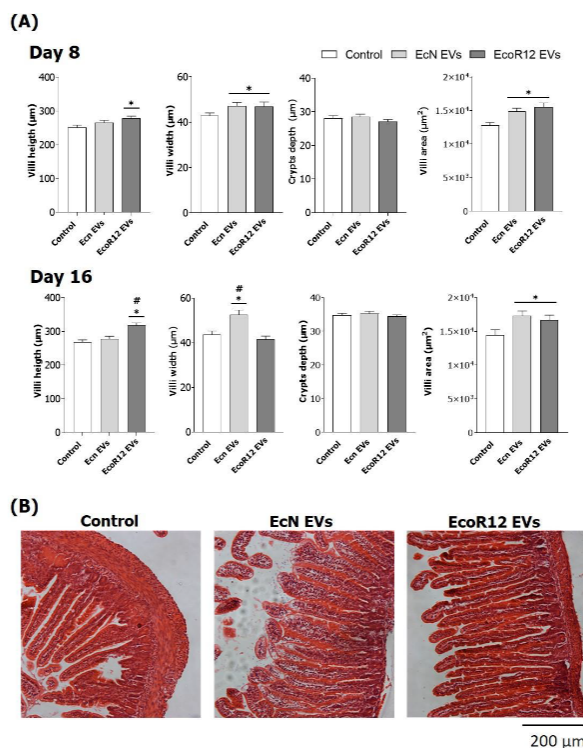


Figure 5. Effect of the interventions with EcN and EcoR12 EVs on intestinal histomorphometry variables on days 8 and 16. **(A)** Crypt depth and villi height, width, and area were measured with ImageJ v1.53t. At least 10 villi per animal were recorded. Results are expressed as mean $\pm$ SEM ($n = 12$ /group). Statistical differences: * $p < 0.05$ compared to the control; # $p < 0.05$ between interventions (by post hoc Bonferroni test). **(B)** Representative images of distal jejunum sections of animals (day 16) stained with hematoxylin and eosin (10 $\times$) are shown.

Pups receiving daily EcN EVs displayed significantly greater villi width and villi area than the control ($p < 0.05$) at both time points. The intervention with EcoR12 EVs also promoted intestinal morphological changes. On day 8, animals receiving EcoR12 EVs showed significantly higher villi width, length, and area values compared to the control group ($p < 0.05$). As the newborn intestine developed (day 16), the increase in the villi height and area was sustained in EcoR12 EVs animals ($p < 0.05$). However, the villi width did not differ from that of the control group. None of the interventions influenced crypt depth.

4. Discussion

Neonate mammals are born with immature intestinal function and immunity. The immature small intestine has altered morphology and barrier properties. The high permeability of the neonatal intestine involves enhanced paracellular and transcellular passage of macromolecules. The transfer of microbial antigens contributes to the development and training of the newborn immature immune system, whereas the transfer of maternal milk IgA and IgGs provides immune protection during the neonatal period [10].

Studies using germ-free animals prove that colonization by gut microbiota plays a key role in intestinal maturation in neonates, affecting both the intestinal architecture and the immune system [13,39,40]. Although the diverse microbiota effects on the development and function of the intestinal barrier and immune system are well known, the molecular actors and mechanisms are not fully understood, particularly in the neonatal period. In the gut, interactions between the microbiota and host epithelial/immune cells are complex. Recent evidence indicates that interkingdom crosstalk in the gut primarily depends on multiple bioactive molecules and factors secreted by the gut microbiota [2,13]. In this context, EVs released by gut-resident microbes serve as vehicles for the secretion and delivery of bacterial effectors to host cells in a protected milieu provided by the vesicle membrane. Numerous studies in adult animals and cellular models of mature intestinal function have confirmed that microbiota EVs mediate the specific effects of gut-resident bacteria on host immune and defense responses [17]. Based on our previous results showing the immunomodulatory and barrier-strengthening activity of EVs from intestinal beneficial *E. coli* strains [33], we hypothesized that in the neonatal period microbiota EVs may contribute to the maturation and development of the newborn immune system.

It is known that at birth, the intestinal maturation stage differs among mammalian species. In particular, rodents are born with an immature gastrointestinal tract, and the development of the functional organ occurs after birth. For this reason, models based on neonatal mice or rats are appropriate for the study of maturation of intestinal barrier function and immunity [41,42].

The present study investigates the influence of interventions involving the oral administration of EVs derived from the probiotic EcN and the commensal EcoR12 on intestinal and immune maturation in the suckling rat model. Daily administration of EVs for two weeks (spanning from day 2 to day 16 of life) did not significantly affect the growth of animals compared to the control group. In general, the interventions did not cause substantial changes in the relative weight of organs (calculated as % of body weight). Nevertheless, the EcN EVs group showed a slight increase in spleen weight, especially on day 8, which might be attributed to enhanced immune activity. However, other factors such as greater irrigation cannot be ruled out. The EcoR12 EVs group displayed a slight enlargement of the empty cecum. Importantly, the stool features were not affected during the interventions, which indicates that administration of microbiota/probiotic EVs during the neonatal period was safe and well tolerated, as evidenced in experimental models with adult animals.

Microbiota EVs are known to influence mucosal and systemic immunity due to their content of numerous MAMPs that engage immune receptors expressed by host epithelial and immune cells of the innate and adaptive immune system [17,43]. Here, we assessed the effects of EVs isolated from beneficial gut resident *E. coli* strains on the humoral and cellular immune responses in neonatal rats. Oral administration of either EcN or EcoR12 EVs promoted a remarkably significant increase in the plasma levels of all Ig types (IgA, IgM, and IgG) after the 16-day intervention period compared to the control group. Overall, these results are in line with the well-known immunostimulatory and immunogenic properties of bacterial vesicles. In fact, bacterial EVs are being explored as native vaccines or vaccine adjuvants. Vaccines based on pathogen-derived EVs are as effective at raising antibody levels as the whole bacterial vaccine [44,45]. Moreover, the use of EVs from non-pathogenic bacterial strains as vaccine adjuvants in immunization processes has yielded promising results. In this context, vaccination with a mixture of bacterial EVs with antigens or EVs loaded with specific antigens triggers robust humoral and cellular immune responses after immunization, without adverse side effects [43,46]. Focusing on the IgG subtypes, our findings demonstrate that both interventions with microbiota EVs promoted a greater increase in Th1-type Igs (IgG2b and IgG2c) than in Th2-type Igs (IgG1 and IgG2a). The immature immune system of the newborn is predominantly directed to a Th2 phenotype, and the immune responses shift towards a Th1 phenotype as the immune system develops [10,47]. Considering this, the increase in the Th1 / Th2 ratio induced by the interventions suggest that microbiota EVs may help immune system maturation in

early life. This observation was supported by the changes induced by EcN and EcoR12 EVs in cellular immunity. Consistent with the increased production of antibodies and the switch to the Th1 phenotype, suckling rats receiving microbiota EVs displayed a higher proportion of splenic cytotoxic Tc, NK, and NKT cells than control animals. The impact of microbiota EVs also increased the proportion of a particular T cell subset expressing TRCγδ, which was considered a primitive immune system that quickly mounts innate responses against pathogens and other injuries [48]. In addition, EVs from the commensal EcoR12 significantly increased the percentage of spleen Treg cells, a fact that is consistent with the previous results of our group, which showed the ability of EcoR12 EVs to activate dendritic cells to drive tolerogenic responses [26]. This Treg expansion in early life is of importance, as tolerance in this period is critical for proper immune development. Overall, the analysis of the spleen lymphoid subsets revealed that interventions with microbiota/probiotic EVs activate balanced immune responses that accelerate immune system maturation in early life, while preserving host tolerance against gut microbes.

It is known that in healthy mammalian adults, microbiota EVs cross the intestinal epithelial barrier and reach systemic circulation [16,49,50]. In the gut, migration of microbiota EVs across the intestinal epithelium is mediated by several pathways including transcellular and paracellular mechanisms. Passage of gut microbiota EVs through the paracellular route is favored under conditions of increased intestinal permeability [51,52]. Thus, the newborn immature intestine should enhance the access of EVs to the antigen-presenting cells of the lamina propria. The stimulation of the neonatal innate immune system by administered microbiota EVs could accelerate immune system development. This is consistent with the results shown in this study.

The results from the gene expression analysis of immune-related genes in intestinal tissue corroborated the immunomodulatory effects of microbiota EVs. Despite considerable variability between individuals within the same group, interventions with EcN EVs and particularly EcoR12 EVs tend to promote upregulation of the immune receptors TLR7 and TLR2, which recognize specific microbial molecules, single-stranded RNA, and bacterial peptidoglycan, respectively, and trigger effective immune responses. By this mechanism, microbiota EVs provide antigens that would contribute to the development and training of the neonatal immune system. However, although microbiota EVs help immune maturation, they do not cause intestinal inflammation, as shown by the mRNA levels of *CD68*, *IL12*, and *COX-2*. The glycoprotein *CD68* is expressed by monocytes and macrophages and is normally used as a histochemical marker of inflammation associated with recruited macrophages [53]. Importantly, interventions with microbiota EVs did not increase *CD68* expression in the intestinal mucosa over control levels. Similarly, none of the interventions upregulated the proinflammatory cytokine *IL-12*. Moreover, EVs from the probiotic EcN specifically exerted a sustained anti-inflammatory effect by downregulating intestinal *IL12* mRNA levels. In this case, significant differences with respect to the control and EcoR12 EVs groups were observed from the first week of intervention and maintained until day 16. Furthermore, animals receiving EcN EVs also displayed reduced intestinal expression of *COX-2* during the intervention period. In the gut, this enzyme is induced in response to inflammatory stimuli and is involved in the production of prostaglandins that support the inflammatory process [54]. Although downregulation of *COX-2* by EcN EVs was already apparent on day 8, EVs from the commensal EcoR12 also reduced *COX-2* mRNA levels on day 16. Overall, these results agree with and support the anti-inflammatory activity of EcN EVs [33]. In addition, they show that interventions with EVs from beneficial gut microbiota potentiate humoral and cellular immunity in early life without causing noticeable inflammatory side effects.

Concerning the barrier properties of the rat immature intestine, one main feature is its high permeability, which allows transfer of Igs, antigens, and bioactive molecules via transcellular endocytosis and paracellular passage. Both routes provide a mechanism to prime immune system development [10]. Accordingly, the neonatal immature intestine displays lower expression of TJ proteins [9] and higher expression of FcRn [11] than the

adult tissue. The maturation of the intestinal epithelial barrier in neonatal rodents has been associated with upregulation of TJ proteins and downregulation of the maternal IgG receptor. In this study, we show that interventions with microbiota EVs influence the expression of these markers towards a mature profile. In particular, the EcoR12 EVs group had significantly more *OCN* expression than the control, and the EcN EVs group had significantly lower *FcRn* mRNA levels. Importantly, differences from the control group were only observed on day 8. On day 16, as the intestinal tract matured in the untreated control animals, the expression of these genes became similar in all groups. These results suggest that interventions with probiotic/microbiota EVs can accelerate intestinal maturation. In this context, the administration of microbiota EVs also seems to promote an intestinal trophic effect by increasing the villi area, primarily due to greater villi width (EcN EVs) or greater villi length (EcoR12 EVs). The positive impact of EV interventions on intestinal development would improve nutrient absorption in immature newborns.

The main advantage of interventions based on probiotic/microbiota EVs, in comparison to live probiotics, lies in the fact that they are non-replicative structures (free from bacteria), yet able to mediate the beneficial effects of the probiotics. This fact avoids the potential risk of bacterial translocation and infection in newborns or immunocompromised patients [55–58]. In this context, probiotic/microbiota EVs could represent a safe postbiotic strategy targeting intestinal immunity and maturation in early life. We are aware that the distinct maturation status of the human and rat neonatal intestine leads to a significant limitation of preclinical assays in suckling rats when transferring the derived results to humans. However, the neonatal rat model could better resemble the gut immaturity of human preterm newborns, a condition that negatively influences digestion, absorption of nutrients, and the pattern of gut microbiota. In turn, this condition contributes to disturbances in neural, functional, and immune development of the intestinal system, which predispose to intestinal complications and infections, such as necrotizing enterocolitis [57–60]. Overall, the findings presented here may suggest that interventions involving probiotic/microbiota EVs could be examined as a postbiotic strategy to prevent intestinal infections and help intestinal maturation to meet adequate nutrient absorption needs in preterm newborns. Also, such interventions could potentially be beneficial for enhancing the immune system and ameliorating allergies in infants or later in life. Clinical trials are needed to prove the benefits and safety of microbiota EV-based interventions in human infants.

5. Conclusions

In the present study, the intervention with probiotic/microbiota EVs in neonatal suckling rats has been shown to be a safe strategy to stimulate immunity and intestinal maturation in the neonatal period. Among the multiple benefits, EcN and EcoR12 EVs: (i) prime the newborn immune system at the cellular and humoral level to better fight against infections, (ii) preserve tolerance against the gut microbiota as depicted by their ability to increase the percentage of spleen Treg cells, (iii) elicit anti-inflammatory effects by reducing *IL12* and *COX-2* expression in the small intestine, (iv) promote intestinal maturation by modulating the expression of genes that affect transcytosis and the paracellular passage of macromolecules across the intestinal epithelium, and (v) promote trophic effects on the developing intestinal morphology by increasing the villi area. To our knowledge, this is the first preclinical assay evaluating the positive effects of microbiota/probiotic EVs on promoting immunity and intestinal maturation in early life.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu15214701/s1>, Table S1: Hematological variables.

Author Contributions: Conceptualization, L.B., J.B. and F.J.P.-C.; methodology, S.M.-R., L.S.-F. and S.C.-C.; formal analysis, S.M.-R. and M.J.R.-L.; investigation, S.M.-R., M.J.R.-L., L.S.-F. and S.C.-C.; data curation, S.M.-R.; writing—original draft preparation, L.B. and S.M.-R.; writing—review and editing, J.B., F.J.P.-C. and M.J.R.-L.; supervision, L.B., J.B. and F.J.P.-C.; funding acquisition, L.B. and J.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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ARTICLE 2

“Microbiota-Derived Extracellular Vesicles as a Postbiotic Strategy to Alleviate Diarrhea and Enhance Immunity in Rotavirus-Infected Neonatal Rats”

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ABSTRACT

Aim: This study investigates the efficacy of EVs derived from probiotic and commensal *E. coli* strains in reducing diarrhea and enhancing immune responses in a preclinical model of RV infection in suckling rats.

Methods: To accomplish this objective pups were supplemented from days 2 to 16 of life, with the EVs or vehicle. At day 5, pups were infected with the RV. Fecal samples were collected during the RV infectious process to calculate the incidence and severity of the RV-induced diarrhea. On days 8 and 16 of life, variables related to humoral and cellular immunity and intestinal function/architecture were assessed.

Results: Both interventions enhanced antiviral immunity by promoting humoral responses (increased serum immunoglobulins) and boosting cellular immunity, as evidenced by higher proportions of splenic natural killer (NK), cytotoxic T (Tc), and TCR $\gamma\delta$ -positive T cells. Additionally, both EV treatments downregulated the expression of the intestinal serotonin receptor 3 (HTR3). Notably, several effects were strain-specific: EVs from *E. coli* EcoR12 upregulated intestinal expression of CD68, TLR2, and IL-12, indicating a pro-inflammatory immune activation, whereas EVs from EcN favored intestinal maturation by enhancing barrier integrity (increased goblet cell numbers and mucin 2 expression) and improving absorptive capacity (villus elongation).

Conclusion: In conclusion, interventions based on probiotic and commensal-derived EVs may represent a safe postbiotic approach to alleviate clinical symptoms and strengthen immune responses during rotavirus infection in early life. Moreover, these EVs hold potential as adjuvants to boost the immunogenicity and effectiveness of anti-rotavirus vaccines.



Article

Microbiota-Derived Extracellular Vesicles as a Postbiotic Strategy to Alleviate Diarrhea and Enhance Immunity in Rotavirus-Infected Neonatal Rats

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Abstract: Rotavirus (RV) infection is a major cause of acute gastroenteritis in children under 5 years old, resulting in elevated mortality rates in low-income countries. The efficacy of anti-RV vaccines is limited in underdeveloped countries, emphasizing the need for novel strategies to boost immunity and alleviate RV-induced diarrhea. This study explores the effectiveness of interventions involving extracellular vesicles (EVs) from probiotic and commensal *E. coli* in mitigating diarrhea and enhancing immunity in a preclinical model of RV infection in suckling rats. On days 8 and 16 of life, variables related to humoral and cellular immunity and intestinal function/architecture were assessed. Both interventions enhanced humoral (serum immunoglobulins) and cellular (splenic natural killer (NK), cytotoxic T (Tc) and positive T-cell receptor $\gamma\delta$ (TCR $\gamma\delta$) cells) immunity against viral infections and downregulated the intestinal serotonin receptor-3 (HTR3). However, certain effects were strain-specific. EcoR12 EVs activated intestinal CD68, TLR2 and IL-12 expression, whereas EcN EVs improved intestinal maturation, barrier properties (goblet cell numbers/mucin 2 expression) and absorptive function (villus length). In conclusion, interventions involving probiotic/microbiota EVs may serve as a safe postbiotic strategy to improve clinical symptoms and immune responses during RV infection in the neonatal period. Furthermore, they could be used as adjuvants to enhance the immunogenicity and efficacy of anti-RV vaccines.

Keywords: rotavirus; extracellular vesicles; microbiota–host communication; probiotics; postbiotics; immunomodulation; intestinal serotonin

1. Introduction

Rotavirus (RV) is a non-enveloped, double-stranded RNA (dsRNA) member of the Reoviridae family that infects enterocytes in the tip of the small intestine, altering fluid secretion and absorptive function [1,2]. RV infection is a major cause of acute gastroenteritis in children under 5 years old worldwide, and is associated with high rates of mortality, principally in low-income countries, caused by excessive loss of fluids through severe diarrhea and vomiting [3,4]. Several non-inflammatory mechanisms contribute to RV-induced watery diarrhea, which include imbalances in intestinal osmosis derived from the loss of absorptive cells, effects of RV enterotoxins on chloride secretion, and activation of the enteric nervous system and neurotransmitters, such as serotonin (5-hydroxytryptamine, 5-HT) [1,5,6].

Serotonin is a crucial mediator of gut functions, having autocrine and paracrine actions acting on several receptors. In the gut, serotonin modulates vagal reflexes, gut motility and barrier permeability [7]. More than 90% of the body's serotonin is produced by the enterochromaffin cells of the intestinal epithelium from dietary tryptophan, which is converted into 5-hydroxytryptophan by the enzyme tryptophan hydroxylase 1 (TPH1) and subsequently transformed into serotonin by a decarboxylase reaction. Free intestinal serotonin levels are controlled by the serotonin transporter (SERT), located at the apical and the basolateral sides of the cell membrane. Once released, extracellular serotonin can be taken up by intestinal epithelial cells through the SERT, and further inactivated by monoamine oxidase, the first enzyme of the serotonin degradation pathway. Serotonin exerts its effects by interacting with specific receptors of the surrounding epithelial, immune and neural cells. There are several families of 5-HT receptors (HTR), and five of them are expressed in the gut [7]. There is evidence that RV activates serotonin secretion via the enterochromaffin cells of the small intestine and that HTR3 is involved in the RV-derived activation of vomiting and diarrhea [6,8,9].

Immunity against RV infection involves innate and adaptive responses. Viral antigens are presented to B and T lymphocytes by dendritic cells and macrophages. Natural killer (NK) lymphocytes are the first line of defense against the virus, and T cytotoxic (Tc) cells also help lysis of infected cells. Finally, B cells produce antibodies (Ab) that confer long-term protection. Immunoglobulin (Ig) A seems to have a critical role in the establishment of immunity against RV infection [10–12]. Although the introduction of oral RV vaccines into global vaccination programs has improved the health burden of RV diarrhea in children, their implementation and efficacy in underdeveloped countries is still limited. In these countries, challenges include the high costs of vaccination programs and the reduced efficacy of the vaccines, most likely due to low standards of hygiene, suboptimal breastfeeding, malnutrition and derived gut microbiota dysbiosis [13–16]. For this reason, new strategies to enhance immunity against both RV infection and vaccines or to ameliorate RV-induced diarrhea are needed.

In this context, interventions with probiotic strains of the *Bifidobacterium*, *Lactobacillus* and *Escherichia* genera have been explored in several neonatal animal models (mouse, rat, piglet) to prove their efficacy in improving immunity against RV and ameliorating diarrhea and clinical markers [17–23]. Comparative studies revealed that the probiotic *E. coli* Nissle 1917 (EcN) is more effective than Gram-positive probiotics in enhancing protective immunity against RV [24].

The probiotic EcN is a good colonizer of the human gut and positively influences gastrointestinal homeostasis and microbiota balance [25,26]. Clinical trials have proved its therapeutic benefits in the remission of inflammatory bowel diseases [27] and acute diarrhea in children [28]. In preclinical assays using gnotobiotic neonatal pigs, colonization with EcN has been shown to efficiently protect against RV infection through several mechanisms that involve the protection of the intestinal epithelial barrier, stimulation of the innate immune system and interference with pathogen binding to intestinal epithelial cells [18,21,22]. Recent studies also prove the beneficial effects of EcN against RV-induced diarrhea and immune responses in a neonatal malnourished pig model colonized with human infant fecal microbiota [29,30]. In addition, colonization of gnotobiotic pigs with probiotic EcN enhances the immunogenicity and protective efficacy of an oral attenuated RV vaccine [31].

Although probiotics are generally recognized as safe (GRAS) biological agents or Qualified Presumption of Safety (QPS) according to the European Food Safety Authority, some concerns about the potential risk of bacterial translocation should be considered, especially in critically immunocompromised individuals and neonates [32,33]. In this context, postbiotics are now being considered as a new health-promoting strategy. Postbiotics are probiotic- or microbiota-derived components that confer a health benefit on the host, including bacterial cell fragments, biomolecules and secreted bioactive compounds [34]. This approach overcomes the disadvantages associated with the administration of live

bacteria. Recent studies show that postbiotics could improve intestinal homeostasis and prevent enteric infections [35,36].

Considering the definition provided by the International Scientific Association for Probiotics and Prebiotics [34], bacterial extracellular vesicles (EVs) fit the postbiotic definition as they are cell-inanimate structures, produced from the bacterial cell membrane, that cannot replicate and mediate beneficial effects on the host. In general, bacterial EVs are spherical membrane-derived nanostructures (typically ranging from 25 to 300 nm in diameter) that contain proteins, lipids, nucleic acids, numerous surface and cytosolic biomolecules, and metabolites produced from their progenitor bacteria. The vesicle components depend on the producer strain, its physiological state and environmental conditions [37,38]. Bacterial EVs, as carriers of biological information, are important messengers in interspecies communication, either between members of the same microbial community or between bacteria and the host [39]. In the gut, microbiota-released EVs diffuse through the mucin layer, interact with epithelial and immune cells of the intestinal mucosa and deliver the cargo molecules upon internalization into target cells. The delivered bacterial bioactive molecules activate signaling pathways that control host responses and functions [40]. There is now scientific evidence that EVs mediate the effects of microbiota and probiotic strains on host physiology and health [41]. Moreover, they are able to alleviate metabolic, immune and neurological disorders in experimental models of human diseases [41–43].

Our group has provided new insights into the role of EVs released by the probiotic EcN and commensal *E. coli* strains. We have reported that EVs isolated from gut-resident *E. coli* strains modulate the integrity and repair of the intestinal epithelial barrier at multiple levels [41,44], and elicit immunomodulatory effects on dendritic cells and derived T cell responses. We have shown that EVs are a central mechanism used by the gut microbiota to steadily prime the immune system in a strain-specific manner through mechanisms that involve, at least in part, regulation of miRNAs and immunomodulatory molecules exported through exosomes released by dendritic cells [45,46]. In addition, we have recently reported that nutritional interventions based on the oral administration of EVs from the probiotic EcN or the commensal EcoR12 in neonatal suckling rats stimulate immunity and intestinal maturation in the neonatal period. Pups receiving EVs displayed higher levels of all plasma Ig types and a greater proportion of Tc, NK and NKT cells in the spleen [47].

Considering this finding together with previous results showing the ability of EcN EVs to both activate dendritic cells towards a prominent T helper 1 (Th1) response [45] and protect the intestinal epithelial barrier against enteropathogenic *E. coli* (EPEC) infection [48], the present study aimed to explore the effectiveness of interventions involving EVs from the probiotic EcN to protect against RV infection in a neonatal rat model. A parallel intervention with EVs from the commensal EcoR12 was carried out to test for strain-specific effects. We assessed the capacity of probiotic/microbiota EVs to ameliorate diarrhea severity and incidence, and to enhance cellular and specific humoral responses.

2. Results

2.1. Evaluation of Body Weight and Morphometric Variables

Infection with RV had no significant impact on animal growth as deciphered by the body weight, body mass index and body height values. The growth parameters were not influenced by the interventions involving EcN EVs or EcoR12 EVs (Figure S1 and Table S1).

Regarding the relative weight of organs, we centered the analysis on immune-related organs (thymus and spleen) and intestinal tissues (small intestine and cecum) at the active diarrhea period (day 8) and at the end of the study (day 16). Differences between groups were only observed on day 8 (Table 1). RV-infected animals exhibited higher spleen weight values than control animals on day 8. Remarkably, differences were statistically significant in the RV + EV-EcN and RV + EV-EcoR12 groups ($p < 0.05$) and close to significance in the RV group ($p = 0.08$).

Table 1. Morphometric variables of intestinal and immune-related organs for the indicated experimental groups. Data corresponding to relative organ weight (g) or length (cm) are presented as percentage of animal body weight (%). The groups were: CON: control group, RV: rotavirus group, RV + EV-EcN: rotavirus group receiving EcN EVs, and RV + EV-EcoR12: rotavirus group receiving EcoR12 EVs.

Day 8	CON	RV	RV + EV-EcN	RV + EV-EcoR12
Intestine weight	3.19 ± 0.06	3.43 ± 0.06	3.41 ± 0.05	3.57 ± 0.11
Intestine length	231.57 ± 7.39	210.64 ± 6.5	226.35 ± 6.42	231.95 ± 10.04
Cecum weight	0.19 ± 0.04	0.20 ± 0.04	0.21 ± 0.04	0.21 ± 0.04
Thymus weight	0.29 ± 0.01	0.30 ± 0.01	0.33 ± 0.03	0.29 ± 0.02
Spleen weight	0.48 ± 0.02	0.59 ± 0.02	0.61 ± 0.04 *	0.60 ± 0.03 *
Day 16	CON	RV	RV + EV-EcN	RV + EV-EcoR12
Intestine weight	2.94 ± 0.04	3.03 ± 0.05	3.10 ± 0.08	2.97 ± 0.05
Intestine length	126.08 ± 5.51	119.74 ± 3.04	128.97 ± 7.26	134.07 ± 4.31
Cecum weight	0.21 ± 0.01	0.21 ± 0.01	0.25 ± 0.02	0.24 ± 0.02
Thymus weight	0.52 ± 0.01	0.50 ± 0.01	0.51 ± 0.02	0.51 ± 0.02
Spleen weight	0.44 ± 0.02	0.50 ± 0.03	0.47 ± 0.03	0.52 ± 0.03

Results are expressed as mean ± SEM ($n = 12$ animals/group). * $p < 0.05$ compared to CON (by post hoc Dunn's multiple comparison test).

2.2. Clinical Evaluation of Diarrhea

RV-induced diarrhea was assessed from day 4 (the day before RV inoculation) until day 11 (last day of diarrhea), considering several variables. The incidence, calculated as the percentage of animals displaying diarrhea (% DA), showed that in the RV group, there was a two-peak shape evolution with maximum % DA on days 6 and 7 (40%), and later, on day 9 (50%). On day 11, none of the animals in the RV group showed diarrhea (Figure 1A). Biphasic disease profiles for DA and diarrhea index (DI) values with two pics on days 2 and 4 post-inoculation have already been described for this experimental model [49]. Also, studies in infant mice reported two peaks of RV replication at the same days post-infection [50]. The interventions involving EcN EVs or EcoR12 EVs were able to reduce the proportion of animals with diarrhea with few differences between them. Consistently, the area under the curve (AUC) of the incidence (iAUC) curve over the evaluation period was significantly lower for the interventional groups (RV + EV-EcN and RV + EV-EcoR12) compared to the RV group (Figure 1B). In addition, both interventions reduced other incidence parameters, such as the maximum percentage of diarrheic animals (MDA) and the maximum percentage of diarrheic feces (MDF), a value referred to the total number of fecal samples per day in each group (Table 2).

Table 2. Clinical variables of the diarrhea process from day 4 to day 11 of life. Results are expressed as mean ± SEM ($n = 12$ –24 animals/group). Statistical differences: # $p < 0.05$ compared to the RV group (by post hoc Dunn's multiple comparison test). The Chi squared test was used for statistical analysis of the incidence variables MDA and MDF.

Clinical Outcome	Variable ¹	RV	RV + EV-EcN	RV + EV-EcoR12
Incidence	MDA	53.85	21.74	23.08
Incidence	MDF	77.77	36.36	50.00
Severity	MDI	2.15 ± 0.12	1.71 ± 0.16 #	1.76 ± 0.15 #
Duration	DP	2.06 ± 0.28	1.75 ± 0.41	1.25 ± 0.25 #
Duration	DwD	1.88 ± 0.22	1.63 ± 0.32	1.10 ± 0.08 #

¹ MDA, maximum percentage of diarrheic animals; MDF, maximum percentage of diarrheic feces; MDI, maximum diarrhea index; DP, diarrhea period; DwD, days with diarrhea.

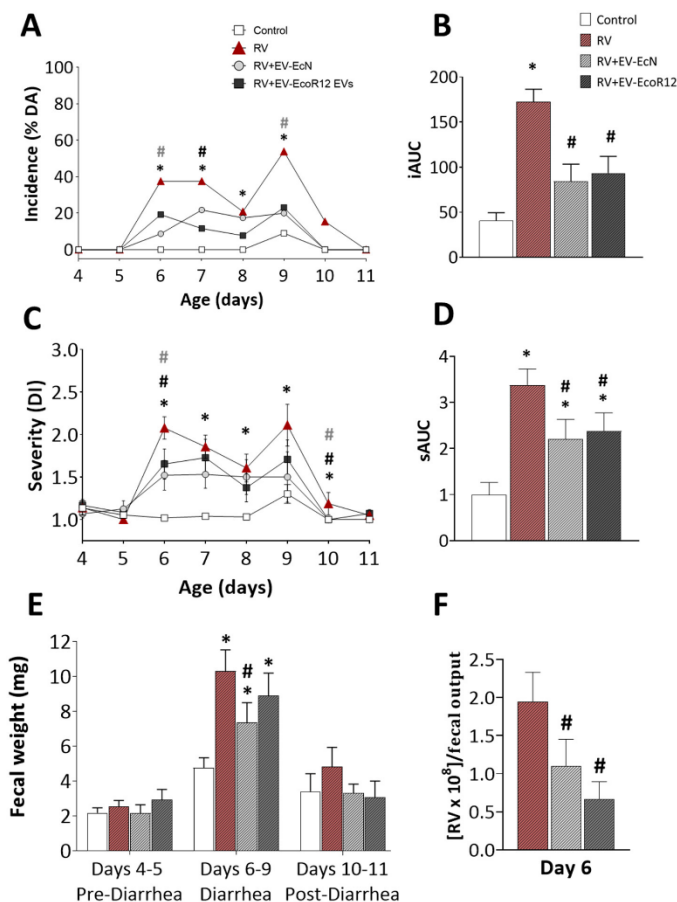


Figure 1. Incidence and severity of RV-induced diarrhea. The clinical variables (A) percentage of diarrheic animals (%DA), (C) diarrhea index (DI) and (E) fecal weight (mg) were evaluated from day 4 (the day before RV inoculation) until day 11 (end of the diarrhea period). (B,D) The area under the curve (AUC) is shown for the DA and DI graphs (iAUC and sAUC, respectively). (F) Viral shedding in feces, expressed as the number of SA-11 particles, referred to the fecal samples pooled on day 6. Viral particles were undetected in feces of control animals. The experimental groups were CON: control group, RV: rotavirus group, RV + EV-EcN: rotavirus group receiving EcN EVs, and RV + EV-EcoR12: rotavirus group receiving EcoR12 EVs. Results are expressed as the mean $\pm$ SEM ($n = 12$ animals/group for days 9–11; $n = 24$ animals/group for days 4–8). Statistical differences: * $p < 0.05$ compared to CON, # $p < 0.05$ compared to the RV group (by post hoc Dunn's multiple comparison test). The Chi squared test was used for statistical analysis of DA% in (A).

The severity of diarrhea was assessed by two approaches: the diarrheic index (DI) and the fecal weight. Inoculation of rotavirus SA-11 resulted in mild diarrhea, with maximum DI values ranging from 2.25 to 3. Both interventional groups, RV + EV-EcN and RV + EV-EcoR12, displayed an overall reduction in DI values compared to the RV group (Figure 1C). For both interventional groups, differences were statistically significant ($p < 0.05$) on day 6 (one day after the RV inoculation). Globally, values of the AUC calculated from the DI

plot revealed that both interventions significantly reduced the diarrhea score ($p < 0.05$) (Figure 1D). Improvement of the diarrhea severity in animals receiving EcN or EcoR12 EVs was also evidenced by their maximum diarrhea index (MDI), which was significantly lower than that of the RV group (Table 2).

The fecal weight was used as an objective variable to characterize the severity of diarrhea (Figure 1E). As expected, no significant differences in the fecal weight output between the CON and RV groups were observed before RV inoculation (days 4–5). The weight of the fecal output during the following four post-infection days, coincident with the acute diarrhea period (days 6–9), was higher in all infected groups compared to the CON animals. However, the intervention with EcN EVs reduced the fecal weight during the acute diarrhea period ($p < 0.05$) compared to the RV group. Similarly, the fecal weight tended to decrease in the RV + EV-EcoR12 group compared to the RV group. In the post-diarrhea period (days 10–11), all groups behaved similarly as diarrhea was already solved.

Regarding the duration of diarrhea, the clinical symptoms started on day 6. The diarrhea period (DP) and the number of days with diarrhea (DwD) were significantly reduced ($p < 0.05$) by the intervention with EcoR12 EVs (Table 2).

2.3. Viral Shedding and In Vitro Blocking Assay

Quantification of RV particles in stool samples was carried out on day 6, according to previous reports showing that, in the suckling rat model, the maximum SA-11 clearance occurs on the first day after inoculation [19]. Consistent with these findings, a high number of SA-11 particles in feces was detected in the RV group (Figure 1F). The number of fecal viral particles was significantly reduced by the interventions involving either EcN EVs or EcoR12 EVs ($p < 0.05$).

It was examined whether lower counts of SA-11 particles in the feces of animals administered EcN or EcoR12 EVs could be attributed to an indirect interference caused by vesicle binding to virus particles (Figure S2). Pre-incubation of SA-11 with different concentrations of EVs isolated from EcN or EcoR12 did not cause any blocking effect in virus detection. This finding ruled out any interference with the immunological quantification of SA-11 in feces.

2.4. Systemic Humoral and Anti-RV Antibody Response

The analysis of the systemic humoral response revealed that RV infection, and specially the interventions with EcN and EcoR12, induced changes in the levels of total circulating Ig (Figure 2A). RV infection affected plasma IgM levels, which were higher than those of the control animals, both on day 8 and day 16 ($p < 0.05$). Although no significant changes in total plasma IgG or IgA levels were observed in the RV group compared to CON, infection with RV increased the Th1/Th2 ratio ($p < 0.05$), estimated from the concentration of the different IgG subtypes (IgG2b + IgG2c/IgG1 + IgG2a) (Figure 2B,C). Minor changes in the levels of IgG subtypes may account for the immune response being biased towards the Th1 phenotype, which is essential to fight against infection. Remarkably, the interventions involving EcN or EcoR12 EV promoted a highly significant increase in the plasma levels of IgM, IgG and all the IgG subtypes compared to the CON and RV groups in both the acute and post-diarrhea periods (days 8 and 16, respectively). Regarding changes in the IgA levels caused by the interventions, differences versus CON and RV were only statistically significant on day 16 ($p < 0.05$) (Figure 2A).

Administration of EcN or EcoR12 EVs stimulated the humoral immune response to better fight against viral infections, as deciphered from the values of the Th1/Th2 Ig ratio (Figure 2C). On day 8, animals in the RV + EV-EcN and RV + EV-EcoR12 groups exhibited Th1/Th2 Ig ratios similar to those of the RV group, whereas on day 16, the ratio values were significantly higher in both interventional groups ($p < 0.05$ vs. RV group). Particularly, EcN-EV-administered animals displayed greater ratio values than EcoR12-EV-treated animals ($p < 0.05$). Although all the IgG subtypes were increased by the interventions, changes

predominantly affected the Th1 isotypes (around an eight-fold increase for IgG2b and a four-fold increase for IgG2c vs. the RV).

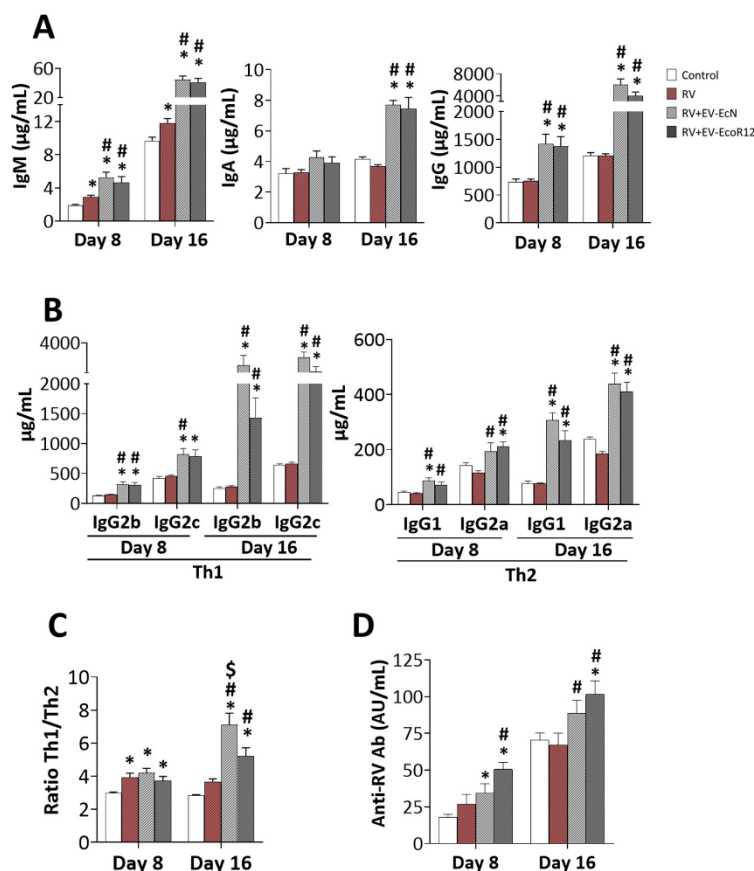


Figure 2. Quantification of anti-RV antibodies and immunoglobulins in plasma on days 8 and 16. The experimental groups are CON: control group, RV: rotavirus group, RV + EV-EcN: rotavirus group receiving EcN EVs, and RV + EV-EcoR12: rotavirus group receiving EcoR12 EVs. (A) Concentration of total IgG, IgM and IgA in plasma (μg/mL). (B) Concentration of IgG subtypes in plasma (μg/mL). (C) The Th1/Th2 Ig ratio refers to the relation (IgG2b + IgG2c)/(IgG1 + IgG2a). (D) The concentration of specific anti-RV Ab (IgG + IgA + IgM) is given in Arbitrary Units/mL. Results are expressed as $\pm$ SEM ($n = 12$ animals/group on both day 8 and day 16). Statistical differences: * $p < 0.05$ compared to CON, # $p < 0.05$ compared to the RV group, \$ $p < 0.05$ between RV-EV-EcN and RV-EV-EcoR12 groups (by post hoc Dunn's multiple comparison test).

To evaluate the impact of the interventions involving EcN EVs or EcoR12 EVs on the anti-RV humoral response, the plasma concentration of total anti-RV Ab (IgG + IgA + IgM) was measured on day 8 and on day 16 (Figure 2D). On day 8 (active diarrheic period), animals in the RV group exhibited a slight increase (not statistically significant) in the level of circulating anti-RV Ab compared to the CON group. This difference was no longer

observed on day 16 (end of the study). Importantly, the interventions with EcN EVs or EcoR12 EVs, although displaying less diarrhea, stimulated the specific anti-RV humoral response. The RV + EV-EcN and RV + EV-EcoR12 groups showed higher levels of anti-RV Ab than the CON and RV groups both on day 8 ($p < 0.05$ vs. CON and RV + EV-EcoR12 also vs. RV) and on day 16 ($p < 0.05$ vs. CON and RV).

2.5. Cellular Immune Response

The cellular immune response was assessed by measuring the relative proportion of the main splenic lymphocyte subsets on day 16 by flow cytometry. Representative dot plots for each lymphocyte subtype gate are shown in Figure S3. The results presented in Table 3 showed that neither the infection with SA-11 nor the interventions involving EcN EVs or EcoR12 EVs significantly altered the proportion of total B and T lymphocytes compared to untreated CON animals on that day. However, a tendency towards increased proportion of T cells was observed in the RV + EV-EcN and RV + EV-EcoR12 interventional groups.

Table 3. Proportion of spleen lymphocytes on day 16 assessed by flow cytometry analysis. The main populations are numbered (1–6) and highlighted in bold. Th, T helper; Tc, T cytotoxic; TCR, T cell receptor; NK, natural killer; NKT, natural killer T; Treg, T regulatory. Data are expressed as mean $\pm$ SEM ($n = 12$ animals/group). Statistical differences: * $p < 0.05$ compared to the CON; # $p < 0.05$ compared to the RV group (by post hoc Dunn’s multiple comparison test).

Lymphocyte Populations in Spleen (%) ¹	CON	RV	Rv + EV-EcN	Rv + EV-EcoR12
1. B cells (CD45+)	29.30 $\pm$ 1.21	25.17 $\pm$ 2.13	30.73 $\pm$ 1.59	27.81 $\pm$ 2.08
2. TCR$\alpha\beta$+ cells	12.48 $\pm$ 1.03	12.20 $\pm$ 1.57	15.00 $\pm$ 2.01	16.93 $\pm$ 1.75
2.1 Th cells (TCR $\alpha\beta$ + CD4+ NK-)	60.45 $\pm$ 1.42	55.46 $\pm$ 1.31 *	55.16 $\pm$ 1.78 *	54.89 $\pm$ 2.56 *
2.2 Tc cells (TCR $\alpha\beta$ + CD8+ NK-)	25.65 $\pm$ 0.84	30.23 $\pm$ 1.1 *	29.97 $\pm$ 0.8 *	31.72 $\pm$ 2.01 *
2.3 Tc/Th ratio	0.44 $\pm$ 0.02	0.54 $\pm$ 0.05 *	0.55 $\pm$ 0.04 *	0.56 $\pm$ 0.03 *
3. TCR$\gamma\delta$+ cells	1.56 $\pm$ 0.05	1.5 $\pm$ 0.17	1.986 $\pm$ 0.11 * #	2.00 $\pm$ 0.21 * #
3.1 TCR $\gamma\delta$ + CD8+	76.54 $\pm$ 1.01	75.99 $\pm$ 2.43	81.60 $\pm$ 1.77 *	85.43 $\pm$ 1.48 * #
3.2 TCR $\gamma\delta$ + CD8-	8.26 $\pm$ 0.26	9.68 $\pm$ 1.12	7.77 $\pm$ 0.59	6.78 $\pm$ 0.74
4. NKT cells	1.88 $\pm$ 0.1	1.95 $\pm$ 0.28	2.45 $\pm$ 0.36	2.67 $\pm$ 0.19 * #
4.1 NKT CD8+	87.82 $\pm$ 0.75	89.41 $\pm$ 0.8	89.25 $\pm$ 1.24	89 $\pm$ 1.15
4.2 NKT CD8-	5.21 $\pm$ 0.3	4.16 $\pm$ 0.52	5.05 $\pm$ 0.68	4.83 $\pm$ 0.67
5. NK cells	5.13 $\pm$ 0.16	4.21 $\pm$ 0.24	5.03 $\pm$ 0.34	4.11 $\pm$ 0.38
5.1 NK CD8+	22.64 $\pm$ 1.18	28.21 $\pm$ 4.48	34.54 $\pm$ 3.15 * (# $p = 0.09$)	43.78 $\pm$ 3.14 * #
5.2 NK CD8-	36.44 $\pm$ 0.82	38.53 $\pm$ 2.74	34.53 $\pm$ 1.91	31.25 $\pm$ 2.67
6. Treg cells	45.17 $\pm$ 3.48	42.33 $\pm$ 5.11	45.81 $\pm$ 3.46	45.28 $\pm$ 3.40

¹ Results are presented as the percentage of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FCs) and side-scatter characteristics (SSCs) (Figure S3) or in a particular main lymphocyte population.

Remarkably, the relative percentage of spleen T $\alpha\beta$ cytotoxic (Tc) cells (TCR $\alpha\beta$ + CD8+ NK-) was increased in all RV-infected groups independently of whether they were treated or not with microbiota EVs ($p < 0.05$ vs. CON). In contrast, the three infected groups showed a minor proportion of Th cells (TCR $\alpha\beta$ + CD4+ NK-). Consistently, the ratio between the spleen Tc/Th cells was higher in the RV-infected groups.

In RV-infected pups, the interventions with EcN EVs or EcoR12 EVs specifically raised the relative percentage of TCR $\gamma\delta$ + ($p < 0.05$ vs. CON and RV), particularly in the CD8+ subpopulation. Moreover, the suckling rats in the two interventional groups exhibited higher proportions of NKT CD8+ and NK CD8+ than those in the CON and RV groups. When compared to the RV group, differences were statistically significant for the RV + EV-EcoR12 ($p < 0.05$) group and close to significance for the RV + EV-EcN ($p < 0.09$) group. Importantly, for NK CD8+ cells, differences between RV + EV-EcN and RV were

significant ($p < 0.05$) if Dunn's multiple test was applied to the three infected experimental groups (RV, RV + EV-EcN and RV + EV-EcoR12), a finding that reinforces the influence of EcN EVs on the differentiation of this lymphocyte subpopulation. No significant differences in the proportion of spleen Treg cells were observed between groups.

2.6. Gene Expression Analysis in Small Intestine

To further examine the effect of the interventions involving EcN and EcoR12 EVs on the intestinal function of RV-infected animals, the expression of several genes involved in host immune responses against infection (TLR2, TLR7, IL12, IGA and CD68), mucin production (MUC2) and intestinal maturation (neonatal constant fragment receptor (FcRn)) was measured with reverse transcription-quantitative PCR (RT-qPCR) in the small intestine on days 8 and 16 (Figure 3).

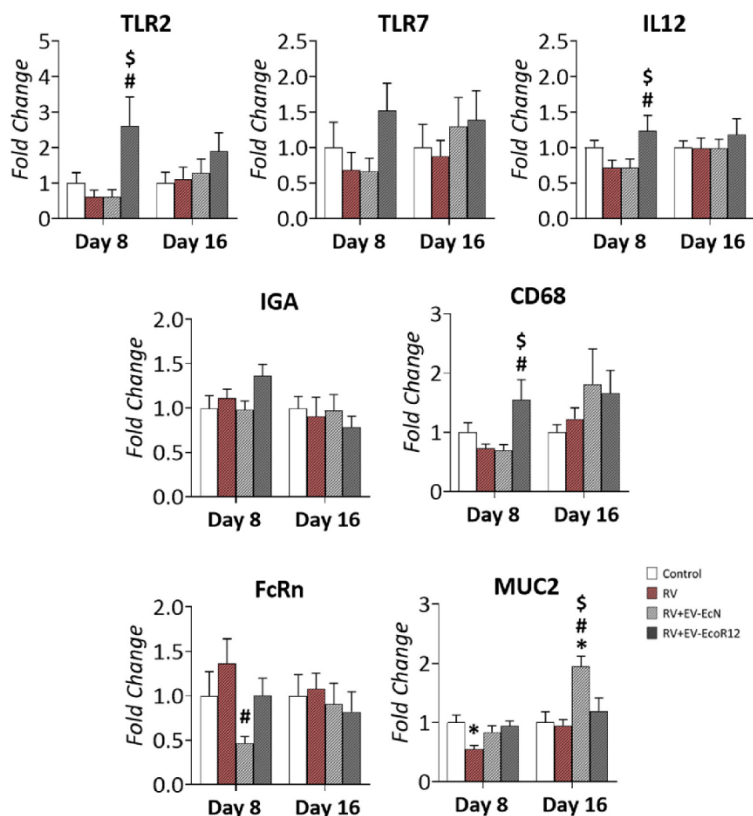


Figure 3. Gene expression analysis in small intestinal tissue collected on days 8 and 16. The experimental groups were CON: control group, RV: rotavirus group, RV + EV-EcN: rotavirus group receiving EcN EVs, and RV + EV-EcoR12: rotavirus group receiving EcoR12 EVs. The transcription levels of the indicated genes were quantified by RT-qPCR in jejunum samples. Relative mRNA levels in the interventional groups were calculated with respect to the CON group (expression value set to

1 at each age). Results are expressed as $\pm$ SEM ($n = 12$ animals/group on both day 8 and day 16). Statistical differences: * $p < 0.05$ compared to CON, # $p < 0.05$ compared to the RV group, \$ $p < 0.05$ between RV-EV-EcN and RV-EV-EcoR12 groups (by post hoc Dunn's multiple comparison test). Abbreviations: TLR, Toll-like-receptor; IGA, immunoglobulin A; CD68, cluster of differentiation 68; FcRn, neonatal constant fragment receptor; IL12, interleukin-12; MUC2, mucin 2.

Infection with SA-11 did not cause significant changes in the expression of the genes analyzed. Nevertheless, the interventions with microbiota EVs modified the expression of some genes in a strain-specific manner.

Concerning the immune-related genes, administration of EcoR-12 EVs drastically upregulated the expression of genes encoding the immune receptors TLR2 and TLR7 ($p = 0.095$) and the macrophage glycoprotein CD68 on day 8 compared to untreated (CON) or infected (RV) animals ($p < 0.05$). In contrast, pups in the RV + EV-EcN group exhibited TLR2, TLR7 and CD68 mRNA levels similar to those of the CON and RV groups. In addition, animals receiving EcoR12 EVs displayed higher IL12 mRNA levels than RV-infected animals ($p < 0.05$). In the RV and RV + EV-EcN groups, the IL12 expression levels tended to be lower than the CON group. No differences were observed in the expression of IGA, although a tendency to increase was observed in the RV + EV-EcoR12 group compared to CON and RV. Importantly, the changes induced by EcoR12 EVs in immune-related genes were not apparent on day 16, after infection resolution.

The expression of the gene *FcRn*, a marker of intestinal immaturity, tended to be higher in the RV group compared to CON. Importantly, only the intervention with EcN EVs promoted a significant reduction in *FcRn* mRNA levels compared to the RV group ($p < 0.05$). This effect was not caused by the intervention with EcoR12 EVs. In this interventional group, *FcRn* expression levels did not significantly differ from those of the CON group. Moreover, EVs from the probiotic EcN specifically upregulated *MUC2* expression on day 16 ($p < 0.05$). On day 8, *MUC2* mRNA levels tended to be reduced in the RV group compared to the CON, but preserved, close to the control levels, in both interventional groups, RV + EV-EcN and RV + EV-EcoR12.

2.7. Intestinal Serotonin (5-HT)

It is known that RV infection induces the release of serotonin, an important mediator of RV-associated diarrhea [6]. To analyze the impact of the interventions involving EcN EVs or EcoR12 EVs on intestinal serotonin levels in RV-infected pups, 5-HT was quantified by ELISA in gut wash samples (Figure 4A).

On day 8, intestinal 5-HT levels were significantly higher in the three RV-infected groups compared to the CON group ($p < 0.05$). Thus, the interventions with EcN or EcoR12 EVs did not prevent RV-induced serotonin release during the acute diarrhea phase. On day 16, all the experimental groups (CON and RV-infected groups) exhibited similar secreted serotonin levels in small intestine-derived samples (Figure 4A).

To further analyze the regulatory mechanisms activated by microbiota EVs, the expression of genes involved in serotonin synthesis (*TPH1*) and transport (*SERT*) was measured with RT-qPCR in small intestinal tissue on day 8 (Figure 4B). No significant differences in the *TPH1* mRNA levels were observed between the four experimental groups, whereas *SERT* expression levels were increased in RV-infected pups administered microbiota EVs. Differences were statistically significant for EcN EVs ($p < 0.05$ vs. CON and RV).

Expression analysis of genes encoding serotonin receptors revealed that the interventions involving EcN EVs or EcoR12 EVs significantly decreased the expression of *HTR3* in RV-infected rats compared to the CON group. The inhibitory effect promoted by EcoR12 EVs was more pronounced, resulting in differences that were statistically significant compared to the RV group. Differences between RV + EV-EcN and RV were close to significant ($p = 0.08$). In contrast, pups in the RV group exhibited *HTR3* mRNA levels that did not statistically differ from those of the CON group (Figure 4B). Concerning the modulation of *HTR4* gene expression, no significant differences were observed between the four experi-

mental groups, although RV and RV + EV-EcoR12 groups displayed a tendency towards lower *HTR4* mRNA levels compared to the CON and RV + EV-EcN groups (Figure 4B).

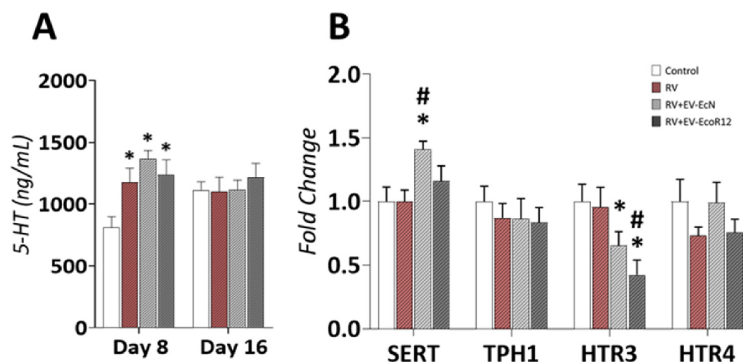


Figure 4. Analysis of serotonin production and regulation by the interventions in RV-infected neonatal rats. The experimental groups were CON: control group, RV: rotavirus group, RV + EV-EcN: rotavirus group receiving EcN EVs, and RV + EV-EcoR12: rotavirus group receiving EcoR12 EVs. (A) Serotonin levels in intestinal wash assessed by ELISA. (B) Gene expression analysis in small intestinal tissue collected on day 8 (diarrhea period) measured by RT-qPCR. The transcription levels of the indicated genes were quantified by RT-qPCR. Relative mRNA levels in the interventional groups were calculated with respect to the CON group (expression value set to 1). Abbreviations: *TPH1*, tryptophan hydroxylase 1; *SERT*, serotonin transporter SLC6A4; *HTR3*, serotonin 5-HT3 receptor; *HTR4*, serotonin 5-HT4 receptor. In both panels, the results are expressed as $\pm$ SEM ($n = 12$ animals/group). Statistical differences: * $p < 0.05$ compared to CON, # $p < 0.05$ compared to the RV group (by post hoc Dunn's multiple comparison test).

2.8. Histological Analysis of Mucosal Morphology and Goblet Cell Numbers

To assess the influence of EV interventions on the RV-induced morphological alterations in the small intestine, jejunum sections collected on day 8 and day 16 were processed for goblet cell staining (Figure 5A). On day 8 (3 days post-infection), the number of goblet cells in the RV group was significantly lower than the CON group ($p < 0.05$). Only the intervention with EVs from the probiotic EcN was able to prevent the reduction in the number of goblet cells in RV-infected animals, yielding similar control numbers. In contrast, the number of goblet cells in the RV + EV-EcoR12 group was similar to that of RV infected animals. On day 16, when the infection was resolved, the number of goblet cells did not differ between the CON, RV and RV + EV-EcoR12 groups. Importantly, significantly higher goblet cell numbers were enumerated in infected pups treated with EcN EVs.

Infection with RV also altered villi morphology, specifically the villus length (Figure 5B). In the RV group, villi became significantly shorter compared to the control group ($p < 0.05$) at both time points. Villus width and crypt depth were not affected by RV infection. The intervention involving EcoR12 EVs did not prevent RV-induced villi atrophy. In contrast, treatment with EcN EVs was capable to preserve the normal villus length in infected animals ($p < 0.05$ vs. RV group).

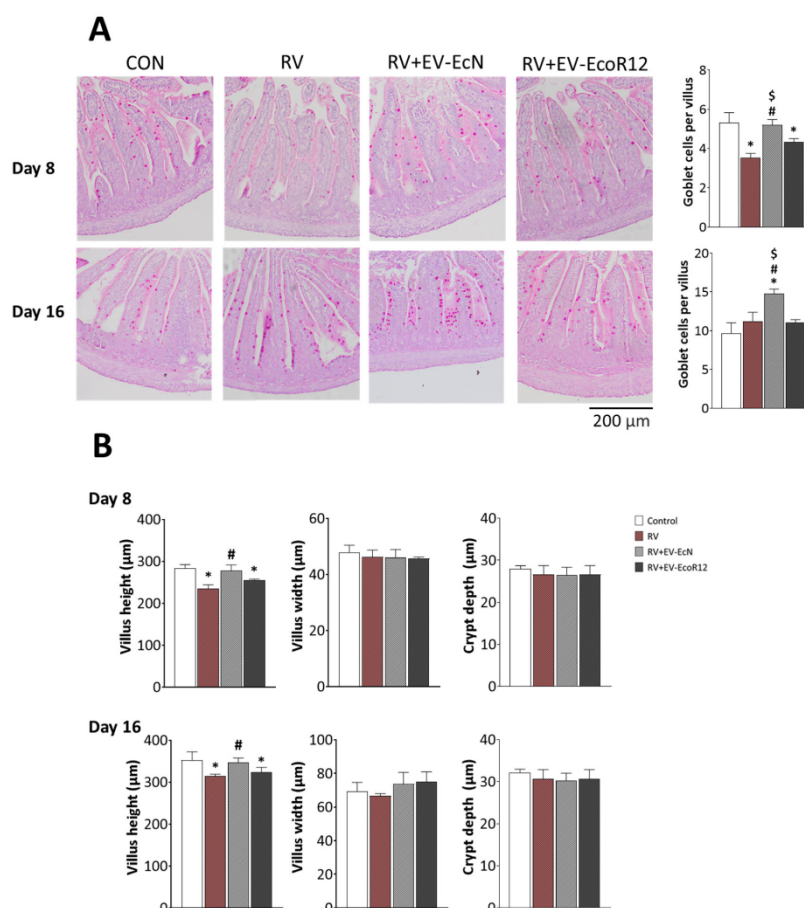


Figure 5. Mucosal morphology of small intestinal tissue collected from all the experimental groups after 3 days of infection (day 8 of life) and at the end of the procedure (day 16). (A) Representative images of distal jejunum sections of animals stained with periodic acid Schiff (PAS) and counterstained with hematoxylin (magnification 10 $\times$). The number of goblet cells (pink dots) per villus is shown on the right graph at both time points. (B) Villi height, width and crypt depth values. Results are expressed as mean $\pm$ SEM ($n = 12$ /group). Statistical differences: * $p < 0.05$ compared to the control; # $p < 0.05$ compared to the RV group; \$ $p < 0.05$ between RV + EV-EcN and RV + EV-EcoR12 groups (by post hoc Bonferroni test).

3. Discussion

Rotavirus infection is one of the main causes of acute gastroenteritis in children that results in severe diarrhea and loss of fluids. Nowadays, the role of the gut microbiota in preventing and modulating viral infections is widely recognized [51]. Thus, besides proper oral rehydration and dietary interventions, the administration of probiotics has been investigated in clinical trials to lessen diarrhea and gastrointestinal signs [52–56]. In addition, oral RV vaccines have been included in childhood immunization programs to prevent

diarrhea and reduce risk of death in low-income countries. However, current vaccines show limited efficacy, especially in children suffering malnutrition and in poor health and hygiene conditions [14,57]. In this context, probiotic intervention represents a potential strategy to enhance immunity and vaccine efficacy, as evidenced by the probiotic EcN in a gnotobiotic piglet model [31]. Also, bacterium-like particles derived from immunobiotic *Lactobacilli* have been shown to improve the intestinal and systemic immune responses triggered by an attenuated RV vaccine [58].

Considering the potential risk of probiotic use in newborns and immunocompromised individuals, the focus on new biotic therapeutic/nutritional strategies is now shifting from viable probiotic bacteria to postbiotics [32,33]. In the present study, EVs from the probiotic EcN and the gut commensal EcoR12 have been analyzed as a postbiotic intervention to ameliorate clinical symptoms and enhance immunity in a preclinical model of early-life RV infection in suckling rats. The dose of EVs was established in accordance with previous studies showing that oral administration of EVs isolated from these microbiota strains in the neonatal period was safe and well tolerated [47].

In the present approach, infection with the simian RV strain SA-11 in rat pups on day 5 of life induced moderate diarrhea without any negative impact on body weight or growth variables. This finding was consistent with previous studies using the RV SA-11 infection model in neonatal rats [19,20,49,59,60]. The interventions based on daily administration of EcN EVs or EcoR12 EVs in the first two weeks of life did not affect the animal weight, length or BMI, but significantly improved clinical symptoms by reducing both the severity and the incidence of RV-associated diarrhea. In addition, the intervention with EcoR12 EVs shortened the diarrhea period. To our knowledge, no studies have previously addressed the *in vivo* effects of microbiota/probiotic EVs on RV infection. In this context, we sought to investigate the mechanisms modulated by the EV interventions to ameliorate diarrhea and improve clinical symptoms.

One of the mediators involved in RV diarrhea is serotonin. It is well established that RV triggers the secretion of serotonin by enterochromaffin cells in the small intestine through the non-structural viral protein (NSP)4 in a Ca^{2+} -dependent manner. The released serotonin activates vagal afferent nerves linked to vomiting brain structures and contributes to diarrhea and intestinal motility. These actions are primarily mediated by the 5-HT₃ receptor [6,8,9]. At the gene expression level, RV promotes downregulation of the gene encoding the serotonin transporter SERT in the ileum of infected mice, without modifying the expression of the gene encoding the rate-limiting enzyme THP1 in the serotonin synthesis pathway [6]. In the neonatal rat model employed in this study, intestinal serotonin levels were also increased in the RV group. However, contrary to what has been described in mice, RV infection did not modify *SERT* expression. This divergence could be attributed, among other factors, to differences in the analysis timing (1–2 days post-infection vs. 3 days post-infection) or the section of the small intestine examined (ileum or jejunum). The interventions with EcN EVs or EcoR12 EVs did not reduce intestinal serotonin levels, although *SERT* mRNA levels were higher in both EV-treated groups compared to the RV group, especially in the RV + EV-EcN group. The serotonin transporter SERT plays an important role in the reuptake and clearance of the serotonin released in the lamina propria, channeling this mediator towards degradation in enterocytes. Reduction/dysfunction of SERT has been observed in enteropathogenic infections [61] and inflammatory bowel disease [7], causing disturbances in intestinal function such as enhanced motility and fluid secretion. Several reports showed that certain probiotics (*Lactobacillus rhamnosus* GC) or gut-beneficial bacteria (*Akkermansia muciniphila* and *Faecalibacterium prausnitzii*) are capable of upregulating *SERT* expression at the intestinal tissue [62–64]. In the case of *A. muciniphila*, the EVs had a more prominent regulatory effect than live bacteria [63]. The higher levels of intestinal serotonin in the RV + EV-EcN and RV + EV-EcoR12 groups are consistent with other studies proving the ability of gut bacteria, including the probiotic EcN, to enhance serotonin synthesis and bioavailability in the gut [65,66]. Besides the evaluation of the effects of EcN or EcoR12 EVs on the main genes of serotonin metabolism, we sought to analyze their impact on the

gene expression of key serotonin receptors involved in intestinal motility and diarrhea, principally *HTR3* and *HTR4*. Importantly, both EV interventions significantly reduced the expression of *HTR3* in RV-infected pups to levels lower than those of the CON or RV groups. Thus, downregulation of *HTR3* may be one of the mechanisms stimulated by EcN and EcoR12 EVs to alleviate diarrhea in neonatal rats. This conclusion is consistent with the pharmacological action of *HTR3* antagonists in improving diarrhea and intestinal inflammation [7]. Regarding *HTR4*, although no statistically significant differences were observed between groups, its expression tended to be reduced in the RV group, whereas in the RV-EV-EcN group, *HTR4* mRNA levels were similar to those of the CON group. The commensal *Bifidobacterium dentium* stimulates production of serotonin by enterochromaffin cells and the released serotonin was shown to activate *HTR4* in goblet cells to promote the secretion of MUC2 and TFF3, which contribute to epithelial repair [67]. In accordance with this finding, *HTR4* agonists alleviate disease severity and reduce inflammation in models of inflammatory bowel disease [7]. Preservation of *HTR4* expression by EcN EVs in RV-infected neonatal rats may help to preserve intestinal epithelium function and repair, thereby mitigating RV-induced dysfunction.

The amelioration of diarrhea observed in the EV-treated groups was accompanied by a decrease in the number of viral particles in fecal samples on day 6 of life (one day post-inoculation). This corresponds to the day with the highest viral elimination [19,59]. The high viral load in feces reflects both the elimination of the viral inoculum and the new viruses produced by replication in infected epithelial cells. Several probiotic strains of the *Lactobacillus*, *Bifidobacterium* and *Escherichia* genera have been shown to reduce viral shedding in RV infection models through several mechanisms, including virus sequestering, interference with RV adhesion to target epithelial cells and activation of antiviral humoral and cell immune responses [18,20,24]. In the case of the probiotic EcN, reduced viral shedding titers in gnotobiotic piglets have been correlated with the ability of this probiotic to bind RV particles, to enhance NK cell function and to regulate intestinal and serum IgA response [18,22,29]. In our study, the interventions with EVs did not involve live bacteria. Therefore, this study can provide new insights into establishing the probiotic beneficial effects that are mediated by EVs. In this context, in vitro studies carried out in polarized human colonic cell lines showed that pre-treatment with EcN culture supernatants reduced RV replication similarly to the live probiotic, although the secreted effector molecules with anti-RV inhibitory properties were not elucidated [68]. Here, we show that the released EVs isolated from culture supernatants act as carriers of such biologically active molecules, not only in the case of the probiotic EcN but also for the commensal EcoR12.

Regarding the antibody response, the level of total anti-RV Ab in the RV group did not significantly increase compared to the CON levels. This finding was previously described in a suckling rat model and attributed to the immaturity of the immune system of neonatal rats [59]. However, pups receiving either EcN EVs or EcoR12 EVs showed higher titers of anti-RV Ab in plasma both at day 8 and 16. During the diarrhea period, reduced RV fecal shedding titers in the RV + EV-EcN and RV + EV-EcoR12 groups correlated with increased levels of serum anti-RV Ab. In addition, both EV interventions also improved the overall systemic humoral response. Administration of either EcN or EcoR12 EVs promoted a significant increase in the plasma levels of all tested Ig types (IgA, IgM and IgG) compared to the CON and the RV groups. For IgM and IgG, this effect was observed at days 8 and 16, whereas the IgA response was only evident at day 16. These results are in line with the immunogenic properties of EVs from EcN and EcoR12 reported previously in healthy neonatal rats following a 16-day intervention period [47], and evidenced that a viral challenge can accelerate the vesicle-induced response as early as day 8. Based on the relationship between the different IgG subtypes (Th1/Th2 ratio), RV inoculation induced a shift towards the Th1 response (on day 8) as an attempt to control infection. The Th1/Th2 ratio returned to normal values once the infection was solved (day 16). The early response was maintained in animals receiving EcN or EcoR12 EVs on day 8 compared to the RV group, and significantly increased on day 16. This finding together with the higher

titers of anti-RV Ab and Ig in pups of the RV + EV-EcN and RV-EV-EcoR12 interventional groups indicate that EVs from these microbiota strains enhance the humoral response in RV-infected animals. Moreover, since high levels of anti-RV IgA and IgG in serum after infection resolution are good indicators of protection [11,12,69], it can be hypothesized that these interventions based on microbiota EVs can confer protection against RV infections later in life.

The interventions also enhanced cellular innate immunity. Independently of the administration of EVs, infection with RV raised the proportion of splenic T cytotoxic cells (TRC $\alpha\beta$ +, CD8+) compared to uninfected animals. In addition, the interventions involving EVs specifically increased the proportion of NK+ CD8+ and TRCY δ + CD8+ cells, this last one being a T cell subset that is considered a primitive immune cell lineage which rapidly drives innate responses against pathogens and other injuries [70]. In the case of the probiotic EcN, the results presented here provide evidence that the enhancement of innate and adaptative immune responses reported in several experimental models of RV infection [18,22,29,31] can be mediated by the released EVs.

Overall, the results related to the modulation of humoral and cellular immunity support the potential of EVs from the probiotic EcN or the commensal EcoR12 to be used as vaccine adjuvants in anti-RV immunization processes involving oral vaccines. There is a need to improve the efficacy of current RV vaccines, especially in low-income countries [57]. The suggested application could yield promising results as it has been described for other vaccination models using a combination of antigens and bacterial EVs [71].

Concerning the impact of EVs on the expression of immune-related genes in the small intestine during the diarrhea period (day 8), the results revealed that interventions based on EcoR12 EVs may confer protection against RV by upregulating genes encoding the immune receptor TLR2 and the pro-inflammatory cytokine IL-12. Activation of TLR2 signaling results in NF- κ B activation and the subsequent expression of pro-inflammatory cytokines. In the presence of specific viral ligands, this receptor can traffic to endosomes in inflammatory monocytes and activate production of antiviral effectors such as type I interferons [72]. Moreover, IL-12 is a pro-inflammatory cytokine produced and released by innate immune cells that plays a key role in the activation of host antiviral immunity and defense mechanisms for virus elimination [73]. The intervention with EcoR12 EVs also promoted upregulation of the glycoprotein CD68, a marker of macrophage recruitment to the infected sites [74]. In contrast, the intervention involving EcN EVs did not activate the expression of these genes involved in mechanisms for virus eradication. Another gene associated with the adaptative immunity that reflects the maturation state of the neonatal intestine is *FcRn*, which encodes the receptor for the uptake of maternal IgG [75]. Animals in the RV group exhibited a trend toward higher levels of *FcRn* mRNA on day 8 compared to the control group. This observation suggests that RV infection in early life may adversely impact intestine maturation by hindering the progressive reduction in *FcRn* during suckling. Importantly, the intervention with EcN EVs, but not with EcoR12 EVs, significantly reduced *FcRn* expression on day 8. This finding was consistent with previous studies showing the modulation of *FcRn* by EcN EVs [47], and indicates that vesicles from this probiotic can protect and accelerate intestinal maturation both under health and infection conditions. On day 16, as the infection was eradicated and the small intestine completed its maturation process, *FcRn* expression became similar in all groups. The benefits of EcN EVs on intestinal maturation were also evidenced by their ability to protect against RV-induced villi atrophy by preserving villi length to values similar to those of non-infected control pups. Again, this effect was not observed in the RV + EV-EcoR12 group. In the jejunum of RV-infected mice, villus shortening and atrophy together with loss of mature enterocytes have been associated with a defective absorptive function, which contribute to RV pathogenesis [76]. The results presented here prove that the beneficial effects of interventions based on microbiota/probiotic EVs on intestinal maturation and absorptive functions altered by RV infection are strain-specific.

Goblet cells and the secreted mucins also play a role as active defense mechanisms against RV infection [50,77]. In response to infection, goblet cells release MUC2 and other proteins, enhancing the mucus barrier as a mechanism to trap virus particles. However, a balanced response is needed as goblet cell exhaustion could result in inflammation [78]. Our study shows that during the acute phase of diarrhea (3 days post-infection), animals in the RV group displayed lower *MUC2* gene expression and number of goblet cells per villus in jejunum samples compared to the CON group. These results were in accordance with those reported in RV infected mice at 2–4 days post-infection [50]. The intervention with EcoR12 EVs did not prevent RV-induced early loss of goblet cells, whereas treatment with EVs from the probiotic EcN was able to preserve *MUC2* expression and the number of goblet cells to values that were indistinguishable from those of uninfected pups from the CON group. After diarrhea resolution (day 16), mucin expression and goblet cell numbers in the RV and RV + EV-EcoR12 groups were normalized to CON values. In contrast, animals receiving EcN EVs displayed significantly higher *MUC2* mRNA levels and goblet cells in jejunum samples in the post-diarrhea period than the other groups. In this interventional group, the increased expression of *MUC2* on day 16 is consistent with the higher number of goblet cells. Therefore, interventions based on EVs from the probiotic EcN can counteract the RV-induced alteration of goblet cell function by preserving mucus production and barrier function, both during the diarrhea period and after infection resolution.

This study provides evidence that microbiota EVs are a safe postbiotic alternative to the utilization of live probiotic bacteria in ameliorating RV clinical symptoms and enhancing innate and adaptive immune responses in the neonatal period. Importantly, the beneficial effects of interventions involving microbiota/probiotic EVs are strain-specific. In addition, this study suggests the healing potential of interventions involving EcN or EcoR12 EVs in protecting newborns against other possible enteric infections. This is especially relevant in the case of preterm newborns, who are highly susceptible to necrotizing enterocolitis (among other infections) due to the functional immaturity of the intestinal system [79]. Clinical trials are needed to prove the benefits of microbiota EV-based interventions in human infants. Moreover, the ability of EcN or EcoR12 EVs to enhance humoral and cell immune responses points to their potential application as adjuvants to boost the immunogenicity and protective efficacy of anti-RV vaccines. In comparison with studies analyzing the efficacy of the probiotic EcN as a vaccine adjuvant in gnotobiotic piglets [31], our experimental model evidences the efficacy of interventions involving the isolated EVs in improving infection symptoms and immunity in the presence of indigenous microbiota.

4. Materials and Methods

4.1. Bacterial Strains and Isolation of Extracellular Vesicles

EVs were obtained from two intestinal *Escherichia coli* isolates: the probiotic EcN (Ardeypharm, GmbH, Herdecke, Germany) and the commensal EcoR12 that is included in the ECOR reference collection [80]. The strains were grown in Luria–Bertani broth (LB).

Bacterial EVs were isolated from the culture supernatants as reported previously [46]. Briefly, the cell-free supernatants obtained after centrifugation of the bacterial cultures were filtered through 0.22 filters (Merck, Millipore, Burlington, MA, USA) to eliminate residual bacteria and then concentrated using Centricon Plus-70 100 kDa filters (Merck, Millipore, Burlington, MA, USA). EVs were then collected using ultracentrifugation at $150,000\times g$ for 1 h at 4 °C, washed twice in phosphate-buffered saline (PBS) and finally resuspended in PBS. The EV samples were tested for sterility using LB plates and quantified in terms of protein concentration with the Pierce BCA protein assay. Samples were also quantified using the lipophilic fluorescent dye FM4-64 (Thermo Fisher Scientific, Barcelona, Spain) that intercalates into the vesicle membrane. This assay allowed us to verify that equal amounts of EcN EVs and EcoR12 EVs were being used in the experimental protocol [47]. For the FM4-64 assay, several dilutions of the stock EV samples (40 µg/mL) were incubated with FM4-64 (5 µg/mL in PBS) for 10 min at room temperature. Reactions containing only EVs or the FM4-64 probe were processed as negative controls. After excitation at 515 nm,

emissions at 640 nm were measured with the modular multimode plate reader Varioskan Lux (Thermo Fisher Scientific, Barcelona, Spain). Samples with equal concentration of EVs, expressed as $\mu\text{g protein/mL}$, yielded similar fluorescence intensity values. EV samples were aliquoted and stored at -20°C until use.

4.2. Virus

The simian rotavirus strain SA-11 was produced by the “Enteric Virus Group-UB” at the University of Barcelona. Rotavirus was propagated in fetal African green monkey kidney cells (MA-104) and titrated as $\text{TCID}_{50}/\text{mL}$ (TCID, tissue culture infection dose) [49].

4.3. Animals

Animal procedures were approved by the Ethical Committees for Animal Experimentation (CEEAA) of the UB and the Catalanian Government (CEEAA-UB Ref.169/20 and Ref.11461, respectively) in compliance with the EU-Directive 2010/63/EU. Twelve G14 pregnant Lewis rats were purchased from Janvier (Le Genest-St-Isle, France) and were individually housed in cages (2184 L Eurostandard Type II L, Tecniplast, West Chester, PA, USA) containing tissue paper and fibrous particle bedding. Rats were monitored daily until natural delivery. The day of birth was assigned as day 1 of life. The next day, litters were randomly distributed into 4 experimental groups with 3 lactating dams each. The number of pups was unified to 8 per dam, maintaining a comparable proportion (40–60%) of males and females per litter. The rats were housed in controlled conditions of temperature, humidity and light/dark cycles of 12 h in an isolated room authorized for work in Biosafety Level 2, at the Animal Experimentation Unit (UEA) of the Diagonal Campus at the University of Barcelona (UB). Dams received a commercial AIN-93 G diet (Teklad Global Diet 2014, INOTIV, Indianapolis, IN, USA) [81] and water ad libitum. Neonatal rats had free access to maternal milk.

4.4. Experimental Design

Litters were assigned to different experimental groups: control (CON), rotavirus infection (RV), RV-infected animals receiving EVs from EcN (RV + EV-EcN) and RV-infected animals receiving EVs from EcoR12 (RV + EV-EcoR12). Each experimental group consisted of 3 litters with 8 pups each ($n = 24/\text{group}$). The experimental design is shown in Figure 6.

Neonatal rats were administered EcN or EcoR12 EVs by oral gavage from the second (day 2) to the sixteenth day of life (day 16). The selected dose of EVs was based on previous studies [47]. Considering the increase in body weight, the EV groups received a concentration of EVs of $2 \mu\text{g protein/animal/day}$ until day 8. From day 9, the dose was increased to $4 \mu\text{g protein/animal/day}$ and maintained until the last day of the procedure. The administered volume was adjusted to $100 \mu\text{L}$. The CON group received an equal volume of the vehicle (PBS). On day 5 of life, rotavirus SA-11 was inoculated to the suckling rats of the RV, RV + EV-EcN and RV + EV-EcoR12 groups by oral gavage ($2 \times 10^8 \text{ TCID}_{50} \text{ RV/rat}$) in $100 \mu\text{L}$ of PBS as previously described [49]. The CON group received the PBS vehicle instead. For animal handling, the mother was separated to a new cage and the pups were kept in the home cage. RV was inoculated 30 min after the administration of the EVs or PBS. Pups were brought together with the corresponding dams after a 45 min period in order to avoid interference between the RV and milk components. On day 8 of life (three days after RV inoculation), half of each litter (4 random pups with a similar proportion of males and females) were euthanized to obtain blood and tissue samples (3 litters/group, $n = 12$). The remaining pups were euthanized at the end of the intervention (day 16 of life).

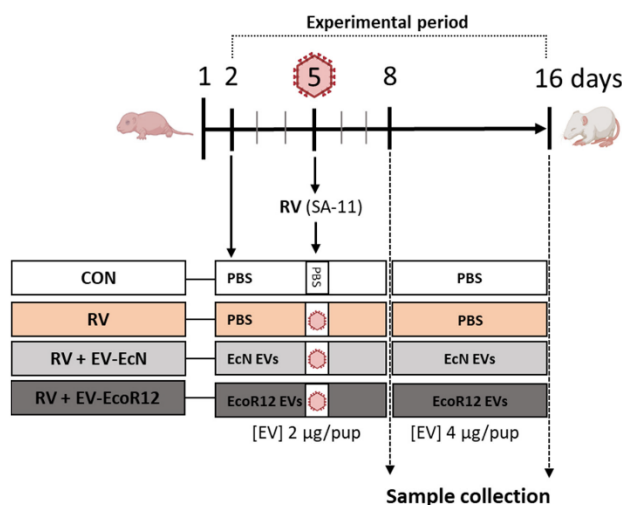


Figure 6. Experimental design. Days are numbered according to the day of birth (day 1). The four experimental groups are CON: non-infected, non-treated animals; RV: animals infected with SA-11; RV + EV-EcN: animals infected with SA-11 and receiving EVs from the probiotic EcN; and RV + EV-EcoR12: animals infected with SA-11 and receiving EVs from the commensal EcoR12. Rotavirus SA-11 was inoculated on day 5 in all experimental groups except in the control. In the interventional groups, pups were intragastrically administered with EVs from day 2 until the end of the protocol at the indicated doses. The other groups (CON and RV) were given the same volume of the vehicle (PBS).

4.5. Clinical Evaluation and Fecal Specimen Collection

Body weight was recorded daily from day 2 of life until the end of the intervention. Fecal samples were obtained once daily (from days 4 to 11) by gently pressing and massaging the abdomen. To assess the severity of diarrhea, fecal specimens were weighed and scored in a blinded manner based on their color, texture and amount to obtain a diarrhea index (DI) as described previously [60]. The scores were (1) normal feces, (2) soft yellow feces, (3) loose yellow-green feces and (4) high amount of watery feces. DI values ≥ 2 indicate diarrhea. Incidence of diarrhea was calculated as the percentage of diarrheic animals (% DA, referred to the number of animals displaying scores of DI ≥ 2 in each group) and by the percentage of diarrheic feces (% DF, which considers the number of total samples collected per day in each group). The area under the curve was calculated for the DA and DI graphs (iAUC and sAUC, respectively) as a global value of the process [19]. Fecal specimens were frozen at -20°C for further analysis.

4.6. Sample Collection

At the end of the experimental procedure (day 8 or day 16 of life), pups were anesthetized with intramuscular injection of ketamine/xylazine (Imalgene 100 mg/mL, Merial Laboratorios, Barcelona, Spain, and Rompun® 20 mg/mL, Bayer Hispania, Sant Joan Despí, Spain). Then, the naso-anal and tail lengths were measured. The body mass index (BMI) was calculated as $\text{body weight}/\text{length}^2$ (g/cm^2) and the Lee index as $3\sqrt{\text{weight}/\text{length} \times 1000}$ ($3\sqrt{\text{g}/\text{cm}}$). Once the animals were fully unconscious, euthanasia was performed by opening the peritoneal cavity and disrupting the diaphragm. Blood was collected via cardiac puncture to obtain plasma samples, which were stored at -20°C . Several digestive and immune-related organs were obtained and weighted to assess morphogenic variables. The length of the small intestine was also recorded. A central portion (1 cm) of the small

intestine was cut and placed immediately in liquid nitrogen. Intestinal tissue samples were stored at -80°C for gene expression analysis. In addition, a fragment of the distal jejunum was collected and processed for histological analysis. The gut wash was obtained as described previously and stored at -80°C for further analysis [59]. The spleens were processed for lymphocyte isolation as described below.

4.7. Fecal Viral Shedding

Fecal samples collected on day 6 were diluted in PBS (10 mg/mL) and homogenized using a FastPrep (MP Biomedicals, Santa Ana, CA, USA). The homogenates were centrifuged ($19,000 \times g$, 3 min) and the supernatants were frozen at -20°C until use. SA11 virus particles were quantified by ELISA using 96-well plates (Nunc MaxiSorp, Wiesbaden, Germany) coated with anti-p42 Ab (Meridian Life Science, Memphis, TN, USA), as previously described [20]. Serial dilutions of inactivated SA11 virus particles (ranging from 2.3×10^8 to 1.8×10^6 /mL) were used as the standard curve.

4.8. In Vitro Blocking Assay

To test the ability of EVs to bind the SA-11 particles, an in-house in vitro blocking assay was performed as previously described [19]. Briefly, RV was diluted in PBS-Tween at a final concentration of 5.8×10^7 /mL. Starting from the concentration administered to the neonatal rats, different dilutions of EVs were prepared to obtain concentrations ranging from 40 $\mu\text{g}/\text{mL}$ to 1.25 $\mu\text{g}/\text{mL}$, and pre-incubated with the virus (1:1) for 30 min. Then, free, noncoated viral particles were quantified by ELISA as described above (fecal SA-11 shedding).

4.9. Quantification of Total Immunoglobulins and Specific Anti-Rotavirus Antibodies in Plasma

Plasma concentration of IgA, IgM and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) were quantified using ProcartaPlex™ Multiplex immunoassay (Thermo Fisher Scientific, Barcelona, Spain), as described in previous studies [47,59]. Briefly, specific color-coded capture beads were bound to the Ig of interest. After adding the different detection antibodies (Abs) conjugated to phycoerythrin (PE), the concentration of each analyte was obtained using the MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB).

Specific anti-RV Abs (IgA + IgG + IgM) in plasma were quantified by ELISA as reported in previous studies [20]. Briefly, 96-well plates were coated with UV-inactivated SA11 particles (10^5 /mL), blocked with PBS-1% BSA for 1 h and incubated with dilutions of sera during 3 h. All the steps were carried out at room temperature. After washing, peroxidase-conjugated polyclonal anti-rat Ig (Dako, Barcelona, Spain) was added and incubated with the substrate following the manufacturer's instructions. The standard used was a pooled serum from RV-inoculated rats available and standardized in the laboratory. The absorbance was measured using a microplate photometer (LabSystem Multiskan, LabX) and analyzed with the ASCENT version 2.6 software (Thermo Fisher Scientific, Barcelona, Spain).

4.10. Lymphocyte Isolation from Spleen and Immunophenotyping by Flow Cytometry

Spleens were mechanically disaggregated using a sterile 40 μm mesh cell strainer (Thermo Fisher Scientific, S.L.U, Barcelona, Spain) as previously described [82]. Erythrocytes were removed with osmotic lysis and lymphocytes were suspended in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL streptomycin–penicillin and 2 mM L-glutamine. After staining dead cells with Trypan Blue, the cell numbers and viability were measured using a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, S.L.U, Barcelona, Spain).

The phenotype of isolated lymphocytes was analyzed using specific anti-rat monoclonal Abs conjugated to different fluorochromes as previously described [47,82]. The Abs used were fluorescein isothiocyanate (FITC)-TCR $\alpha\beta$, brilliant violet 786 (Bv786)-CD8 β , FITC-CD25, PE-CD161a, brilliant violet 605 (Bv605)-TCR $\gamma\delta$, PE-CD4, peridinin chlorophyll protein (PerCP)-CD8 α , allophycocyanin (APC)-CD4 brilliant violet 421 (BV421)-CD45RA (all from BD Biosciences) and APC-FoxP3 (eBioscience, Frankfurt, Germany). Briefly, lymphocytes were incubated with saturating concentrations of fluorochrome-conjugated Ab, fixed with 0.5% p-formaldehyde and stored (4 °C, in darkness) until flow cytometry analysis. For Treg analysis, lymphocytes were incubated with anti-CD4 (PE) and anti-CD25 (FITC) Ab (20 min, 4 °C, in darkness), treated with a fixation–permeabilization solution (eBioscience, Frankfurt, Germany) (30 min, 4 °C, in darkness) and then incubated with APC-conjugated anti-Foxp3 Ab (30 min, 4 °C, in darkness) as previously described [45]. A negative control staining with an isotype-matched monoclonal Ab was included in each cell sample.

Analyses were carried out with an AURORA™ Cytometer (Beckman Coulter, Miami, FL, United States) in the Flow Cytometry Unit of the Scientific and Technological Centers of the UB (CCiT-UB), and data were analyzed with Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA). The results are expressed as percentage of positive cells in a particular lymphocyte population selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) (Figure S3).

4.11. Gene Expression Analysis by Reverse Transcription–quantitative PCR (RT-qPCR)

Fragments of the small intestine tissue were homogenized in lysing matrix tubes (MP Biomedicals, Illrich, France) using Fast-Prep-24 equipment (MP Biomedicals, Illrich, France). RNA was isolated with the RNeasy® Mini Kit (Qiagen, Madrid, Spain). RNA concentration and purity were assessed using the Thermo Scientific Varioscan Lux modular multimode reader (Thermo Fisher Scientific, Barcelona, Spain). Synthesis of cDNA was carried out using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany) following the manufacturer's protocol.

Quantitative PCR was performed using ABI Prism 7900 HT equipment (Life Technologies, Madrid, Spain). The specific PCR TaqMan® primers (AB) were *TLR2* (Rn02133647_s1), *TLR-7* (Rn01771083_s1), *IGA* (Rn01511082_m1), *CD68* (Rn00566655_m1), *FcRn* (Rn00583712_m1), *MUC2* (Rn01498206_m1), *IL12* (Rn00584538_m1), *SERT1* (Rn00564737_m1), *TPH1* (Rn01476867_m1), *HTR3a* (Rn00667026_m1) and *HTR4* (Rn00563402_m1). The endogenous housekeeping gene *Gusb* (Rn00566655_m1) was used as the internal control gene. The relative gene expression was expressed as fold change compared to the CON group and calculated by means of the $2^{-\Delta\Delta C_t}$ formula [83].

4.12. Serotonin Quantification by ELISA

Serotonin levels were quantified in gut wash samples with a commercial 96-well Rat Serotonin ELISA kit (Cat. No MBS725497, MYBioSource, San Diego, CA, USA) following the recommendations and procedures indicated by the manufacturer (sensitivity 1 ng/mL).

4.13. Histomorphometry Analysis/Mucin Staining

Paraffin-embedded tissues from distal jejunum fragments were cut into 6 μ m sections and processed for histochemical Periodic Acid Schiff (PAS) staining. The sections were deparaffinized with xylene (Merck) and rehydrated in graded ethanol solutions as described elsewhere [76]. After PAS staining (15 min), slides were washed and counterstained with hematoxylin for 30 s. Images were captured by bright-field microscopy (Olympus BX41, Olympus Corporation, Tokyo, Japan) at 100 $\times$ magnification. At least 10 villi per animal were recorded and analyzed with ImageJv1.53t. The number of goblet cells were counted in ten well-defined villi from each animal.

4.14. Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS v22.0) (IBM, Chicago, IL, USA). Data were tested for normal distribution (by Shapiro–Wilk) and variance equality (by Levene’s test). A conventional one-way ANOVA test was carried out followed by the post hoc Bonferroni when data were homogeneous and had a normal distribution, considering the experimental group as the independent variable. The non-parametric Kruskal–Wallis test followed by the post hoc Dunn’s multiple comparison test was applied for the analysis of non-parametric variables (such as feces scoring) or when data displayed non-normal distribution or dissimilar variances. Differences were considered significant at $p < 0.05$. The results were expressed as mean $\pm$ standard error of the mean (SEM).

5. Conclusions

This study provides evidence that the beneficial effects of interventions involving microbiota/probiotic EVs are strain-specific. EVs from both the probiotic EcN and the commensal EcoR12 have a preventive effect in RV-infected neonatal rats by different mechanisms. Both interventions potentiate the humoral (Ig) and cellular (NK, Tc and TCR $\gamma\delta$ cells) immunity against viral infections to similar levels. In addition, EcoR12 EVs mainly activate macrophage recruitment to the infected sites (*CD68* expression) and TLR signaling towards increased production of pro-inflammatory IL-12, whereas EcN EVs improve intestinal maturation (*FcnR* expression), absorptive function (villus length) and barrier properties (goblet cell numbers and *MUC2* expression). Concerning serotonin modulation, EVs from both strains can alleviate diarrhea by downregulating the expression of the intestinal receptor HTR3.

Our results suggest that microbiota EVs are a safe postbiotic alternative to the utilization of live probiotic bacteria in ameliorating RV clinical symptoms and enhancing innate and adaptative immune responses in the neonatal period. Moreover, EVs isolated from beneficial Gram-negative microbiota/probiotic strains could be used as adjuvants to enhance the immunogenicity and protective efficacy of anti-RV vaccines and prevent RV outbreaks in developing countries.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25021184/s1>.

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ARTICLE 3

“Extracellular vesicles from Escherichia coli strains of the gut microbiota trigger hepatic antioxidant and anti-lipogenic effects via the gut-liver axis in healthy neonatal rats”

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ABSTRACT

Aim: This study explored the impact of EVs from two beneficial *Escherichia coli* strains, the probiotic EcN and the commensal EcoR12, on hepatic metabolism and oxidative stress in healthy neonatal rats.

Methods: EVs were administered orally during the first 16 days of life, and blood and liver samples were collected on days 8 and 16.

Results: The results demonstrated that EVs significantly reduced intestinal permeability, as evidenced by decreased plasma zonulin levels. In the liver, EVs enhanced redox homeostasis by downregulating CYP2E1 and upregulating key antioxidant genes (SOD1, CAT, GPX). Furthermore, the treatment shifted liver metabolism toward an anti-lipogenic profile by inducing fatty acid oxidation genes (PPARA, CPT1A) and suppressing genes involved in de novo lipogenesis (SREBP1c, ACC1, FASN, CNR1). Importantly, markers of hepatic inflammation remained unchanged, indicating the safety of the intervention. *In vitro* experiments using human HepG2 cells supported these findings, further validating the antioxidant and metabolic effects of the EVs.

Conclusion: Our results underscore the role of microbiota-derived EVs as important mediators of hepatic metabolic programming in healthy individuals via the gut-liver axis, and highlight their potential as therapeutic postbiotic agents for management of fatty liver diseases.

Article

Extracellular vesicles from *Escherichia coli* strains of the gut microbiota trigger hepatic antioxidant and anti-lipogenic effects via the gut-liver axis in healthy neonatal rats

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Abstract: The gut-liver axis is essential for maintaining liver physiology, with the gut microbiota playing a central role in this bidirectional communication. Recent studies have identified microbiota-derived extracellular vesicles (EVs) as key mediators of inter-organ signaling. This study explored the impact of EVs from two beneficial *Escherichia coli* strains, the probiotic EcN and the commensal EcoR12, on hepatic metabolism and oxidative stress in healthy neonatal rats. EVs were administered orally during the first 16 days of life, and blood and liver samples were collected on days 8 and 16. The results demonstrated that EVs significantly reduced intestinal permeability, as evidenced by decreased plasma zonulin levels. In the liver, EVs enhanced redox homeostasis by downregulating CYP2E1 and upregulating key antioxidant genes (SOD1, CAT, GPX). Furthermore, the treatment shifted liver metabolism toward an anti-lipogenic profile by inducing fatty acid oxidation genes (PPARA, CPT1A) and suppressing genes involved in de novo lipogenesis (SREBP1c, ACC1, FASN, CNR1). Importantly, markers of hepatic inflammation remained unchanged, indicating the safety of the intervention. *In vitro* experiments using human HepG2 cells supported these findings, further validating the antioxidant and metabolic effects of the EVs. Our results underscore the role of microbiota-derived EVs as important mediators of hepatic metabolic programming in healthy individuals via the gut-liver axis, and highlight their potential as therapeutic postbiotic agents for management of fatty liver diseases.

Keywords: Intestinal microbiota; bacterial extracellular vesicles; postbiotics; gut-liver axis; liver metabolism; inflammation; oxidative stress; early-life.

1. Introduction

The intestine, particularly its resident microbial community, plays a crucial role in regulating human health. The gut microbiota supports a wide range of essential physiological functions, including nutrient absorption and metabolism, immune system development, and the maintenance of intestinal barrier integrity [1,2]. Moreover, it actively participates in complex communication networks that link the gut to distant organs throughout the body [3,4].

The composition of the gut microbiome is shaped by a multitude of factors, such as genetics, diet, and lifestyle-related environmental exposures. Any disturbance of the intestinal environment can lead to an imbalance in the microbial community, a condition known as dysbiosis. Increasing evidence suggests that dysbiosis not only contributes to gastrointestinal disorders but also exerts pathophysiological effects on extraintestinal tissues [5,6].

The intestinal mucosa forms a critical barrier to face the huge number of microbes and luminal antigens in close proximity. The intestinal barrier comprises three structurally and functionally distinct layers that work coordinately to preserve gut integrity. The outermost layer is the mucus layer, which is rich in mucins secreted by goblet cells. This layer not only provides a habitat for commensal bacteria but also acts as a physical coating that prevents direct interaction between microbes and the underlying epithelial monolayer [7]. Underneath the mucus layer lies the intestinal epithelium, formed by specialized cells connected by tight intercellular junctions, which seal the paracellular space to impede passage of luminal bacteria to the underlying tissue. The deepest layer is the immune system located in the lamina propria, which shapes and mount defensive responses against pathogens that succeed in crossing the epithelial boundary. Together, these three layers work synergistically to regulate the transport of molecules and compounds from the gut to the liver and subsequently into the systemic circulation. Under healthy conditions, the epithelial tight junctions remain intact, permitting the absorption of water and nutrients while effectively preventing harmful intestinal contents from entering the bloodstream and internal organs [8,9]. However, dysregulation of the gut microbiota can compromise the gut barrier, leading to a leaky gut characterized by increased intestinal permeability.

Since the gut microbiota access to the host is physically and chemically restricted by the intestinal barrier, microbiota–host communication primarily occurs through microbiota-secreted factors such as metabolites, bioactive molecules, and extracellular vesicles (EVs) [10,11]. EVs are released by all bacterial species and serve

as a crucial tool for intercellular communication. These vesicles are nanosized, double-layered structures that originate from bacterial membranes and encapsulate various biomolecules found in the parent cells, including proteins, lipids, metabolites, and genetic material like DNA and RNA [12]. Numerous studies on probiotic and gut microbial strains have shown that EVs mediate microbiota functions by delivering functional molecules into host cells, thus influencing signaling mechanisms and cellular processes [10].

A substantial body of evidence now indicates that microbiota-derived EVs can cross cellular barriers and disseminate throughout the body [13]. Experimental support for the systemic distribution of microbiota EVs in healthy individuals has been obtained from studies using mouse models [14,15] as well as from blood samples of healthy human donors [16]. In this context, EVs released by the gut microbiota are emerging as key components of the communication networks linking the gut to distant organs [17,18].

The gut-liver axis refers to the bidirectional relationship between the gut, its microbiota, and the liver. The interaction is established primarily through the portal vein, which carries gut-derived products to the liver. By this route, metabolites such as short-chain fatty acids and EVs released by a balanced and healthy microbiota community can reach the liver and modulate hepatic immune and metabolic processes [19]. However, risk factors such as unbalanced diet, alcohol abuse, virus infection, or immune dysfunction disrupt the intestinal epithelial barrier facilitating the translocation of inflammatory microbial products that activate the immune system and cause liver dysfunction and injury [6,20]. Alterations in lipid and glucose metabolism, steatosis, oxidative stress, and inflammation are common traits in chronic alcoholic- and non-alcoholic fatty liver diseases [21,22]. In fact, increased intestinal permeability is a common hallmark of a wide range of metabolic and neurological disorders [6].

Given the critical involvement of intestinal dysbiosis in the development of chronic liver diseases, emerging therapeutic strategies are increasingly focusing on the gut, and in particular on the gut microbiome [22,23]. This includes innovative approaches utilizing next-generation probiotics and postbiotics [24,25]. Among postbiotics, EVs produced by beneficial gut bacteria are emerging as promising tools for intervention [26,27].

Previous studies from our group have provided evidence that EVs derived from probiotic and gut beneficial *E. coli* strains exhibit immunomodulatory and barrier

strengthening effects in various models of epithelial and immune cells [10,28]. Furthermore, preclinical studies in rodent models of inflammatory bowel disease and rotavirus infection have shown that oral administration of EVs derived from these strains alleviates diarrhea, clinical symptoms, and inflammation [29,30]. Overall, our research has broadened the understanding of how probiotic and beneficial gut microbes modulate immune responses and safeguard intestinal homeostasis.

Here, we investigated whether EVs from microbiota-derived *E. coli* strains could modulate hepatic metabolism and inflammation through the gut-liver axis in healthy neonatal rats. EVs from the probiotic *Escherichia coli* Nissle 1917 (EcN) and the commensal EcoR12 were administered daily to healthy suckling rats during the first 16 days of life. This experimental approach has previously been shown to be a safe intervention for stimulating immunity and intestinal maturation during the neonatal period [31]. In the present study, we assessed the presence of EVs in liver samples and analyzed the impact of the intervention on intestinal permeability and hepatic gene expression related to inflammation, oxidative stress response and lipid metabolism. Additionally, we used the HepG2 hepatic cell line to confirm that direct stimulation of liver cells with EVs produced effects consistent with those observed *in vivo*.

2. Materials and Methods

2.1. Bacterial Strains and Isolation of EVs

The probiotic *E. coli* strain EcN was obtained from Ardeypharm (GmbH, Herdecke, Germany) and the commensal *E. coli* strain EcoR12 was isolated from a fecal sample of a healthy human adult [32].

Bacterial EVs were isolated from the culture supernatants as reported previously [33]. Briefly, cell-free supernatants, obtained after centrifugation of the bacterial cultures, were filtered through 0.22 filters (Merck, Millipore, Burlington, MA, USA) to eliminate residual bacteria and then concentrated using Centricon Plus-70 100 kDa filters (Merck, Millipore, Burlington, MA, USA). EVs were then collected using ultracentrifugation at 150,000× g for 1 hour at 4 °C, washed twice in phosphate-buffered saline (PBS) and finally resuspended in PBS. The EV samples were tested for sterility using LB plates and quantified in terms of protein concentration with the Pierce BCA protein assay. Samples were also quantified using the lipophilic fluorescent dye FM4-64 (Thermo Fisher Scientific, Barcelona, Spain) that intercalates into the vesicle membrane. This assay allowed us to verify that equal amounts of EcN EVs and

EcoR12 EVs were being used in the experimental protocol [31]. For the FM4-64 assay, several dilutions of the stock EV samples were incubated with FM4-64 (5 µg/mL in PBS) for 10 min at room temperature. Reactions containing only EVs or the FM4-64 probe were processed as negative controls. After excitation at 515 nm, emissions at 640 nm were measured with the modular multimode plate reader Varioskan Lux (Thermo Fisher Scientific, Barcelona, Spain). EV samples with equal protein yielded comparable fluorescence intensity values. EV samples were aliquoted and stored at -20 °C until use.

2.2. Animals and experimental design

The present study builds upon previous experiments in which newborn rats were used to investigate the effects of interventions based on EVs derived from the probiotic EcN and the commensal EcoR12 on intestinal maturation and immunity. The methodology for animal housing, experimental design, and EV administration have been previously described [31]. Briefly, a total of nine pregnant Lewis rats (G14), purchased from Janvier (Le Genest-St-Isle, France), were individually housed in cages (2184 L Eurostandard Type II L, Tecniplast, West Chester, PA, USA). The rats were housed in controlled conditions of temperature, humidity, and light: dark cycles of 12 hours, in an isolated room, designed and authorized for working under biosecurity level 2 conditions, at the Animal Experimentation Unit (UEA) of the Diagonal Campus at the University of Barcelona. Rats were fed the commercial diet AIN-93G (Teklad Global Diet 2014, INOTIV, Indianapolis, IN, USA), recommended by the American Institute of Nutrition [34], and water ad libitum.

Rats were monitored daily until natural delivery. Litters were randomly distributed into three experimental groups (with 3 lactating dams each) and the number of pups was unified to 8 per dam, keeping a similar proportion (40–60%) of males and females per litter. Pups had free access to maternal milk. For the interventions, neonatal rats were daily administered by oral gavage with EVs from day 2 to day 16 of life, with dosage adjustments based on body weight (2 µg/pup until day 8, and 5 µg/pup from day 9 to day 16). Control animals received an equivalent volume of the vehicle, PBS.

Blood and tissue samples were collected on day 8 and day 16. The methodology for animal euthanasia, biometric measurements, and organ collection were described in our previous study [31]. Blood was collected by cardiac puncture and processed to obtain plasma samples, which were aliquoted and stored at -20 °C. Liver samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent molecular and biochemical assessments.

Animal procedures were approved by the Ethical Committees for Animal Experimentation (CEEAA) of the University of Barcelona and the Catalan Government (CEEAA-UB Ref.169/20 and Ref.11461, respectively) in compliance with the EU Directive 2010/63/EU, which establishes the rules for the protection of animals used in experimental work.

2.3. Cell culture and stimulation conditions

Human hepatocellular carcinoma cells (HepG2; 85011430, human liver, Epithelial sigma Aldrich) were maintained in 75 cm² flasks (ref. 3290; Corning Glass Works, Corning, NY, USA) at 37°C in a 5% CO₂ environment. The cells were cultured in DMEM/F12 medium (Sigma Aldrich, ref. D8900 – 10 X 1 L), supplemented with 10% fetal bovine serum (FBS; Cultilab, ref. 0521–500) and 1% penicillin-streptomycin (Corning, Fisher Scientific, Inc. Barcelona, Spain, Cat. #30-002CI). Cell proliferation was monitored daily under an inverted optical microscope, and all procedures were conducted within a laminar flow hood. Once the cultures reached approximately 80% confluence, the cells were washed with PBS, detached from the flask using trypsin, and subsequently plated into appropriate plates, depending on the experiment. For cell viability assays, cells were transferred into 96-well plates and cultured for 24 hours before the addition of EVs. For gene expression analysis, HepG2 cells were plated at a density of 2×10^5 cells/mL in 12-well plates and maintained in culture until 85-90% of confluence. The cells were then exposed to EcN EVs or EcoR12 EVs at a concentration of 5 µg/mL for either 8 or 24 hours. As a control, a parallel culture was maintained without bacterial EV treatment. Additionally, the impact of a higher concentration of EVs (50 µg/mL) on HepG2 monolayers was also evaluated. Following the incubation period, culture supernatants and cells were collected for ELISA and quantitative RT-PCR analyses, respectively.

2.4. Cell viability assays

Following treatment of HepG2 cells with EcN or EcoR12 EVs, cell viability was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described previously [35]. The results were expressed as the percentage of cell survival relative to untreated control cells.

2.5. Gene expression analysis by reverse transcription–quantitative PCR (RT-qPCR)

Fragments of the liver tissue were homogenized in lysing matrix tubes (MP Biomedicals, Illrich, France) using Fast-Prep-24 equipment (MP Biomedicals, Illrich, France). RNA was isolated by using the RNeasy® Mini Kit (Qiagen, Madrid, Spain). RNA

concentration and purity were assessed with the Thermo Scientific Varioscan Lux modular multimode reader (Thermo Fisher Scientific, Barcelona, Spain). Quantitative real-time PCR (qPCR) was performed using the ABI Prism 7900 HT system (Life Technologies, Madrid, Spain).

For gene expression analysis in rat liver samples, synthesis of cDNA was carried out using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany) following the manufacturer's protocol. Quantitative PCR was performed using TaqMan® assays (Applied Biosystems), targeting genes related to fatty acid synthesis and oxidation (SREBP-1c, FASN, ACC1, PPARA), oxidative stress (CYP2E1, INOS, COX2, SOD111, CAT, GPX), and inflammation (TNFA, TGFB, IL12). The endogenous gene GUSB was used as internal control. Primer and probe details are listed in Supplemental Table S1.

For the *in vitro* gene expression analysis using the HepG2 human cell line, RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and SYBR® Green PCR Master Mix (Applied Biosystems) was used with specific oligonucleotides to examine the same set of genes as in the *in vivo* study. The housekeeping TBP gene was used as a normalizing gene. The corresponding primer sequences are provided in Supplemental Table S2. Relative gene expression was presented as fold change compared to the control group and calculated by means of the $2^{-\Delta \Delta Ct}$ formula (Livak & Schmittgen, 2001).

2.6. Quantification of cytokines, LPS and zonulin by enzyme-linked immunosorbent assays (ELISA)

For HepG2 cells, culture supernatants were collected after the indicated treatments, centrifuged at 10,000×g for 20 minutes at 4 °C, and stored at -80 °C until further analysis. The concentrations of secreted IL-8 and TNF-α were quantified using commercial ELISA kits (BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions.

Lipopolysaccharide (LPS) was quantified in liver extracts using the High-Sensitivity ELISA Kit for Lipopolysaccharide (Cloud-Clone Corp., Katy, TX, USA; Cat. No. HEB526Ge-96T), which displays a detection range spanning from 12.35 to 1,000 ng/mL. Crude extracts were prepared from liver tissue using fresh lysis buffer provided by the same supplier (Cat. No. CC-IS007-1). Protein concentration was measured by the Pierce BCA

protein assay. Zonulin concentration was assessed in plasma samples using a commercial 96-well Rat Zonulin ELISA kit (MYBioSource, San Diego, CA, USA; Cat. No. MBS2606662), according to the manufacturer's protocol. The range of detection spans from 1.56 ng/mL to 100 ng/mL.

2.7. Dot blot assay

The presence of EVs in liver samples was analyzed by dot blot on a nitrocellulose membrane. Liver extracts were prepared and quantified in terms of protein concentration as described for the ELISA-based measurement of LPS. A total 20 µg protein amount was spotted on the membrane for each sample. The membrane was blocked for 30 min in EveryBlot blocking buffer (Bio-Rad, Cat. 12010020), and then incubated overnight with rabbit polyclonal anti-*E. coli* OmpA antibody (Invitrogen, Ref. ZK4543559, 1/2000 dilution), mouse monoclonal anti-*E. coli* LPS antibody (Abcam, Ref. AB55654, 1/2000 dilution) or mouse monoclonal anti-vinculin antibody (Santa Cruz Biotechnology, Ref. 73614, 1/10,000 dilution) in Tris-buffered saline (TBS)-Tween. After two washes in TBS-Tween, the membranes were incubated for 1 hour with anti-rabbit or anti-mouse HRP-conjugated antibodies (dilution 1/10,000) in TBS-Tween. After two washes, the membranes were revealed by chemiluminescence using the Amersham ECL Prime Western detection reagent, Ref. RPN2236.

2.8. Lipid and glucose determination in blood samples

Plasma glucose and triglyceride levels were determined using commercial colorimetric assay kits. Glucose concentration was measured with the Glucose Kit (Cat. No. 11229010, LINEAR Chemicals, S.L., Barcelona, Spain), while triglyceride levels were assessed using the Triglycerides Kit (Cat. No. 41032, Spinreact, Girona, Spain), following the manufacturers' protocols.

2.9. Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS v22.0) (IBM, Chicago, IL, USA). Data were tested for normal distribution (by Shapiro-Wilk) and variance equality (by Levene's test). A conventional one-way ANOVA test was carried out followed by the post hoc Tukey when data were homogeneous and had a normal distribution, considering the experimental group as the independent variable. The non-parametric Kruskal-Wallis test followed by the post hoc Dunn's multiple comparison test was applied when data displayed non-normal distribution or

dissimilar variances. Differences were considered significant at $p < 0.05$. The results were expressed as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Orally administered EVs reach the liver while enhancing gut barrier integrity

As evidence of the gut-liver axis function, we sought to prove that orally administered EVs reached the liver. First, the presence of EVs in the liver was indirectly assessed by quantifying LPS by ELISA in liver extracts obtained from tissue samples collected on days 8 and 16 (Figure 1A). Neonatal rats receiving daily EcN or EcoR12 EVs exhibited significantly higher LPS levels in the liver than control pups at both time points. In addition to its distribution via bacterial EVs, LPS can also be released as a soluble product during the turnover of intestinal Gram-negative bacteria. Therefore, quantification of LPS is not sufficient to prove the presence of bacterial EVs. To confirm that the elevated LPS levels detected in the liver of treated rats were associated with EVs, we performed dot blot assays in liver extracts (20 μ g total protein) using specific antibodies against the *E. coli* outer membrane protein OmpA (Figure 1B). This protein is a typical transmembrane protein of the Enterobacteriaceae outer membrane that has been widely used as a marker of bacterial vesicles [16,37]. Dot blot immunodetection of LPS was performed in parallel as a control. The analysis revealed that livers from rats receiving treatment (EcN-EVs or EcoR12-EVs) exhibited a higher OmpA signal than that of control animals, while the intensity of the hepatic protein marker vinculin was similar in all groups (Figure 1B). A strong correlation was observed between hepatic LPS levels and OmpA signal across all experimental groups. These results suggest that the differences in hepatic LPS and OmpA content between control and treated animals may be attributed to the orally administered EVs.

The passage of gut microbiota EVs through the paracellular route and their subsequent entry into the bloodstream are facilitated by increased intestinal permeability. Previous studies from our group have shown that oral gavage of EcN or EcoR12 EVs in neonatal rats upregulates the expression of occludin in the small intestine by day 8 of life [31]. This finding suggested that EVs could contribute to barrier protection, particularly in the neonatal period, where the immaturity of the small intestine is linked to enhanced paracellular permeability [38]. However, the impact of EcN or EcoR12 EVs on intestinal permeability had not been assessed until now. Here, we quantified plasma zonulin levels as a marker of barrier dysfunction [39,40]. Neonatal rats administered with EVs from either the probiotic EcN or the commensal EcoR12

exhibited significantly lower circulating zonulin levels than control animals on day 8, indicating that EV treatment enhanced intestinal barrier integrity (Figure 1C). By day 16, statistical significance between experimental groups was only observed for the EcN-EV treated group.

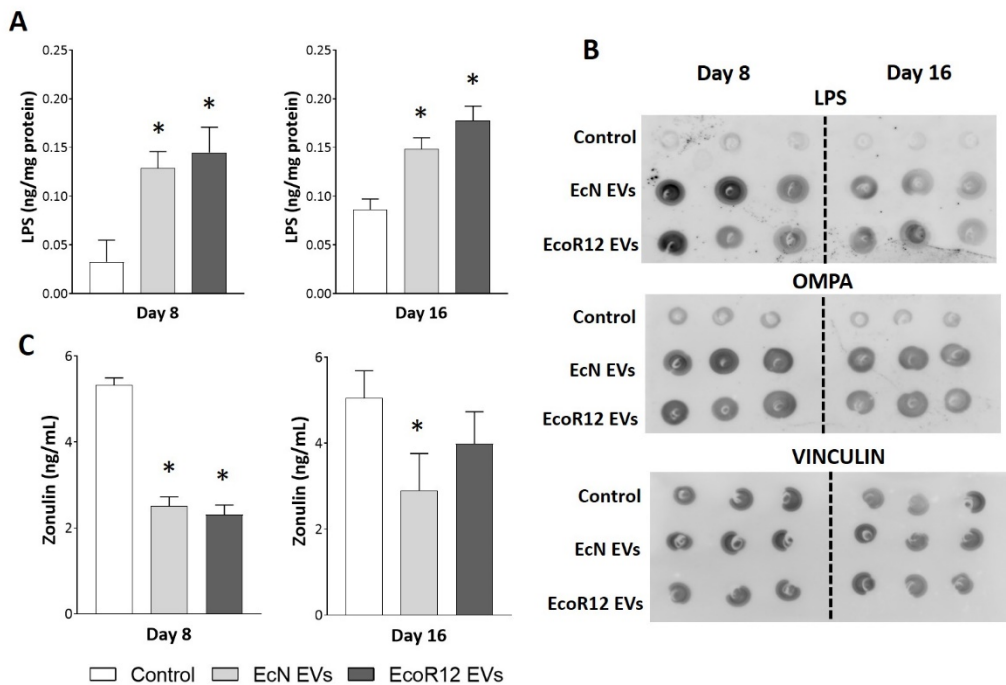


Figure 1. Detection of orally administered EVs in liver samples and their impact on plasma zonulin levels. Neonatal rats were administered PBS as the control or EVs of the indicated microbiota strains (from days 2 to 16 of life). By days 8 and 16, liver and plasma samples were collected. **(A)** The presence of bacterial EVs in the liver was assessed by means of LPS quantification by ELISA. **(B)** Representative dot blot assays of LPS, OMPA and vinculin as an endogenous control of loaded protein (20 μ g protein). **(C)** Quantification of the intestinal permeability marker zonulin in plasma samples using ELISA. Statistical differences: * $p < 0.05$ compared to control (by post hoc Dunn's multiple comparison test).

Overall, the results confirmed that orally administered EVs reach the liver through the portal vein as part of the normal gut-liver communication network, rather than as a consequence of increased paracellular passage induced by the treatment.

3.2. Interventions with EcN and EcoR12 EVs reduce liver inflammation and improve the antioxidant response in neonatal rats

LPS induces hepatic oxidative stress and inflammation, and elevated LPS levels have been associated with the onset and progression of chronic inflammation-related diseases [41,42]. In the liver, the inflammatory response induced by LPS predominantly

involves resident macrophages, known as Kupffer cells, which release pro-inflammatory cytokines that exacerbate liver damage [42,43]. Since, in our model, the elevated LPS levels detected in the liver of treated rats were associated with the administered EVs, we assessed the effects of the interventions with EcN and EcoR12 EVs on liver inflammation by analyzing the expression of genes encoding TNF- α and IL-12 (Figure 2A). By day 8, none of the treatments led to an increase in the mRNA levels of these pro-inflammatory cytokines compared to control. Moreover, administration of EVs from the probiotic EcN significantly downregulated the expression of TNF- α and IL-12 at this time point. This finding aligns with previous reports on the anti-inflammatory effects of this probiotic and its EVs in various inflammation models [29,35,44,45]. After 16 days of treatment, TNF- α and IL-12 mRNA levels in the EV-treated groups were comparable to those in the control group. Moreover, interventions based on EcN or EcoR12 EVs did not alter the expression of transforming growth factor β (TGF- β), even after 16 days of treatment. This pleiotropic cytokine plays a role in both suppressive and inflammatory immune responses, depending on the pathophysiological condition. In the liver, high levels of TGF- β contribute to liver injury, causing inflammation, fat accumulation, and fibrosis [46,47]. Overall, the results presented in Figure 2A indicate that EV administration did not induce adverse effects on liver inflammation.

Oxidative stress and inflammation are closely related pathophysiological processes that can be triggered by common mediators through TLR signaling pathways. Moreover, these processes mutually influence each other. Since microbiota EVs enclose various microbial-associated molecular patterns (MAMPs) that are recognized by host Toll-like receptors (TLRs), we analyzed the expression of genes encoding enzymes involved in the antioxidant response, namely superoxide dismutase (SOD1), catalase (CAT) and glutathione peroxidase (GPX), as well as enzymes that generate reactive oxygen or nitrogen species, such as cytochrome P-450 2E1 (CYP2E1) and inducible nitric oxide synthase (iNOS), respectively. The study also included the gene encoding the inducible cyclooxygenase isoform (COX2). In addition to its role in synthesizing the inflammatory mediator prostaglandin E2 from arachidonic acid, COX2 is an important source of ROS generation. Gene expression was assessed by RT-qPCR in liver samples collected on days 8 and 16 (Figure 2B).

By day 8, interventions with EcN or EcoR12 EVs significantly upregulated mRNA levels of the antioxidant enzymes SOD1, CAT, and GPX while downregulating CYP2E1 expression. Except for SOD1, this expression pattern was maintained after 16 days of treatment. At this time point, SOD1 mRNA levels were higher in the group treated with

EVs from the probiotic EcN compared to the control and EcoR12-EV-treated groups, although the differences did not reach statistical significance. Oral administration of EVs did not influence INOS or COX2 expression. However, long-term treatment with EcN EVs tended to reduce COX2 mRNA levels (Figure 2B), a finding consistent with their anti-inflammatory properties.

The results showed that in healthy animals, beneficial gut bacteria may positively modulate the hepatic antioxidant response through their released EVs.

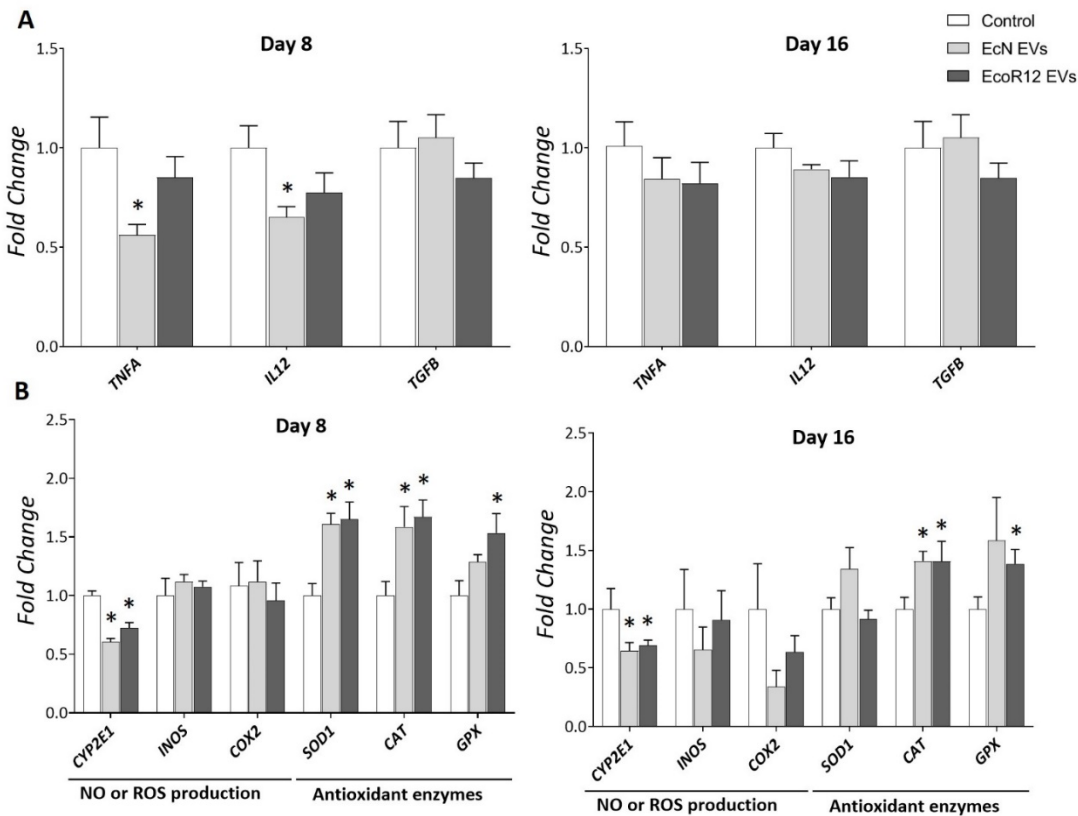


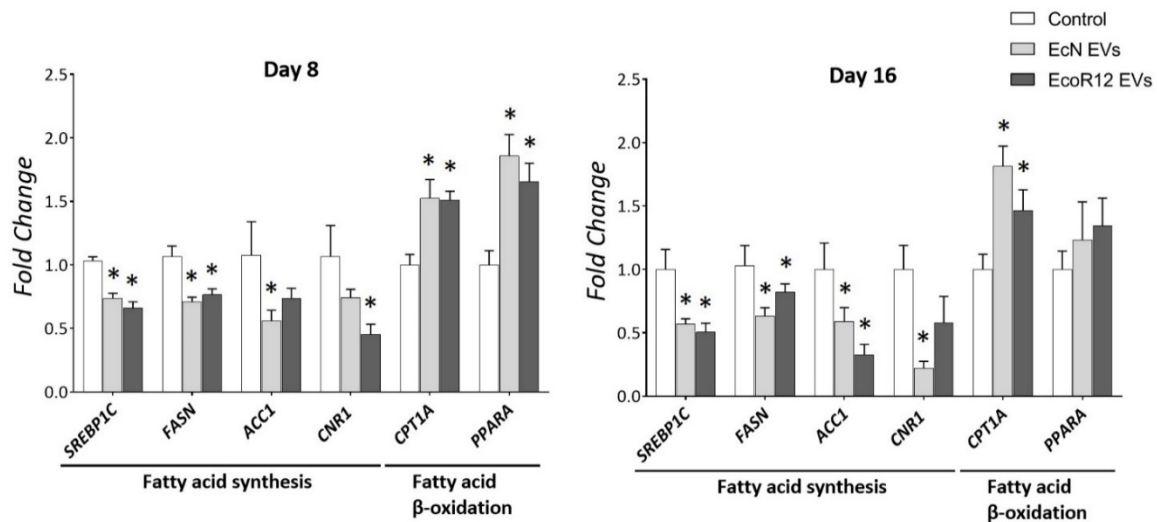
Figure 2. Regulation of genes involved in liver inflammation (A) and oxidative stress balance (B) by orally administered EcN and EcoR12 EVs. Neonatal rats were administered PBS as the control or EVs of the indicated gut microbiota strains (from days 2 to 16 of life). By days 8 and 16, liver samples were collected and processed for RNA isolation. The transcription levels of the indicated genes were quantified by RT-qPCR using GUSB as the reference gene. Relative mRNA levels in the interventional groups were calculated with respect to the CON group (expression value set to 1). Results were expressed as $\pm$ SEM ($n = 8$ animals/group and time point). Statistical differences: * $p < 0.05$ compared to CON group (by post hoc Dunn's multiple comparison test). Abbreviations: TNFA, tumor necrosis factor- α ; IL12, interleukin-12; TGF- β , transforming growth factor- β ; CYP2E1, cytochrome P-450 2E1; COX2, cyclooxygenase-2; INOS, inducible nitric oxide synthase; SOD1, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase

3.3. *EcN and EcoR12 EVs regulate hepatic lipid metabolism via the gut-liver axis by promoting lipid oxidation and inhibiting lipid synthesis*

After demonstrating the beneficial influence of EcN and EcoR12 EVs on hepatic inflammation and oxidative stress markers, we examined their impact on lipid metabolism by analyzing the expression of key metabolic enzymes and the transcription factors involved in their regulation in liver samples collected from the three experimental groups. The analysis included genes encoding enzymes involved in fatty acid synthesis, namely acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN), as well as the hepatic transcriptional activator sterol regulatory element-binding protein 1c (SREBP1c). For the analysis of the fatty acid oxidation pathway, we selected the gene encoding carnitine palmitoyl-transferase 1A (CPT1A), which mediates acyl-CoA transport across the mitochondrial inner membrane, and the regulatory protein peroxisome proliferator-activated receptor alpha (PPARA) (Figure 3). RT-qPCR results showed that EVs from either EcN or EcoR12 significantly downregulated SREBP1c and its target genes, FASN and ACC1, throughout the intervention period (days 8 and 16). Simultaneously, EV treatment, regardless of the producer strain, significantly upregulated genes associated with fatty acid oxidation (CPT1A and PPARA). Notably, the mRNA levels of the transcriptional activator PPARA increased only at day 8, returning to near-control levels with prolonged treatment. In contrast, EV-induced upregulation of CPT1A expression was sustained throughout the 16-day treatment period. The above data indicate that orally administered EVs from the beneficial microbiota strains EcN and EcoR12 regulate fatty acid metabolism in the liver favoring their oxidation while suppressing the novo synthesis.

In the liver, fatty acid synthesis is also regulated by the endocannabinoid system. Activation of the CB1 receptor (CNR1) influences hepatic fat metabolism by increasing de novo fatty acid synthesis through the upregulation of lipogenic genes [48,49]. Cumulative evidence indicates that CNR1 is upregulated in the liver under steatogenic conditions, such as a high-fat diet and ethanol exposure, and that CNR1 blockade or knockdown exerts anti-lipogenic effects on hepatocytes [50]. Gene expression analysis of CNR1 in the liver of neonatal rats showed that oral administration of EcN or EcoR12 EVs significantly downregulated the mRNA levels compared to the control group. This regulatory effect was sustained throughout the treatment period (Figure 3). Notably, this finding aligns with the inhibitory effect exerted by these microbiota EVs on the expression of the regulator SREBP1c and its targets involved in fatty acid synthesis.

We further examined whether the regulatory mechanisms activated by EcN or EcoR12 EVs could influence circulating plasma triglycerides (TAG) or glucose. The plasma concentrations of both metabolic markers were not altered by any of the EV interventions throughout the treatment period (Supplemental Figure S1). This finding suggests that in the absence of liver inflammation and steatosis, the metabolic



regulation elicited by microbiota EVs has no impact on normal lipid or glucose circulating levels in healthy suckling rats.

Figure 3. Regulation of hepatic genes involved in fatty acid synthesis and oxidation by orally administered EcN and EcoR12 EVs. Neonatal rats were administered with PBS as the control or EVs of the indicated gut microbiota strains (from days 2 to 16 of life). By days 8 and 16, liver samples were collected and processed for RNA isolation. The transcription levels of the indicated genes were quantified by RT-qPCR using GUSB as the reference gene. Relative mRNA levels in the interventional groups were calculated with respect to the CON group (expression value set to 1). Results were expressed as $\pm$ SEM ($n = 8$ animals/group and time point). Statistical differences: * $p < 0.05$ compared to CON group (by post hoc Dunn's multiple comparison test). Abbreviations: SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; CPTA1, carnitine palmitoyl-transferase 1A; PPARA, peroxisome proliferator-activated receptor alpha; CNR1, cannabinoid receptor 1.

3.4. Gene regulation by EcN or EcoR12 EVs in HepG2 cells improves anti-inflammatory, antioxidative, and lipid oxidation pathways

To prove the direct effects of microbiota EVs on hepatocytes we used the human hepatic cell line HepG2, which is commonly used as an *in vitro* model to study human liver function and disease. In this study, HepG2 cells were exposed to EVs isolated from the probiotic EcN or the commensal EcoR12 to analyze the expression of genes related to fatty acid metabolism, inflammation and antioxidative pathways. We selected two

incubation times, 8 and 24 hours, to assess the short- and long-term effects of the treatment.

Initially, we determined the optimal dose of EVs for the experiments. HepG2 cells were incubated with EcN or EcoR12 EVs at two different concentrations, 5 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$, for 8 and 24 hours. MTT assays were carried out to assess whether the selected doses were cytotoxic for this hepatic cell line. The results showed that cell viability was not affected by any of them up to 24 hours of incubation (Supplemental Figure S2). Additionally, the inflammatory response was chosen as a marker of potential harmful effects. To this end, the expression of the proinflammatory cytokines TNF- α and IL-8 was analyzed by RT-qPCR (Figure 4).

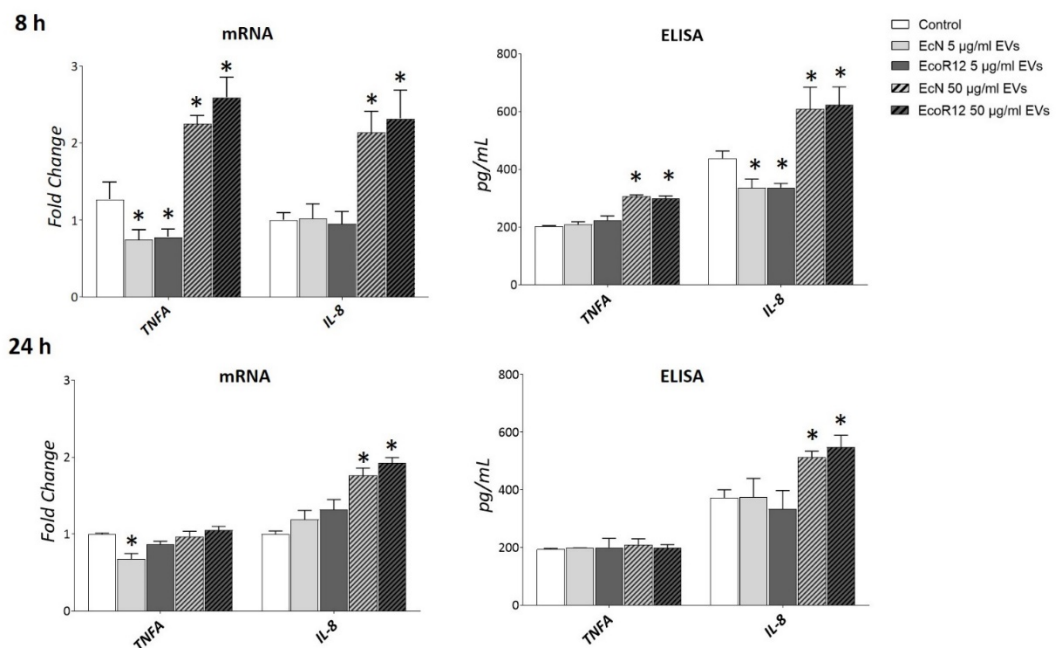


Figure 4. Expression analysis of inflammation markers in HepG2 cells exposed to different doses of bacterial EVs. HepG2 cells were incubated with EVs of the microbiota strains EcN or EcoR12 at 5 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$, for 8 or 24 hours. The transcription levels of the proinflammatory cytokines TNF- α and IL-8 were assessed by RT-qPCR using TBP as the reference gene. Relative mRNA levels were calculated with respect to the CON group (expression value set to 1). Secreted levels of both cytokines were quantified by ELISA in the culture supernatants. In all panels, data were expressed as $\pm$ SEM from three independent experiments. Statistical differences: * $p < 0.05$ compared to CON group (by post hoc Tukey's test).

At the highest concentration of EVs (50 $\mu\text{g/mL}$), a significant increase in the mRNA levels of both genes was observed after 8 hours of incubation compared to untreated control cells. In contrast, at the lower dose (5 $\mu\text{g/mL}$), IL-8 expression levels remained unchanged, while TNF- α expression was even lower than those of the control. After

24 hours of incubation with the higher EV dose, TNF- α expression returned to control levels, whereas IL-8 expression remained elevated. Notably, the short-term downregulation of TNF- α by low doses of EcN EVs in HepG2 cells was consistent with the anti-inflammatory effects observed in rat liver by day 8 of treatment. Quantification of IL-8 and TNF- α secreted levels by ELISA confirmed the inflammation profile of HepG2 cells exposed to a high dose of bacterial EVs (Figure 4). Based on these results we selected the dose of 5 μ g/mL EVs for further analysis.

The antioxidative effects of EcN and EcoR12 EVs were evaluated by analyzing the expression of genes encoding SOD1, CAT, GPX, COX2 and iNOS (Figure 5A). After 8 hours of incubation, EVs from both strains significantly upregulated the mRNA levels of the three antioxidant enzymes analyzed. However, this effect was not sustained at longer incubation times, except for catalase. The CAT mRNA levels remained elevated in cells treated with EVs from the probiotic strain EcN after 24 hours of incubation. Regarding the influence of bacterial EVs on the *in vitro* generation of oxygen or nitrogen reactive species, CYP2E1 was not included in the analysis because HepG2 cells exhibit very low or undetectable expression levels of this cytochrome P450 family enzyme [51,52]. In the *in vitro* HepG2 cell model, the effects of EVs were strain-specific. Consistent with the *in vivo* results, only EVs from the probiotic EcN reduced INOS mRNA levels compared to untreated control cells. This was a short-term effect, as mRNA levels returned to baseline after 24 hours. Although differences did not reach statistical significance, EcN EVs also tended to reduce COX2 expression at 8 hours. These findings confirm the antioxidative potential EVs from probiotic and commensal *E. coli* strains by enhancing the antioxidative response in hepatic cells. Additionally, EVs from EcN may protect from oxidative stress by downregulating genes encoding enzymes associated with prooxidant reactive species.

Regarding the impact of gut bacteria-derived EVs on lipid metabolism in HepG2 cells, we analyzed the same genes involved in fatty acid catabolic and anabolic pathways as in the *in vivo* suckling rat model. The gene CNR1 was excluded from this *in vitro* analysis because its Ct values were below the detection limit. This finding was consistent with previous reports in the literature on the low CNR1 basal expression in HepG2 cells [53]. The results closely aligned with the *in vivo* data. After 8 hours of incubation, EVs from both strains downregulated the expression of lipogenic genes SREBP1c, FASN and ACC1, while upregulating the expression the fatty acid oxidation-related genes CPT1A and PPARA (Figure 5B). However, the modulatory effects of bacterial EVs on the expression of the regulatory genes SREBP1c and PPARA, as well as the metabolic gene FASN were not apparent at longer incubation times. In contrast, the regulation (upregulation or downregulation) of genes encoding the rate-limiting

enzymes for lipid oxidation (CPT1A) and synthesis (ACC1) was sustained throughout 24 hours of incubation with EcN or EcoR12 EVs (Figure 5B).

Importantly, the gene expression profile of HepG2 cells stimulated with a higher dose of EVs (50 $\mu\text{g/mL}$) closely resembled that observed with the lower concentration of 5 $\mu\text{g/mL}$, except for CPT1A (Supplemental Figure S3). This finding suggests that the metabolic regulatory effects of microbiota-derived EVs are achieved at very low concentrations, potentially reflecting physiological conditions *in vivo*.

Overall, the *in vitro* results from the direct stimulation of hepatic cells with gut microbiota-derived EVs support the role of these vesicles in modulating liver function through the gut-liver axis.

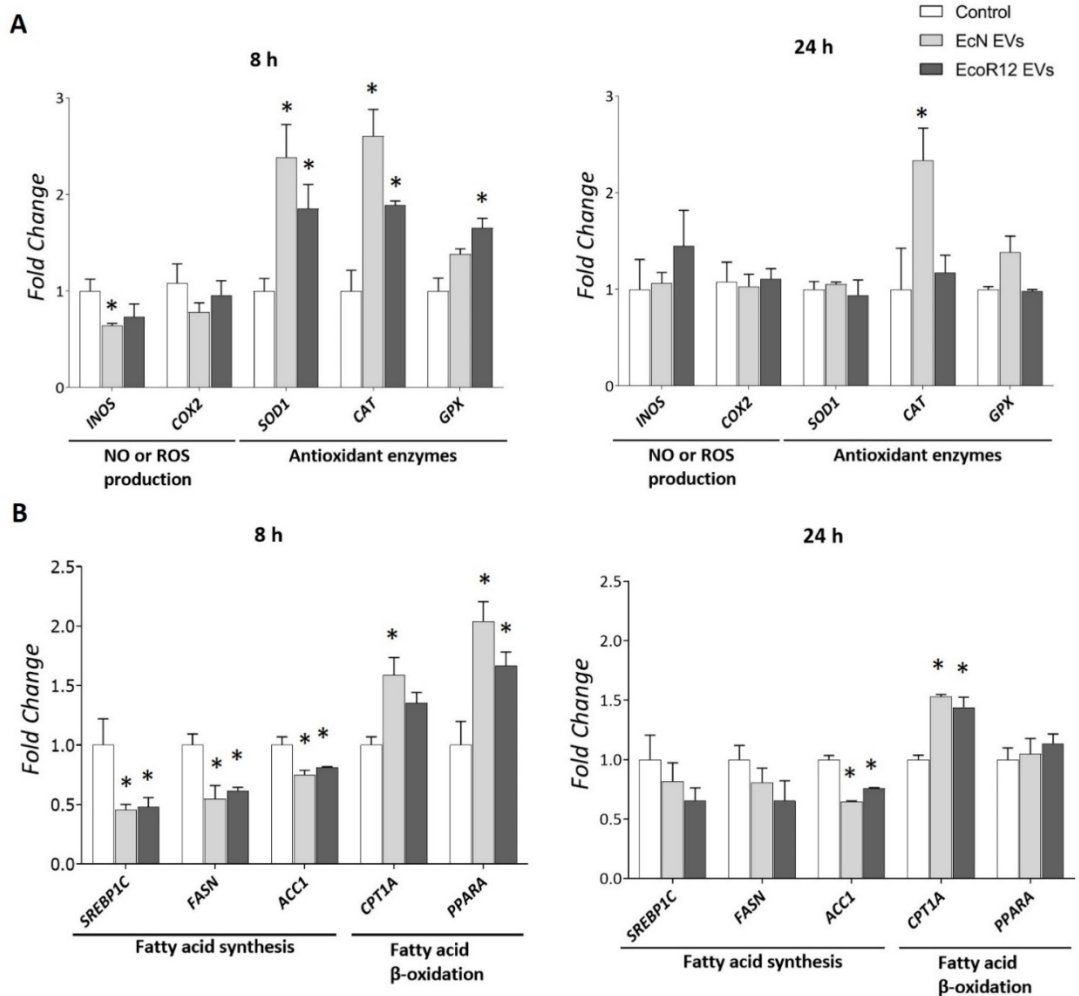


Figure 5. Expression analysis of genes related to oxidative stress (A) and lipid metabolism (B) in HepG2 cells exposed to EcN or EcoR12 EVs. HepG2 cells were incubated with EVs of the indicated microbiota

strains (5 µg/mL) for 8 or 24 hours. The transcription levels of the selected genes were assessed by RT-qPCR using TBP as the reference gene. Relative mRNA levels were calculated with respect to the CON group (expression value set to 1). In all panels, data were expressed as $\pm$ SEM from three independent experiments. Statistical differences: * $p < 0.05$ compared to CON group, # $p < 0.05$ between cells treated with EcN or EcoR12 EVs (by post hoc Tukey's test). Abbreviations: INOS, inducible nitric oxide synthase; SOD1, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase. SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; CPTA1, carnitine palmitoyl-transferase 1A; PPARA, peroxisome proliferator-activated receptor alpha.

4. Discussion

The gut microbial community plays a crucial role in regulating multiple host functions. Notably, most interactions between the host and the microbiota are indirect, mediated by a wide range of bacterial secreted factors that can enter the bloodstream and reach extraintestinal tissues and organs [23,25,54]. In this context, EVs released by the gut microbiota are emerging as key players in the communication networks linking the gut to distant organs [10,18,55]. Given that microbiota-derived EVs are transported through the systemic circulation and carry bioactive molecules that facilitate cell-to-cell communication, they play a crucial role in maintaining body homeostasis and well-being under healthy conditions, while also contributing to disease progression under dysbiosis and the resulting leaky gut. Despite growing evidence supporting the beneficial properties of microbiota-derived EVs in the gut environment, particularly their role in modulating intestinal barrier integrity and host immune responses, research into their broader systemic effects remains limited. In this context, few studies have investigated the role of microbiota-derived EVs in major inter-organ axes within physiologically healthy models.

The liver is the first organ to receive intestinal metabolites and microbial compounds, absorbed or translocated across the gut epithelial barrier, via the portal vein. The influence of gut beneficial bacteria and their derived EVs on liver function has been primarily studied in cellular and experimental models of fatty liver disease, including metabolic dysfunction-associated fatty liver disease (MAFLD) and alcohol-associated liver disease [24,26,27,56]. These studies revealed that oral administration of EVs derived from *Akkermansia muciniphila*, *Lactobacillus rhamnosus* GG or *Enterococcus faecium* improved intestinal permeability and protected liver against metabolic or alcohol-induced liver injury, fibrosis and metabolic dysfunction, with the effects being dependent on the producing bacterial strain.

The novelty of our study lies in investigating the effects of interventions based on the oral administration of EVs derived from gut-beneficial *E. coli* strains on liver function in healthy neonatal rats. This *in vivo* model is particularly relevant, as neonatal rodents

are born with immature intestinal function and immunity. The high permeability of the neonatal intestine facilitates the transfer of luminal contents into the lamina propria and, subsequently, into the portal vein and systemic circulation. This condition enables the transmission of microbiota-derived compounds and antigens that support the development and education of the newborn immune system, as well as maternal immunoglobulins that confer immune protection to the pups [38]. Therefore, the early postnatal stage represents a critical window for gut development, during which host-microbiota interactions can have a long-lasting impact on health. In this context, the model used in this study enables the investigation of the physiological role of gut microbiota-derived EVs in modulating liver metabolism under healthy conditions, in the absence of liver dysfunction or injury. Previous studies by our group using the same suckling rat model demonstrated the safety and efficacy of interventions based on EVs from the probiotic EcN and the commensal strain EcoR12 in promoting intestinal maturation and enhancing innate and adaptive immune responses during the neonatal period, under both healthy conditions and rotavirus infection [30,31].

The results presented here demonstrate that orally administered EVs reach the liver. After normalizing by the total protein concentration in liver samples, elevated LPS levels were observed in treated rats compared to controls, as measured by both ELISA and Western blot. Hepatic LPS content correlated with the presence of the outer membrane protein OmpA, a component of EVs released by Gram-negative bacteria. The basal levels of LPS and OmpA observed in liver samples from control animals likely reflect EVs derived from the indigenous gut microbiota, whereas the elevated levels in treated groups are attributable to the administered EVs. The results from the control group are consistent with previous studies reporting the presence of LPS-containing EVs in the hepatic portal vein and liver tissue of both lean and obese mice [15]. Concerning the fate of administered EVs, other studies have reported their rapid trafficking to the liver [14,57]. Importantly, after seven days of treatment (day 8 of life), plasma zonulin levels were reduced by EVs from either the probiotic EcN or the commensal EcoR12. This finding confirms the efficacy of administered EVs in counteracting the increased intestinal permeability associated with intestinal immaturity in early life, and suggests their potential applicability in other conditions characterized by a leaky gut. Despite reducing intestinal permeability, the administered EVs reach the liver, confirming the natural trafficking of EVs released by gut bacteria through the gut-liver axis in healthy individuals.

Since EVs derived from Gram-negative bacterial strains carry LPS and other MAMPs that interact with cell surface and intracellular PRRs, which activate inflammatory signaling cascades [10,58,59], we aimed to assess whether the amount of

administered EVs reaching the liver could induce hepatic inflammation or disrupt redox balance. The results revealed strain specific-anti-inflammatory effects. At the administered doses, EVs from the commensal strain EcoR12 did not trigger adverse inflammatory responses, as the expression levels of pro-inflammatory cytokines (IL-12 and TNF- α) and inflammation-associated enzymes (INOS, COX2) remained comparable to those in control animals at both day 8 and day 16. In contrast, EVs from the probiotic EcN induced anti-inflammatory effects by suppressing the expression of these pro-inflammatory cytokines. Notably, this effect was observed only on day 8, corresponding to the phase of intestinal immaturity and enhanced permeability, which is consistent with previous reports describing the anti-inflammatory properties of EcN EVs *in vivo* and *in vitro* models of intestinal barrier disruption and inflammation [29,35]. By day 16, IL-12 and β α - α mRNA levels were comparable between control and EcN-EV-treated animals; however, the expression of COX2 and INOS showed a decreasing trend in animals receiving EcN EVs. In addition to their effects in the liver, downregulation of both enzymes by EcN EVs in intestinal tissue has previously been reported in experimental models of ulcerative colitis [29]. Inflammation and oxidative stress are closely interconnected processes. The enzymes COX2 and iNOS are important mediators of inflammation, catalyzing the formation of prostaglandin PGE2 and nitric oxide, respectively. Additionally, they generate reactive oxygen and nitrogen species that can damage tissues by oxidizing lipids, proteins and DNA. Another major source of ROS in the liver is CYP2E1. This inducible enzyme plays an important role in liver homeostasis, as it is involved in the metabolism of endogenous metabolites such as acetone and fatty acids, as well as xenobiotics. Increasing evidence indicates that the upregulation of CYP2E1 contributes significantly to the progression of chronic fatty liver diseases [60]. Notably, interventions involving EcN- or EcoR12-derived EVs led to a sustained downregulation of CYP2E1 throughout the treatment period. This regulation was accompanied by a robust induction of key enzymes involved in the cellular antioxidant response, namely SOD1, CAT, and GPX. By reducing hepatic ROS production and enhancing the antioxidant enzyme arsenal, EVs released by microbiota beneficial *E. coli* strains can protect liver from oxidative stress and subsequent tissue damage. In addition, EVs derived from the probiotic EcN may contribute to the attenuation of liver inflammation.

To assess the liver metabolic effects of the administered EVs, we focused on lipid metabolism, specifically examining the expression of genes encoding the rate-limiting enzymes of de novo fatty acid synthesis (ACC1 and FASN) and fatty acid oxidation (CPT1) pathways, as well as their key hepatic transcriptional regulators, SREBP1c and PPARA, respectively. Our results revealed that both EcN and EcoR12 EVs significantly

modulate the expression of these genes, downregulating those involved in lipid synthesis while upregulating genes associated with fatty acid degradation. In the liver, CNR1 plays a critical role in regulating lipid metabolism under physiological conditions by upregulating the expression of lipogenic genes involved in fatty acid synthesis. Its expression is influenced by nutritional status and is particularly elevated in fatty liver diseases [50]. Notably, pharmacological inhibition of CNR1 using specific antagonists has been shown to reduce hepatic steatosis, improve lipid profiles and attenuate oxidative stress and proinflammatory cytokine production [50,61]. Consistent with their anti-lipogenic and antioxidant effects, oral administration of either EcN or EcoR12 EVs led to a significant downregulation of CNR1 compared to control animals. Overall, these findings demonstrate that EVs from both beneficial *E. coli* strains exert a substantial influence on hepatic lipid metabolism via the gut-liver axis. Specifically, they trigger a gene expression profile that favors fatty acid degradation pathways while suppressing lipid synthesis, thus preventing their subsequent accumulation in the liver. As stated above, the neonatal period represents a critical stage for gut development, during which microbiota-host interactions can have profound and long-term effects on health. In this context, EVs derived from a healthy, balanced microbiota may positively influence metabolic programming across various gut-organ axes by promoting the establishment of healthy metabolic profiles.

Despite inherent differences between the *in vivo* neonatal rat model and the *in vitro* human HepG2 cell model, both experimental approaches yielded comparable results. Exposure of HepG2 cells with low doses of EcN EVs or EcoR12 EVs (5 µg/mL) increased antioxidant capacity, suppressed the expression of de novo lipogenic genes, and activated genes involved in fatty acid oxidation. These effects were most prominent at early incubation times. Notably, EVs from these *E. coli* strains of the gut microbiota showed greater efficacy in modulating lipid metabolism in HepG2 cells compared to EVs derived from well-characterized gut beneficial bacteria such as *A. muciniphila*, a species currently receiving significant attention as a next-generation probiotic [62]. In the HepG2 cell model, *A. muciniphila*-derived EVs did not significantly alter CNR1 expression relative to the control, and upregulation of PPARA was only observed at a vesicle concentration of 100 µg/mL, which is 50 times higher than that used in our study with EcN or EcoR12 EVs [63]. The ability of EcN or EcoR12 EVs to exert metabolic regulatory effects at very low concentrations aligns with the number of vesicles that may reach the liver via the portal vein under healthy gut conditions, reinforcing their physiological role as mediators of systemic homeostasis.

Overall, the results from this study demonstrate that microbiota-derived EVs present in the intestinal tract act as regulators of liver oxidative balance and metabolism under

physiological conditions in healthy individuals, confirming their role as key mediators of communication in the gut-liver axis. In addition, they provide a basis for the potential application of EVs derived from the probiotic EcN and the commensal strain EcoR12 as postbiotics for preventing chronic liver diseases involving inflammation, steatosis and oxidative stress-induced injury. Preclinical assays are essential to establish the efficacy of these EVs in experimental models of liver disease prior to their translation into clinical settings.

The ongoing obesity epidemic and the rise of metabolic syndrome have significantly contributed to the increasing prevalence of MAFLD, now recognized the most common form of liver disease worldwide. This condition represents a major public health concern, with a substantial economic burden due to frequent hospitalizations [64]. In 2024, the US FDA approved resmetirom as the first drug for the treatment of severe forms of MAFLD. However, there is currently no effective treatment for the initial stages of disease [65]. Also, the global burden of alcohol-associated liver disease underscores the urgent need for new therapeutic strategies to mitigate long-term complications [66].

In this context, probiotics have garnered attention as potential interventions due to their ability to counteract gut dysbiosis, reduce intestinal permeability and its impact on liver diseases. However, their potential risk in immunocompromised individuals and technological limitations have hampered their full potential applications in the food and pharmaceutical industries. As a result, the focus gradually shifts from viable probiotic bacteria to postbiotics, defined as non-viable bacterial components with health benefits [67]. This group of biotic therapies pose no risk of bacterial translocation, offer enhanced bioavailability of molecules for interaction with host-specific receptors to trigger targeted biological responses [68–70]. Among the most promising postbiotic candidates are EVs derived from beneficial gut bacteria. These EVs are emerging as innovative tools to prevent and manage metabolic, neurological, and inflammatory diseases across various gut-organ axes [71,72].

5. Concluding remarks

This study confirms that, in healthy individuals, gut microbiota-derived EVs play a central role in the communication network between the gut and the liver. The findings provide compelling evidence that oral administration of EVs from the probiotic EcN and the commensal EcoR12 can beneficially modulate liver function through multiple mechanisms. In the gut, EVs from both beneficial *E. coli* strains help reduce intestinal permeability, thereby preventing the translocation of luminal antigens and harmful

compounds to the liver. Upon reaching the liver via the portal vein, these EVs influence gene expression, promoting antioxidant and lipid-lowering profiles that protect against oxidative stress and metabolic damage. Additionally, EVs from the probiotic EcN exert anti-inflammatory effects within the liver. The continuous trafficking of EVs released by a balanced and healthy gut microbiota along the gut-liver axis may support homeostatic mechanisms that promote and maintain liver health. This programming may be important during the neonatal period to achieve long-lasting benefits for well-being.

Author Contributions: L.B, J.P and S.M-R were involved in the conception and design of the study. S.M-R conducted the study, performed the experiments and the statistical analysis of data. L.B, J.P and S.M-R were involved in the interpretation of results and wrote the first draft of the manuscript. Funding acquisition was obtained by L.B. and J.B. All authors edited, reviewed, and approved the final version for publication. L.B. is the guarantor of this work, and had full access to all data in the study.

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Conflicts of Interest: The authors of this study disclose that they have no conflicts of interest to declare.

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Supplementary figures

Table S1. TaqMan Assays (Applied Biosystems) used to analyze gene expression in the neonatal rat model.

Gene	reference
PPARG	Rn00440945_m1
PPARA	Rn00566193_m1
ACC1	Rn00573474_m1
GUSB	Rn00566655_m1
CPT1	Rn00580702_m1
FASN	Rn00569117_m1
SREBP1c	Rn01495763_g1
IL12	Rn00584538_m1
TNFA	Rn99999017_m1
SOD	Rn00566938_m1
CAT	Rn00560930_m1
GPX	Rn00577994_g1
COX2	Rn01483828_m1
iNOS	Rn00561646_m1
CYP2E1	Rn00580624_m1

Table S2. Nucleotide sequences of primers used to analyze gene expression in HepG2 cells.

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
PPARG	GGGATCAGCTCCGTGGATCT	TGCACTTTGGTACTCTTGAAGTT
PPARA	CGGTGACTTATCCTGTGGTCC	CCGCAGATTCTACATTCGATGTT
ACC1	CATGCGGTCTATCCGTAGGTG	GTGTGACCATGACAACGAATCT
TBP	GTTCAGCAGTCAACGTCCCA	TCATGGGGGAGGGATACAGT
CPT1	TTTCCTTGCTGAGGTGCTCT	TCTCGCCTGCAATCATGTAG
FASN	TCGTGGGCTACAGCATGGT	GCCCTCTGAAGTCGAAGAAGAA
SREBP1c	CGGAGCCATGGATTGCACT	TAGGCCAGGGAAGTCACTG
IL8	CTGGCCGTGGCTCTCTTG	GGGTGGAAAGGTTTGGAGTATG
TNFA	AACTAGTGGTGCCAGCCGAT	CTTCACAGAGCAATGACTCC
SOD	ATCCTCTATCCAGAAAACACG	ACACCACAAGCCAAACGAC
CAT	TGTTGAAGATGCGGCGAG	ATGAGAGGGTAGTCCTTGTG
GPX	GCCTTCCCGTGTAACCACT	GCGAACTCTTTGATCTCTTCGT
COX2	GGGTTGCTGGGGGAAGAAATG	GGTGGCTGTTTTGGTAGGCTG
iNOS	GTTGAAGACTGAGACTCTGG	ACTAGGCTACTCCGTGGA

Figure S1. Concentration of triglycerides and glucose in plasma samples from control and vesicle-treated neonatal rats collected at the indicated times. Neonatal rats were administered PBS as the control or EVs of the indicated gut microbiota strains (from days 2 to 16 of life). By days 8 and 16, blood samples were collected and processed for plasma obtention. No statistical differences were observed by the post hoc Dunn's multiple comparison test.

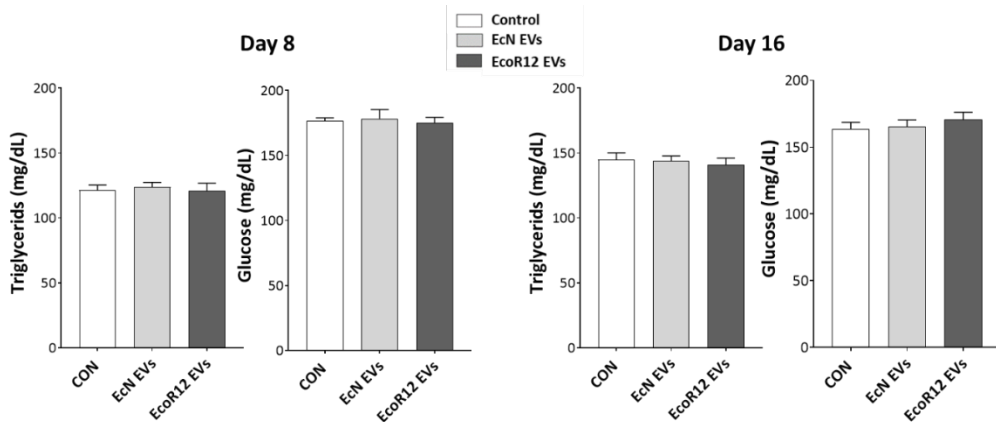


Figure S2. Cell viability of HepG2 cells exposed to different doses of bacterial EVs. HepG2 cells were exposed to EVs of the microbiota strains EcN or EcoR12 at 5 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$, for 8 h or 24 h. Cell viability was assessed by the MTT assays. Data were expressed as percentage of viability when compared to untreated control cells (value set to 100%). No statistical differences were observed by the post hoc Tukey's test.

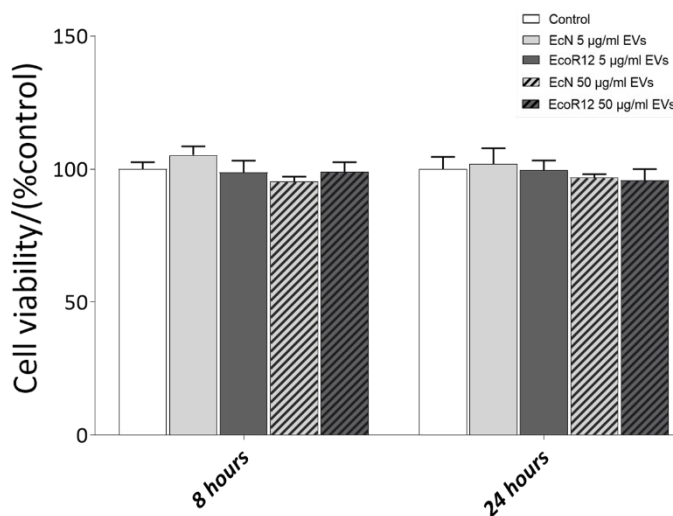
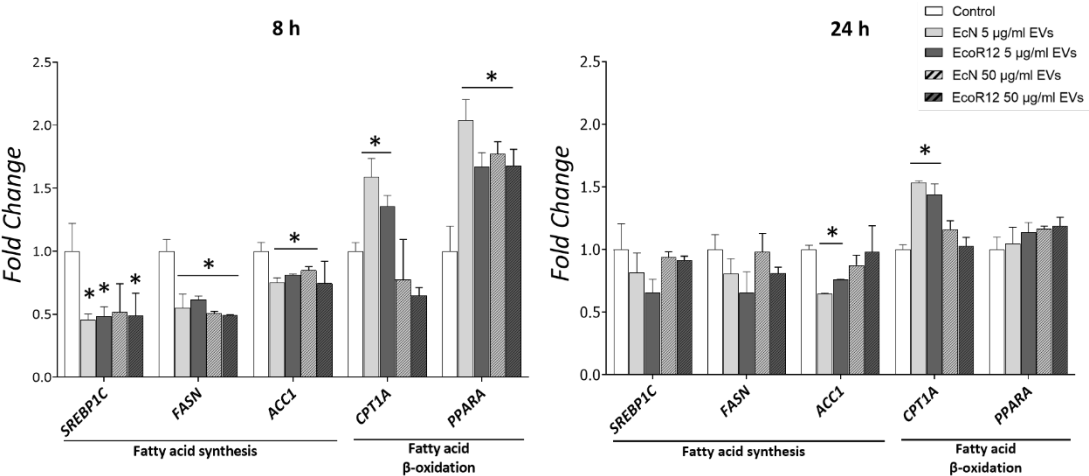


Figure S3. Regulation of genes involved in fatty acid synthesis and oxidation by different doses of bacterial EVs in HepG2 cells. HepG2 cells were incubated with EVs of the microbiota strains EcN or EcoR12 at 5 µg/mL and 50 µg/mL, for 8 h or 24 h. The transcription levels of the indicated genes were measured by RT-qPCR using TBP as the reference gene. Relative mRNA levels were calculated with respect to the CON group (expression value set to 1). Data were expressed as ± SEM from three independent experiments. Statistical differences: * $p < 0.05$ compared to CON group (by post hoc Tukey's test). Abbreviations: *SREBP1c*, sterol regulatory element-binding protein 1c; *FASN*, fatty acid synthase; *ACC1*, acetyl-CoA carboxylase 1; *CPT1A*, carnitine palmitoyl-transferase 1A; *PPARA*, peroxisome proliferator-activated receptor alpha; *CNR1*, cannabinoid receptor 1.



ARTICLE 4

“Gut-brain axis: Role of microbiota-derived extracellular vesicles in the modulation of intestinal barrier and neuronal development”

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Manuscript in preparation

ABSTRACT

Aim: This study examines the effects of extracellular vesicles (EVs) derived from *Escherichia coli* Nissle 1917 (EcN) and the commensal strain EcoR12 on intestinal barrier function and neuronal development, using human induced pluripotent stem cell (iPSC)-derived intestinal organoids and neurons as experimental models.

Methods: Human iPSCs were cultured and differentiated into neurons and intestinal organoids, which were then stimulated with EVs from EcN and EcoR12. Immunostaining, confocal microscopy, and Sholl analysis were used to assess protein expression and neuronal morphology.

Results: EVs stimulation of intestinal organoids enhanced epithelial barrier integrity, increasing the expression of ZO-1, claudin-3, E-cadherin, and villin-1. Claudin-3 upregulation was dose-dependent, requiring higher EV concentrations. In neurons, EcoR12 EVs reduced cell numbers and dendritic length but increased nuclear size and dendritic branching, indicating altered neuronal morphology.

Conclusion: These findings highlight microbiota-derived EVs as promising postbiotic agents capable of modulating gut and brain cellular responses. Their dual action on intestinal integrity and neuronal morphology suggests potential for therapeutic strategies targeting the gut-brain axis, pending *in-vivo* validation.

Article

Gut-brain axis: Role of microbiota-derived extracellular vesicles in the modulation of intestinal barrier and neuronal development

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Abstract: The gut-brain axis (GBA) is a complex communication network linking the gastrointestinal system with the central nervous system and plays a pivotal role in regulating homeostasis, immunity, and brain function. Recent studies have highlighted the emerging role of extracellular vesicles (EVs) derived from gut microbiota as key mediators in this axis. These nano-sized particles can cross biological barriers and carry bioactive molecules that influence intestinal epithelial integrity and neurodevelopment. This study investigates the impact of EVs isolated from *Escherichia coli* Nissle 1917 (EcN) and the commensal strain EcoR12 on intestinal barrier function and neuronal development using human induced pluripotent stem cell (iPSC)-derived intestinal organoids and neurons. EV treatment of intestinal organoids resulted in the upregulation of the tight junction proteins ZO-1 and claudin-3, the adherens junction protein E-cadherin, and the cytoskeletal protein villin-1, indicating improved epithelial barrier integrity. Notably, claudin-3 expression was dose-dependent, requiring higher EV concentrations to elicit a significant response. In parallel, iPSC-derived neuronal cultures exposed to EcoR12 EVs showed reduced cell numbers and dendritic length, but a marked increase in nuclear size and dendritic branching. These findings suggest that EcoR12 EVs may promote neuronal maturation and complexity. Together, these results support the concept of microbiota-derived EVs as functional postbiotics capable of modulating both gut and brain cellular responses. Their dual action on intestinal integrity and neural morphology positions these vesicles as promising tools for therapeutic strategies targeting the GBA, particularly in conditions associated with gut dysbiosis and neurodevelopmental disorders. Future *in vivo* validation is necessary to confirm these findings and explore clinical applications.

Keywords: Extracellular vesicles, Gut-brain axis, Intestinal organoids, Microbiota-derived postbiotics, Neuronal development

1. Introduction

The gut-brain axis (GBA) is a complex, bidirectional communication network that connects the gastrointestinal tract with the central nervous system, integrating neural, immune, endocrine, and metabolic pathways. This axis regulates homeostasis, behavior, cognition, and immune function. The interaction between gut microbiota and the host is fundamental to GBA function, as gut microbes produce a variety of bioactive compounds, including neurotransmitters, short-chain fatty acids (SCFAs), and inflammatory mediators that can influence brain function and behavior. Increasing evidence indicates that alterations in gut microbiota composition and barrier permeability impair GBA signaling, contributing to the development and progression of neurological and psychiatric disorders such as anxiety, depression, and neurodegenerative diseases [1–4].

In this context, EVs have emerged as key mediators of gut-brain communication. These nano-sized, membrane-bound particles are secreted by both Gram-negative [5] and Gram-positive [6] bacteria and are capable of transporting diverse molecular cargo — including proteins, lipids, nucleic acids, and metabolites — to distant host tissues. Bacterial EVs can cross biological barriers such as the intestinal epithelium and the blood-brain barrier (BBB), modulating barrier integrity and influencing immune responses [7]. Their ability to affect physiological barriers positions them as important players in the gut-brain axis and potential contributors to neuroinflammatory processes.

A growing interest in this field has led to more studies exploring the effects of gut microbiota-derived EVs on host physiology. Recent studies have suggested that EVs derived from pathogenic bacteria may contribute to neurodegeneration by triggering pro-inflammatory responses, while those from beneficial bacteria may exert protective effects by enhancing gut barrier function and modulating neuroimmune signaling [8]. In particular, EVs from probiotics (e.g., *Lactobacillus* spp., *Bifidobacterium* spp.) and beneficial gut commensals (e.g., *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, *Escherichia coli*) exhibit immunomodulatory and barrier-strengthening effects in both *in vitro* and *in vivo* models. Depending on the bacterial strain, EVs can influence the expression of tight junction (TJ) proteins, stimulate antimicrobial peptide production, enhance mucus

secretion, and modulate both innate and adaptive immune responses [6,9,10]. Furthermore, recent findings indicate that EVs from beneficial gut bacteria can carry neuroactive molecules such as γ -aminobutyric acid (GABA), serotonin precursors, and microRNAs that influence neural function, highlighting their potential role in neuroprotection, synaptic plasticity, and cognitive health [11].

Within this context, our group has contributed to the field by studying *E. coli*-derived EVs, particularly from the probiotic *E. coli* Nissle 1917 (EcN) and commensal *E. coli* strains isolated from stool samples of healthy individuals. We have demonstrated that EVs from these strains can modulate immune responses and reinforce epithelial barrier integrity in intestinal epithelial cell cultures, colonic explants, and *in vivo* models [12–16].

The present study aims to evaluate the capacity of EVs derived from EcN and EcoR12 to modulate gut barrier function and neuronal development, specifically focusing on their role within the gut-brain axis. We employed human inducible pluripotent stem cell (iPSC)-derived intestinal organoids and iPSC-derived neurons as *in vitro* models to investigate vesicle uptake, barrier integrity, and downstream cellular responses. This integrative approach can provide new insights into how commensal bacteria may influence neuroimmune communication and brain homeostasis. Ultimately, our findings may contribute to developing novel microbiota-based therapeutic strategies for neurological disorders associated with gut dysbiosis and compromised GBA function.

2. Materials and Methods

2.1. Bacterial Strains and Isolation of EVs

The probiotic strain EcN was obtained from Ardeypharm GmbH (Herdecke, Germany). The commensal *E. coli* strain EcoR12 was isolated from the fecal sample of a healthy adult human and is part of the ECOR reference collection originally described by Ochman and Selander (1984) [17].

EVs were isolated from culture supernatants following previously established protocols [18]. Briefly, bacterial cultures were centrifuged to remove cells, and the resulting cell-free supernatants were filtered through 0.22 μ m filters (Merck, Millipore, Burlington, MA, USA) to eliminate any remaining bacteria. The filtered supernatants were then concentrated using Centricon Plus-70 100 kDa filters (Merck Millipore, Burlington, MA, USA). Subsequently, EVs were collected via ultracentrifugation at 150,000 $\times$ g for 1 hour at 4°C. The pelleted EVs were washed

twice with phosphate-buffered saline (PBS) and resuspended in PBS. To ensure sterility, EV samples were plated on LB agar. The protein concentration was quantified using the Pierce BCA Protein Assay.

Additionally, EV quantification was performed using the lipophilic fluorescent dye FM4-64 (Thermo Fisher Scientific, Barcelona, Spain), which integrates into the vesicle membrane. This method allowed for verification that equal amounts of EcN EVs and EcoR12 EVs were used in the experimental protocol [14]. For the FM4-64 assay, several dilutions of the stock EV samples were incubated with FM4-64 (5 µg/mL prepared in PBS) for 10 minutes at room temperature. Control reactions included samples containing only EVs or solely the FM4-64 dye. Fluorescence was measured at an excitation wavelength of 515 nm and an emission wavelength of 640 nm using the modular multimode plate reader Varioskan Lux (Thermo Fisher Scientific, Barcelona, Spain). EV samples with equal protein concentrations (expressed as µg protein/mL) exhibited similar fluorescence intensity values. All EV samples were aliquoted and stored at -20 °C until further use.

2.2. Culture of human inducible pluripotent stem cells (iPSC)

SCTi003-A cells are human pluripotent stem cells initially derived and isolated from a healthy female donor's peripheral blood mononuclear cells (STEMCELL Technologies; #200-0511). They were obtained from StemCell Technologies, Vancouver, Canada) and cultured according to StemCell Feeder-Independent Pluripotent Stem Cell Protocols. Briefly, SCTi003-A cells were seeded onto Matrigel-coated 6-well plates (Matrigel™; Corning, New York, USA) in mTeSR™1 medium (StemCell Technologies, Vancouver, Canada) and maintained in a humidified incubator at 37°C with 5% CO₂. The culture medium was replenished daily, and cells were passaged every 5 days at a 1:6 ratio using Dispase (MilliporeSigma, Burlington, Massachusetts, USA). Before passaging, SCTi003-A cells were examined under a microscope for any signs of differentiation, as differentiated cells exhibit distinct morphological traits compared to undifferentiated embryonic stem cell colonies. According to the manufacturer's guidelines, when the differentiation rate exceeded 5%, differentiated cells were selectively removed using a micropipette tip. H1 cells were continuously maintained under these conditions and routinely screened for the expression of pluripotency markers using immunofluorescence analysis.

2.3. Culture of human iPSC-derived neurons

The iPSC line SCTi003-A was utilized for neural progenitor cells (NPC) and neuronal monocultures. The iPSC lines were maintained under feeder-free conditions on vitronectin XF-coated surfaces (STEMCELL Technologies) using supplemented StemFlex medium (Thermo-Fisher Scientific). To prepare the coating, 40 μ L of vitronectin XF was diluted in 1 mL of Cell Adhere dilution buffer (STEMCELL Technologies) and added to each well of a 6-well plate. Cells were cultured as adherent monolayers with media changes every 2–3 days and were passaged weekly at a 1:6 ratio using ReLeSR (STEMCELL Technologies) according to the manufacturer's instructions. Cultures were incubated at 37°C in a humidified incubator with 5% CO₂. A previously established protocol was used to differentiate iPSCs into neural precursor cells (NPCs). iPSC cells were detached with Accutase cell detachment solution (STEMCELL Technologies) according to the manufacturer's instructions and seeded at a density of 3×10^5 cells per well on Matrigel-coated (Corning) 6-well plates. Cells were cultured in Rapid Differentiation Media (RDM), consisting of a 1:1 mixture of DMEM/F12 and Neurobasal medium (NBM) (Gibco, Thermo-Fisher Scientific), supplemented with 1X Gibco N-2 supplement (Thermo-Fisher Scientific), 1X MACS NeuroBrew-21 (B21) without Vitamin A (Miltenyi Biotec), 1X GlutaMAX, 100 units/mL penicillin, and 0.1% w/v streptomycin. Small molecules and inhibitors were included in RDM to promote neural lineage commitment, namely 4 mM CHIR99021, 3 mM SB 431542, 10 ng/mL human LIF, and 0.1 mM Compound E (all from STEMCELL Technologies). Cells were cultured under these conditions for 7 days and then passaged at a 1:3 ratio onto Matrigel-coated plates. Induced NPCs were cultured in RDM basal media supplemented with 3 mM CHIR99021, 2 mM SB 431542, and 10 ng/mL human LIF (STEMCELL Technologies) on Matrigel (Corning) coated 6-well plates. These cells were cultured as adherent monolayers and passaged at a 1:6 ratio using Accutase (STEMCELL Technologies) according to the manufacturer's instructions, and incubated at 37°C in a humidified incubator with 5% CO₂. NPCs from Passage 3 to Passage 8 were used for neuronal differentiation. An adapted differentiation protocol was employed to generate mature neurons. Using Accutase (STEMCELL Technologies), NPCs were detached and dissociated into single cells by passing through a 40 μ m cell strainer. Cells were then seeded at a density of 3×10^4 cells per well onto 8-well slide chambers (iBidi) pre-coated with 20 mg/mL poly-L-ornithine (Sigma-Aldrich) and 20 mg/mL laminin (Gibco). Neuronal cultures were maintained in NBM (Gibco, Thermo-Fisher Scientific) supplemented with 1X Gibco N-2 supplement (Thermo-Fisher Scientific), 1X MACS NeuroBrew-21 (B21) (Miltenyi Biotec), 1X GlutaMAX, 100 U/mL penicillin, and 0.1% w/v streptomycin (N2B21 media). To support neuronal maturation, the N2B27 medium

was further enriched with 20 ng/mL BDNF, 20 ng/mL GDNF, 100 mM dibutyl-*c*-AMP, 200 nM ascorbic acid, and 200 nM DAPT (all from STEMCELL Technologies). Neurons were cultured as adherent monolayers for up to 28 days at 37°C in a humidified incubator with 5% CO₂.

2.4. Culture of human iPSC-derived Intestinal organoids

Human intestinal organoids (HIOs) were developed from SCTi003-A stem cells using the STEMdiff™ Intestinal Organoid Kit (STEMCELL Technologies, Vancouver, Canada), following the manufacturer’s guidelines (Figure 1). Briefly, SCTi003-A cells were seeded onto Matrigel-coated 24-well plates and cultured in mTeSR™1 medium (STEMCELL Technologies, Vancouver, Canada) until they reached the appropriate confluency. To induce differentiation, SCTi003-A cells were grown in an endoderm basal medium supplemented with Activin A, with daily media changes for three days to generate definitive endoderm (DE). The DE was then maintained in an endoderm basal medium containing Wnt3A and fibroblast growth factor 4 (FGF4) for 5–6 days until mid-/hindgut (MH) spheroids were released into the supernatant. The MH spheroids were collected, counted, and embedded in Matrigel (Corning, New York, USA) domes, where they were cultured in intestinal organoid basal (IOB) medium supplemented with epidermal growth factor (EGF) and Noggin until HIOs were finally formed.

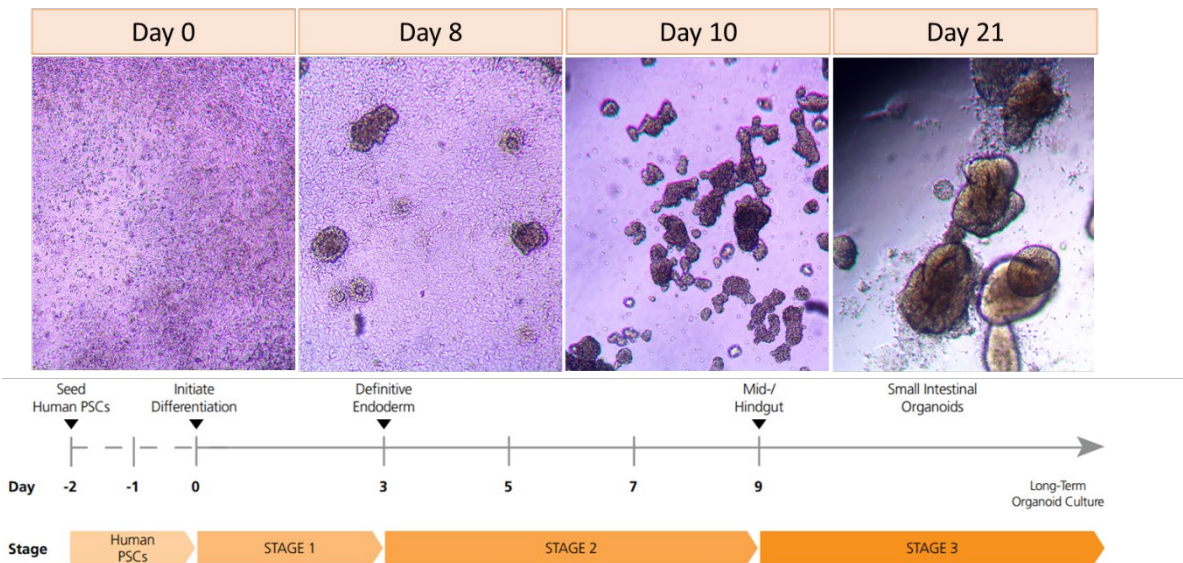


Figure 1. Protocol for the differentiation of 3D organoids from iPSC. The diagram shows the different stages of the differentiation process. Images were obtained from cultures on the indicated days.

Once established, the organoids were maintained in EGF- and Noggin-enriched IOB medium, with media changes every 3–4 days and passaging every 10 days at a 1:3 ratio. HIOs and their intermediate developmental stages were characterized using immunofluorescence to assess their progression and structural integrity. HIOs were cultured to passages 4–5, with stable intestinal architecture observed throughout this period.

2.5. Immunohistochemistry of Intestinal organoids

Primary antibodies were obtained from Abcam (Anti-Claudin-3 polyclonal antibody, ab15102; Anti-GFAP monoclonal antibody [GF5], ab10062), Thermo-Fisher-Scientific (ZO-1 polyclonal antibody, 61–7300), and Santa Cruz (E-cadherin, sc-8426; villin sc-58897). Secondary antibodies were sourced from Dako (HRP conjugated) and ThermoFisher (AlexaFluor conjugated).

Organoids were released from the Matrigel domes by incubating in 2 mM EDTA for 5 minutes on ice. Then, the organoids were centrifuged at 1000 rpm for 5 minutes. The resulting pellet was resuspended in 4% paraformaldehyde (PFA) and fixed at room temperature for 15 minutes. After fixation, the organoids were washed three times with 1X PBS to remove unreacted PFA. For blocking and permeabilization, the organoids were incubated at room temperature for 1 hour in PBS containing 10% horse serum and 0.1% saponin. After incubation, the organoids were centrifuged (1000 rpm, 5 minutes), resuspended in a primary antibody solution prepared in 10% horse serum and 0.1% saponin in PBS, and incubated overnight at 4°C. The next day, the organoids were washed three times with 1X PBS, incubated with secondary antibodies in the dark at room temperature for 1 hour, and washed three times with 1x PBS. Finally, the organoids were resuspended in mounting medium (F4680, Sigma-Aldrich), transferred onto microscope slides, and imaged using the ImageXpress Micro XL Confocal 50 µm slit High Content Screening System (Molecular Devices), equipped with a Nikon 20X S Plan Fluor air objective.

2.6. Immunostaining of neurons

Primary antibodies were obtained from Abcam (anti-MAP2; Aveslabs Cat. no. MAP-0020; ICC: 1:1000), while secondary antibodies were sourced from Dako (HRP conjugated) and ThermoFisher (AlexaFluor conjugated).

Cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and were washed with phosphate-buffered saline (PBS). Next, the cells were incubated in a blocking solution containing 10% fetal calf serum (FCS) in PBS and subsequently

permeabilized with 0.3% Triton X-100 for 30 minutes at room temperature. The primary antibodies were diluted in a blocking solution and incubated overnight at 4°C. After PBS washes, the secondary antibodies were added and incubated for 1 hour at room temperature in darkness. Finally, the nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, Missouri, USA) and visualized under a fluorescent microscope (Leica DM2000; Leica Microsystems GmbH, Germany). A minimum of 50 cells from three separate experiments were counted.

2.7. Morphometric analysis of neurons

Sholl analysis was performed using ImageJ Fiji software v1.51 to quantitatively assess dendritic morphology. Images of MAP2- and DAPI-positive neurons were acquired using an LSM880 confocal microscope (Carl Zeiss, Oberkochen, Germany). Neurons were selected based on the following criteria: low dendrite entanglement with other neuronal dendrites or no aggregation with adjacent cells. A blinded method was employed during selection and analysis to minimize bias. Neurons matching the selection criteria were systematically analyzed. The coverslip was scanned in a structured pattern starting from the top-left corner to the top-right and then to the bottom-right and bottom-left. Morphometric analysis followed the same scanning direction and continued until twenty neurons were analyzed per coverslip. Each treatment group included 4–5 coverslips, all obtained from a single dam dissection. Each neuron was manually traced on a computer using a transparent overlay layer superimposed on the neuron's image. These traced outlines were then used for Sholl analysis, where equidistant concentric circles (10–300 μm) were centered on the soma. The number of dendrite intersections with each concentric circle was recorded to assess dendritic complexity. Additionally, the number of primary, secondary, and tertiary dendrites per neuron was quantified. Dendritic branches were manually counted from high-resolution immunofluorescence images, as described in the previous section. To ensure consistency and accuracy, only distinguishable branches were included, and each branch category was defined based on its order of emergence from the soma.

2.8. Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS v22.0) (IBM, Chicago, IL, USA). Data were tested for normal distribution (by Shapiro–Wilk) and variance equality (by Levene's test). A conventional one-way ANOVA test followed Tukey's post-test when data were homogeneous and had a normal

distribution, with the experimental group as the independent variable. Differences were considered significant at $p < 0.05$. The results were expressed as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Regulation of intestinal barrier permeability in iPSC-derived gut organoids by EcN and EcoR12 EVs

Disturbances in intestinal permeability and low-grade chronic inflammation have been linked to blood-brain barrier dysfunction via the microbiota–gut–brain axis [19]. In this study, we investigated the potential of EVs from EcN and EcoR12 to regulate the expression of proteins involved in gut barrier function using human iPSC-derived intestinal organoids.

Organoids were cultured and maintained under standard conditions with daily monitoring and medium changes every three days until day 30 (passage 7). On day 30, organoids were treated with EVs isolated from EcN or EcoR12 strains at two concentrations (5 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$) for 24 hours. Untreated organoids served as the control. As no significant effects were observed at 25 $\mu\text{g/mL}$, results are presented only for the 5 $\mu\text{g/mL}$ condition (Figure 2). Following treatment, samples were collected for protein analyses.

To evaluate the impact of EcN- and EcoR12-derived EVs on intestinal permeability, we examined the expression of three proteins that are key components of the epithelial barrier: the tight junction protein ZO-1 (red), the adherens junction protein E-cadherin (yellow), and the apical cytoskeletal protein villin-1 (green). Immunofluorescence staining, followed by confocal laser scanning microscopy, was used to assess both protein localization and expression levels. Representative confocal images are presented in Figure 2A.

Protein signal intensity was quantified as described in the *Materials and Methods* section, using data from three independent biological replicates, each comprising 10–15 organoids across multiple fields of view. Quantitative analysis revealed a significant increase in E-cadherin and villin-1 fluorescence intensity ($p < 0.05$) in both EcN EcoR12 EV-treated groups compared to the control. Notably, ZO-1 expression also increased in both EV-treated groups; however, statistical significance ($p < 0.05$) was only observed in organoids treated with EcN-derived EVs (Figure 2B).

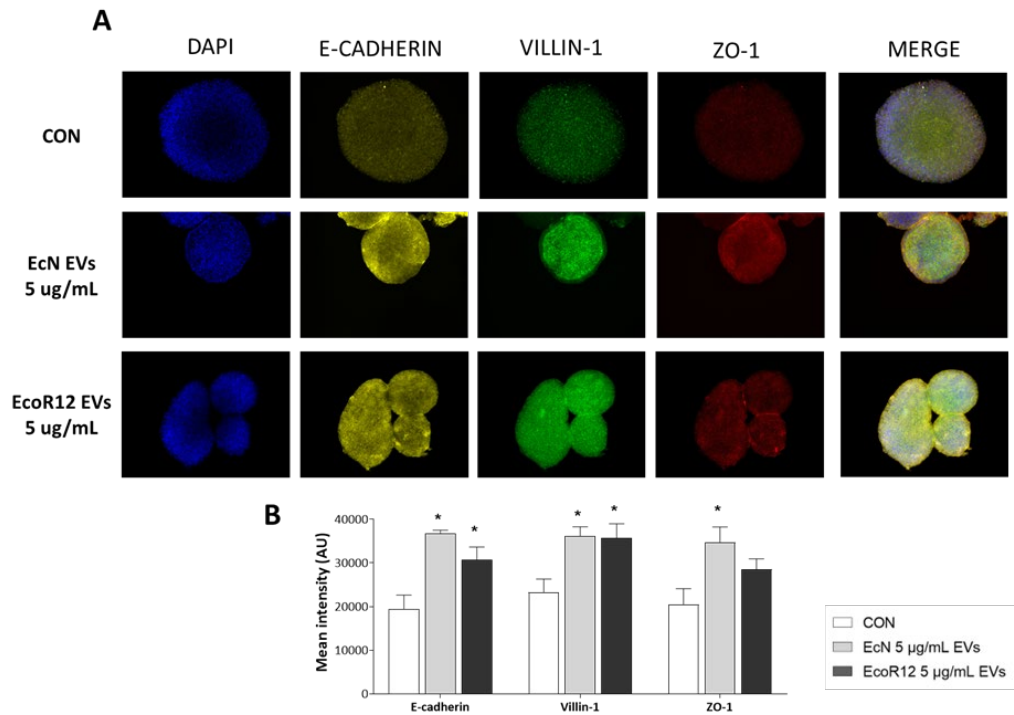


Figure 2. Expression and localization of epithelial barrier-associated proteins in iPSC-derived intestinal organoids treated with EcN or EcoR12-derived EVs. (A) Immunofluorescence staining of intestinal organoids after 24-hour exposure to EVs derived from EcN or EcoR12 at a 5 µg/mL concentration. The proteins analyzed included ZO-1 (red), E-cadherin (yellow), and villin-1 (green), and the nuclei were counterstained using DAPI (blue). Merged images showed the colocalization of these proteins in the epithelial monolayer. **(B)** The bottom panels display a quantitative analysis of fluorescence intensity for each marker under the indicated conditions. Data are expressed as mean ± SEM from three independent biological replicates. Statistical differences were assessed with one-way ANOVA, followed by post hoc Tukey's. * $p \leq 0.05$, vs. control untreated organoids.

3.2. Dose-dependent regulation of claudin-3 in IPSC-derived gut organoids by EcN and EcoR12 EVs

To further evaluate the effect of EcN and EcoR12 EVs on intestinal epithelial tight junctions, we analyzed the expression of claudin-3, a key transmembrane component of the tight junction complex (Figure 3). In contrast to ZO-1, which primarily functions as a scaffolding adaptor linking transmembrane proteins to the actin cytoskeleton, claudin-3 plays a direct role in forming the paracellular barrier by regulating ion selectivity and sealing the intercellular space between epithelial cells. This structural

function positions claudin-3 as a central determinant of epithelial barrier integrity and permeability [20,21].

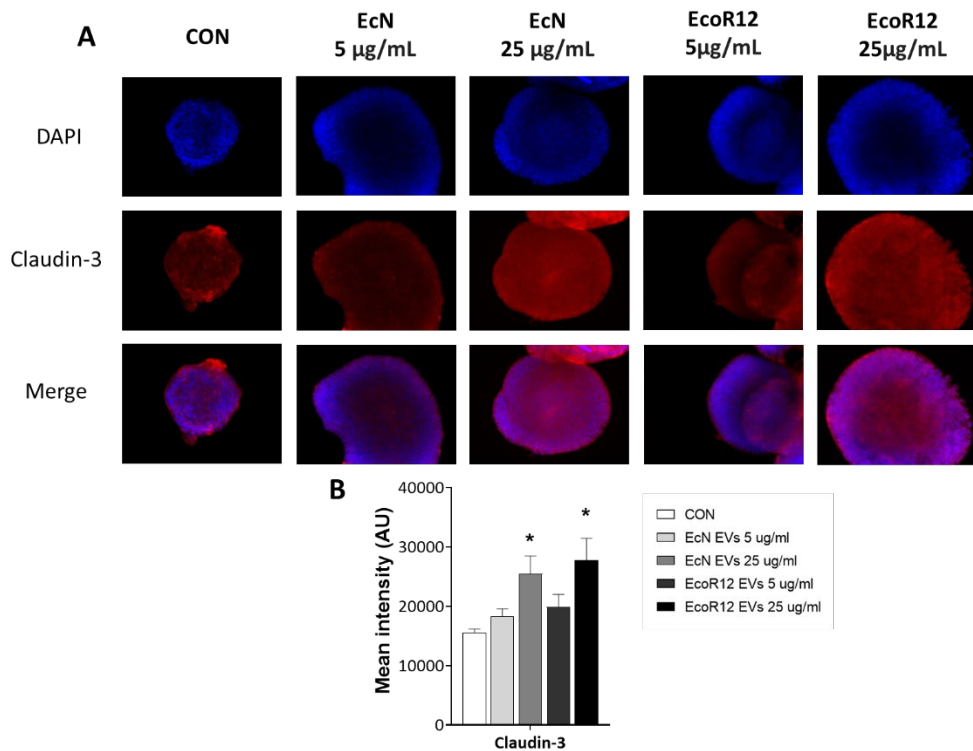


Figure 3. Expression and localization of claudin-3 in iPSC-derived intestinal organoids treated with EcN or EcoR12 EVs. (A) Representative immunofluorescence images of intestinal organoids stained for claudin-3 (red) after 24-hour exposure to EVs derived from EcN or EcoR12 at 5 µg/mL and 25 µg/mL concentrations. Nuclei were counterstained with DAPI (blue). Claudin-3 was localized at the cell borders, indicating tight junction formation and epithelial monolayer organization. **(B)** Quantification of claudin-3 fluorescence intensity under each condition. Data are expressed as mean ± SEM from three independent biological replicates (10–15 organoids per condition). Statistical differences were assessed with one-way ANOVA, followed by post hoc Tukey's. * $p \leq 0.05$, vs. control untreated organoids.

Immunofluorescence analysis revealed a significant increase in claudin-3 signal intensity following a 24-hour treatment with the higher dose (25 µg/mL), regardless of whether the EVs were generated by the probiotic EcN or the commensal EcoR12, compared to untreated controls ($p < 0.05$) (Figure 3). Claudin-3 upregulation correlated with an enhanced fluorescent signal, suggesting a reinforcement of tight junction integrity. In contrast, no significant changes were detected at the lower EV concentration (5 µg/mL) compared to the control, indicating a dose-dependent regulatory effect specific to claudin-3.

3.3. Regulation of neuron development by EcoR12 EVs using the *in vitro* model of iPSCs

To evaluate the impact of EcoR12 EVs on neuronal morphology and dendrite organization, cultures of iPSC-derived neurons were treated with two concentrations of EVs (1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$) throughout the 20-day maturation period. EVs were replenished with a media change every 3–4 days. Morphometric analysis was conducted to quantify changes in dendritic size, cell number, and nuclear area (Figure 4).

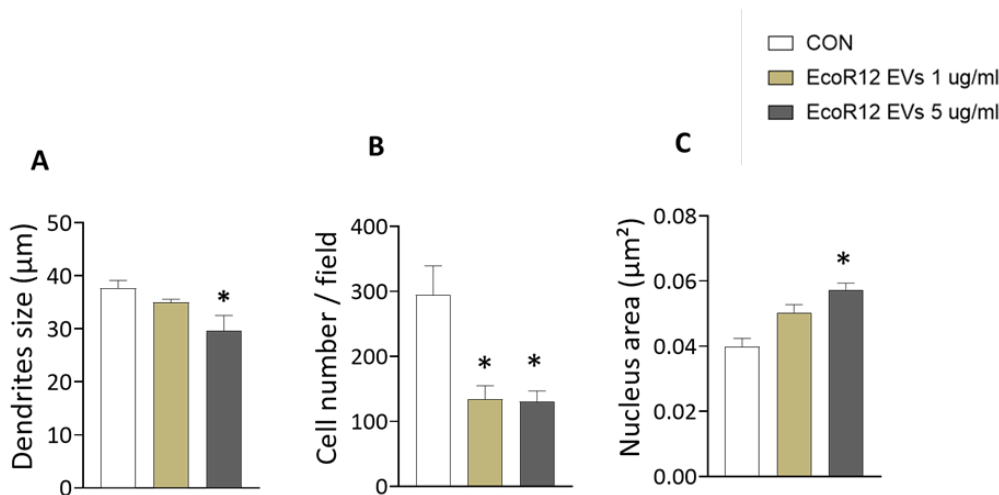


Figure 4. Morphological analysis of iPSC-derived neurons treated with EcoR12-derived EVs. Neurons were exposed to EcoR12 EVs at concentrations of 1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ for 24 hours. Morphological parameters were quantified and compared to untreated control neurons. **(A)** Quantification of dendritic length in control and EV-treated neurons. **(B)** Quantification of total cell numbers per field of view under each condition. **(C)** Quantification of the nuclear area in control and EV-treated neurons. Data are presented as mean $\pm$ SEM from three independent experiments. Statistical differences were assessed with one-way ANOVA, followed by post hoc Tukey's. * $p \leq 0.05$, vs. control untreated neurons.

Treatment with EcoR12 EVs resulted in a significant ($p < 0.05$) dose-dependent reduction in dendritic size and length compared to untreated control neurons, with the most pronounced decrease observed in the 5 $\mu\text{g}/\text{mL}$ -treated group. Similarly, EV treatment led to a significant reduction in cell numbers at both EV concentrations tested ($p < 0.05$) (Figure 4B). In contrast, the neuronal nuclear area increased following EV treatment, with the greatest effect observed in the 5 $\mu\text{g}/\text{mL}$ condition ($p < 0.05$) (Figure 4C).

We applied the Sholl analysis method to evaluate dendritic morphology and development [22]. Neuronal arborization was assessed through immuno-fluorescence

staining, using MAP-2 (red) as a dendritic marker and DAPI (blue) to visualize neuronal nuclei (Figure 5).

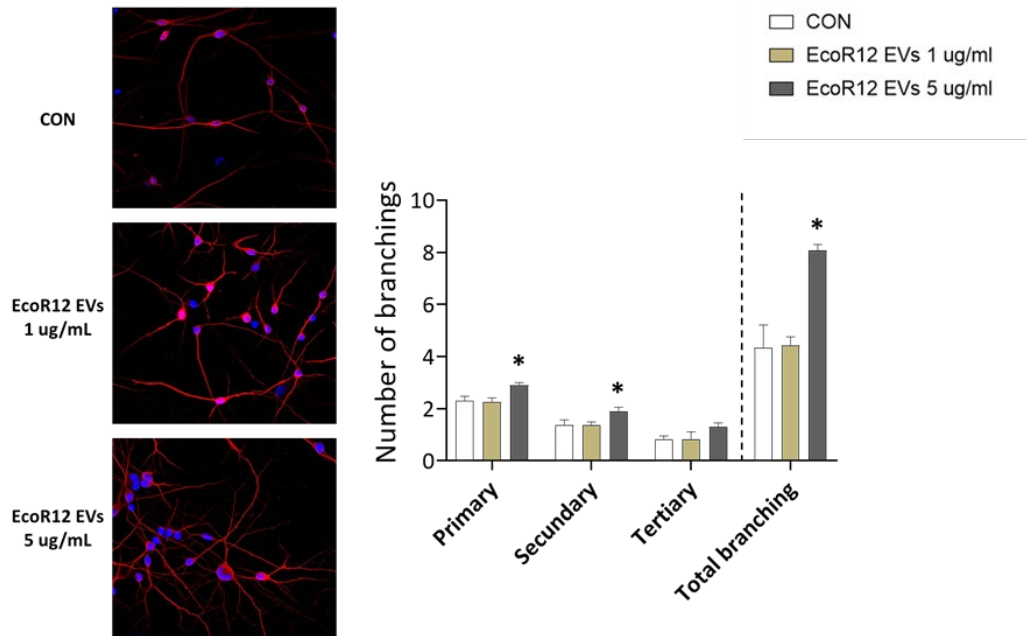


Figure 5. Morphological analysis of neuronal dendrites. Neurons were fixed and stained with MAP-2 (red) and DAPI (blue) to assess dendritic structural features. (A) Representative fluorescence images showing neuronal morphology under different experimental conditions. (B) Quantification of dendritic branching in control and EV-treated neurons. Data are presented as mean $\pm$ SEM from three independent experiments. Statistical differences were assessed with one-way ANOVA, followed by post hoc Tukey's. * $p \leq 0.05$, vs. control untreated neurons.

Stimulation with EcoR12 EVs induced a dose-dependent increase in dendritic complexity. Quantitative analysis revealed that exposure to a higher dose (5 $\mu\text{g/mL}$) significantly increased both primary and secondary branching. The total number of dendritic branch points per neuron was significantly higher in the high-dose group than in the control and low-dose conditions ($p < 0.05$), indicating that EcoR12 EVs promote dendritic outgrowth and arborization.

4. Discussion

Modern lifestyle factors such as chronic stress, unbalanced diets, physical inactivity, and aging are significant contributors to a growing number of disorders, including

inflammatory, metabolic, and neurological conditions. A common underlying factor in many of these pathologies is the disruption of the gut microbiota. Changes in microbial composition and diversity are frequently linked to increased intestinal permeability, systemic low-grade inflammation, and the spread of microbial-derived molecules beyond the gut.

Loss of intestinal barrier integrity allows for the translocation of microbial components into the lamina propria, triggering immune activation and promoting inflammation at both local and systemic levels. This process affects gastrointestinal homeostasis and contributes to distant organs' dysregulation [23]. Circulating microbial metabolites and pro-inflammatory mediators have been shown to interfere with immune and metabolic functions in tissues such as the liver, adipose tissue, and the brain [24–26]. Consequently, preserving the structure and function of the intestinal barrier has become a central goal in strategies aimed at preventing or mitigating complex, multi-organ disorders. The gut-brain axis has emerged as a key regulatory pathway linking intestinal health to neurological outcomes. Increasing evidence suggests that microbiota-derived signals play a pivotal role in modulating brain physiology, including neurodevelopment, synaptic function, and behavior [27].

The well-established link between gut microbiota and host health has led to the development of targeted nutritional and therapeutic strategies aimed at restoring microbial balance and supporting barrier function. Dietary interventions are among the most studied approaches due to their significant influence on microbiota composition. However, short-term modifications often fail to induce lasting effects, while long-term outcomes depend on the duration of the intervention and individual adherence [28]. Probiotics have emerged as a widely explored option for modulating gut microbiota and counteracting intestinal dysfunction. Nevertheless, their use is not without limitations. In immunocompromised individuals, introducing live bacteria may pose a health risk, and the viability of probiotic strains during storage and delivery remains a challenge for consistent application in food and pharmaceutical contexts. These concerns have stimulated the search for safer and more controlled alternatives.

In this regard, postbiotics have gained momentum [29,30]. Postbiotics refer to non-viable microbial products and metabolic byproducts that retain the capacity to interact with host cells and modulate physiological processes. This group includes various compounds such as bacterial surface structures, enzymes, peptides, organic acids, and secreted signaling molecules. Their favorable stability, safety, and targeted bioactivity make them attractive candidates for developing microbiome-based therapies [31,32]. Among postbiotic candidates, microbiota EVs have emerged as potent mediators of

host–microbiota communication. These nanoscale structures, naturally secreted by both probiotic and commensal bacteria, transport a diverse molecular cargo capable of penetrating the mucus layer, engaging with epithelial and immune cells, and eliciting systemic responses. Importantly, due to their ability to circulate beyond the gut, microbial EVs may also participate in interorgan signaling, including direct communication with the brain [33].

Several *in vivo* studies have demonstrated that oral administration of EVs derived from probiotic and commensal bacteria, including EcN, *Clostridium butyricum*, *Bacteroides fragilis*, and *Akkermansia muciniphila*, can enhance intestinal barrier function [16,34–36]. These effects are mediated through the modulation of immune and inflammatory responses, reinforcement of tight junction proteins, and regulation of mucin-associated pathways. The efficacy of *A. muciniphila* EVs in improving gut permeability has also been confirmed in a mouse model of diet-induced metabolic dysfunction [37].

Based on these findings, we explored the ability of EVs derived from the probiotic EcN and the commensal strain EcoR12 to modulate gut barrier integrity by analyzing the expression of ZO-1, claudin-3, villin-1, and E-cadherin in intestinal organoids derived from iPSCs. Treatment with EVs from both strains resulted in a significant upregulation of ZO-1, villin-1, and E-cadherin at low concentrations, suggesting that microbiota-derived EVs can reinforce epithelial integrity at multiple levels. These proteins play key roles in the structural and functional maintenance of the intestinal barrier [38,39]. The coordinated upregulation of these components reinforces the notion that EVs activate epithelial remodeling processes involved in junctional integrity and tissue stability. Notably, ZO-1 downregulation has been reported in an irritable bowel syndrome-like mouse model subjected to chronic stress, highlighting the involvement of tight junction disruption in gut-brain axis dysregulation [40].

Additionally, we observed that claudin-3 expression was increased by EV treatment from both strains, but only at the higher dose, indicating a dose-dependent effect. This dose-responsive modulation suggests that distinct EV concentrations may selectively engage signaling pathways, potentially influencing the balance between epithelial maintenance and plasticity. Claudin-3 is a major sealing component of TJs, and its loss has been associated with impaired barrier function, increased paracellular permeability, and microbiota-driven dysbiosis [41]. These findings support the hypothesis that specific EV-associated signals modulate tight junction composition and could act as regulators of epithelial plasticity.

After evaluating the capacity of EcN and EcoR12 EVs to reinforce the intestinal epithelial barrier, we next assessed whether these vesicles could also influence

neuronal development. For this purpose, we analyzed human iPSC-derived neurons treated with EVs from the commensal strain EcoR12, focusing on key morphological parameters related to neuronal maturation. Although we observed a reduction in the number of neurons following treatment, dendritic branching and nuclear size significantly increased in EV-treated cells. These findings suggest that bacterial EVs may modulate the early stages of neuronal differentiation, potentially promoting neuronal maturation and functional organization. Dendritic arborization is a critical process during neurodevelopment, as it determines the ability of neurons to integrate synaptic inputs and form functional networks. It has been shown that synaptic activity regulates dendritic branching, contributing to the refinement and maturation of neuronal circuits [42]. Similarly, nuclear enlargement has been associated with increased transcriptional activity and metabolic demand in developing neurons, reflecting changes in gene expression programs required for differentiation and structural complexity [43].

The observed morphological changes may reflect an EV-driven activation of intracellular pathways involved in cytoskeletal remodeling, neurite outgrowth, or transcriptional regulation. Gene expression analysis will provide more detailed insights into the molecular mechanisms involved in microbiota EV-mediated regulation. Components present in the EV cargo, such as microbial proteins, small RNAs, or metabolites, could influence these processes by engaging host receptors or modulating epigenetic regulators; however, the specific mediators remain to be identified.

Together, these results highlight the dual capacity of microbiota-derived EVs to enhance intestinal barrier integrity and promote structural features associated with neuronal maturation. This dual action supports the growing concept of bacterial EVs as key effectors in gut-brain communication. Given the central role of intestinal permeability and neurodevelopment in the pathophysiology of various disorders, including those involving chronic inflammation, dysbiosis, or neuroinflammation, our findings provide a foundation for future research exploring microbiota-derived EVs as postbiotic therapeutic tools targeting the gut-brain axis. While our findings in iPSC-derived models are promising, further *in vivo* studies are needed to confirm the systemic and neurological effects of microbiota EVs under physiological conditions.

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Conflicts of Interest: The authors of this study disclose that they have no conflicts of interest to declare.

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DISCUSSION

The lifestyle of modern societies has a profound impact on human health. Stress, social habits, Western diet, physical inactivity, and aging are among the main factors predisposing individuals to a wide spectrum of inflammatory, metabolic, neurological, and infectious diseases. Intestinal homeostasis and overall health are strongly dependent on the gut microbiota and the integrity of the intestinal epithelial barrier. Disruptions in microbial composition and diversity, commonly referred to as dysbiosis, along with increased intestinal permeability are recurring features across these disease states [323].

Beyond chronic disorders, dysbiosis and barrier dysfunction are increasingly recognized as key contributors to infectious diseases. Viruses, in particular, can both result from and further aggravate intestinal imbalance. Rotavirus, one of the leading causes of severe diarrhea in infants and young children worldwide, exemplifies this interaction. Upon infection, rotavirus induces epithelial damage, villus atrophy, and a disruption of tight junction proteins leading to enhanced paracellular permeability [161]. This barrier dysfunction contributes to malabsorption, diarrhea, and systemic immune activation.

Importantly, dysbiosis not only affects local gut immunity but also has systemic repercussions through different gut-organ axes, such as the gut-liver and gut-brain axes. These bidirectional communication pathways allow microbial metabolites and pro-inflammatory mediators to influence liver function and neuroinflammatory processes, contributing to the progression of hepatic and neurological disorders, respectively. In this context, the strong connection between gut microbiota and host health has stimulated the development of novel nutritional and therapeutic strategies aimed at modulating the microbiome to restore dysbiosis and support intestinal homeostasis. Such interventions seek to promote anti-inflammatory profiles, enhance mucosal integrity, and reinforce the epithelial barrier, thereby improving systemic resilience to infection and reducing the severity of microbial translocation and immune activation. Biotic therapies including the use of prebiotics, probiotics, and symbiotic

have emerged as promising tools not only for maintaining intestinal health, but also for modulating immune responses and mitigating the impact of viral infections [324].

Probiotics are among the earliest biotic therapies used as treatment options for the manipulation of the gut microbiota and its impact on disorders associated with intestinal permeability. However, potential risks for immunocompromised individuals and neonates, due to their immature immune systems, along with technological limitations related to maintaining bacterial viability, are considered critical barriers to their widespread application as functional foods. To minimize safety concerns, future microbiome-based therapies targeting the gut are increasingly focusing on the use of postbiotics [209,325,326]. The term postbiotic includes non-replicative bioactive compounds such as metabolites, bacterial components, and secreted factors produced by probiotics or beneficial bacteria in the gut. These molecules are characterized by efficient absorption and distribution, and a strong capacity for signaling across different organs and tissues to elicit specific host responses [211].

Within this framework, microbiota- and probiotic-derived EVs are emerging as next-generation postbiotics, as they meet the criteria and provide the benefits required for inclusion in this category of biotherapy [222,327–329]. Due to their nanometric structure, high stability, and diverse molecular cargo, microbiota-derived EVs can transport and deliver effector molecules to host cells, modulating key signaling pathways and cellular processes, including immune and defense responses. Notably, the effects exerted by microbial EVs are strain-specific, depending on their origin and bacterial content [77]. Importantly, these interventions hold potential far beyond the gut. Microbiota EVs have been shown to cross not only the intestinal barrier but also other biological barriers, which enables them to reach peripheral organs and exert systemic effects [77]. By modulating the gut-liver axis, they can reduce endotoxemia and hepatic inflammation, which are often associated with microbiota-derived metabolites and increased intestinal permeability [243]. Similarly, through the gut-brain axis, they may influence neuroimmune and neuroendocrine signaling pathways,

potentially alleviating inflammation-driven neurocognitive effects of chronic conditions [314,315].

Several *in vivo* studies in murine models have demonstrated that the oral administration of EVs derived from beneficial gut microbes such as *Clostridium butyricum* [297], *Bacteroides fragilis* [330], and *Akkermansia muciniphila* [331] can enhance the integrity and function of the intestinal epithelial barrier. These effects are mediated through diverse mechanisms, including the modulation of immune and inflammatory responses, as well as the regulation of tight junction proteins and mucin-associated molecules [242]. Reinforcement of the intestinal barrier is particularly relevant in the context of viral infections, where increased permeability facilitates pathogen translocation and systemic inflammation.

In this regard, our group has shown that EVs derived from probiotic and commensal *Escherichia coli* strains modulate epithelial barrier function [202] and activate regulatory mechanisms that maintain a balance between pro-inflammatory and anti-inflammatory responses [236,332]. These vesicles also stimulate dendritic cells to shape virus-relevant T cell responses through multiple mechanisms, including TLR signaling, modulation of microRNA expression, and the differential release of immune mediators via host exosomes [241,333]. Such immunomodulatory capacities highlight the potential of microbiota-derived EVs not only in local gut immunity but also in systemic defense. Moreover, according to results from our group, EVs derived from probiotic EcN are able to modulate the expression of serotonin-related genes in intestinal epithelial cells under inflammatory conditions, suggesting a novel mechanism by which they may influence the gut-brain axis [334]. In a complementary study, we demonstrated that EcN-derived EVs reduce the expression of the peptide transporter PepT1 in IL-1 β -treated Caco-2 cells, revealing their role in regulating nutrient transport and inflammatory signaling at the intestinal barrier [335]. Additionally, oral administration of EcN EVs improved colitis progression in DSS-treated

mice, further supporting their protective role in maintaining epithelial integrity and reducing intestinal inflammation [240].

Despite the increasing evidence supporting the beneficial properties of EVs derived from commensal and probiotic bacteria in modulating intestinal barrier integrity and host immune responses, research into their broader systemic effects remains limited. Initial studies have primarily focused on their local role in the gut, where EVs have been shown to influence epithelial cell function and immune activation. In this context, the neonatal period represents a critical window for the development of the intestinal and immune systems, and EVs have emerged as promising postbiotic candidates to promote immune maturation and epithelial barrier development in a safe manner. However, beyond the local gut environment, few studies have explored the systemic regulatory capacity of bacterial EVs, particularly in relation to their involvement in inter-organ communication through the gut-liver and gut-brain axes in physiologically healthy models.

The main objective of this thesis was to investigate the role EVs derived from probiotic and commensal *Escherichia coli* strains in modulating intestinal function and systemic signaling pathways. This objective was addressed through a series of experimental approaches designed to explore their effects at both local and systemic levels. First, we evaluated the ability of EVs isolated from the probiotic EcN and the commensal EcoR12 to enhance immune and epithelial maturation and to modulate antiviral responses during the neonatal period using two *in vivo* models involving suckling rats: (i) a healthy model (**Article 1**) and (ii) a model of rotavirus infection (**Article 2**). Second, in the healthy neonatal rat model we assessed the biodistribution and presence of EVs in the liver following oral administration, as well as their impact on liver function through the gut–liver communication axis. This was achieved by analyzing the expression of hepatic genes involved in fatty acid metabolism, inflammation, and oxidative stress (**Article 3**). Finally, we investigated the potential role of EVs in gut–brain communication using iPSC-derived models. Specifically, we analyzed the influence of EVs on tight junction

protein expression in iPSC-derived intestinal organoids and on neuronal development using human iPSC-derived neurons (**Article 4**).

EVs derived from EcN and EcoR12 enhance immune development and intestinal maturation in healthy neonatal rats.

The role of microbiota-derived EVs in modulating essential host functions, such as mediating the immunological and defense effects of gut-resident bacteria, has been increasingly recognized. While numerous studies in adult animals and *in vitro* models of mature intestinal systems have confirmed the ability of microbial EVs to influence host immunity and barrier function [77], the molecular mediators and mechanisms involved remain incompletely understood, particularly during the neonatal period. The early postnatal stage represents a critical window for immune and epithelial development, during which host–microbiota interactions may have long-lasting impacts on health.

Building upon our previous findings demonstrating the immunomodulatory and barrier-strengthening properties of EVs released by beneficial *E. coli* strains from the gut microbiota [240], we hypothesized that these vesicles could contribute to the maturation of intestinal and immune functions in early life. Therefore, the work presented in **Article 1** of this thesis investigates the effects of oral administration of EVs derived from the probiotic EcN and the commensal strain EcoR12 on intestinal architecture, immune development, and barrier maturation in a healthy neonatal rat model (Results summarized in Figure 15).

Firstly, the results of this study showed that the body weight, animal behavior and stool characteristics remained unaltered throughout the interventions, indicating that the administration of microbiota- or probiotic-derived EVs during the neonatal period was safe and well tolerated. This is consistent with previous findings in experimental models using adult animals. Having confirmed the safety of the intervention, we next evaluated the effects of EVs isolated from beneficial gut-resident *E. coli* strains on both humoral and cellular immune responses in neonatal rats.

Regarding the humoral response, oral administration of either EcN- or EcoR12-derived EVs led to a significant increase in plasma levels of all immunoglobulin isotypes (IgA, IgM, and IgG) after the 16-day intervention period compared to control animals. These findings align with the well-established immunostimulatory and immunogenic properties of bacterial EVs. Indeed, bacterial vesicles are currently being explored as natural vaccines or as adjuvants in vaccine formulations. EVs derived from pathogenic bacteria have been shown to elicit antibody responses comparable to those generated by whole-cell vaccines [336,337]. Moreover, the use of EVs from non-pathogenic bacterial strains as adjuvants has shown promising results, as immunization with EVs combined with free antigens or EVs loaded with specific antigens induces robust humoral and cellular immune responses without notable adverse effects [338,339]. Focusing on the IgG subclasses, our data showed that both microbiota-EV interventions promoted a greater increase in the Th1-associated IgGs (IgG2b and IgG2c) compared to the Th2-associated subclasses (IgG1 and IgG2a). It is well established that the neonatal immune system exhibits a Th2-biased profile, which progressively shifts towards a Th1-dominant phenotype during postnatal development [340,341]. In this context, the increased Th1/Th2 ratio observed following EV administration suggests that microbiota-derived EVs may contribute to the maturation of the neonatal immune system by promoting a more balanced immune profile.

Consistent with enhanced antibody production and Th1 polarization, animals receiving EVs displayed a higher proportion of splenic cytotoxic Tc, NK, and NKT cells than control animals. Notably, microbiota EVs also increased the proportion of TCR $\gamma\delta$ ⁺ T cells, a primitive T-cell subset that contributes to early innate-like immune responses [342]. In addition, EcoR12-derived EVs significantly promoted the expansion of regulatory T cells (Tregs), in line with previous data from our group showing that EcoR12 EVs can induce tolerogenic dendritic cells [241]. Given the importance of Treg expansion for establishing tolerance during early immune development, these results indicate that microbiota-derived EVs promote balanced immune activation while preserving regulatory mechanisms.

To further investigate immune regulation, we analyzed the expression of immune-related genes in small intestinal tissue. Despite some inter-individual variability, both EcN and particularly EcoR12 EVs upregulated the expression of Toll-like receptors TLR2 and TLR7, which recognize bacterial peptidoglycan and single-stranded RNA, respectively. This activation suggests that EVs may contribute to immune system training by delivering microbial antigens that stimulate the innate PRR-signaling pathways. Importantly, this immune stimulation did not lead to intestinal inflammation. No increases in the expression of the macrophage marker CD68 or the pro-inflammatory cytokine IL-12 were observed. On the contrary, EcN EVs significantly downregulated IL-12 mRNA expression from day 8, with effects sustained through day 16. Similarly, EcN EVs also reduced intestinal COX-2 expression—an enzyme involved in prostaglandin synthesis and inflammation—by day 8, while EcoR12 EVs reduced COX-2 expression by day 16. These findings support the anti-inflammatory properties previously described for EcN-derived EVs [240] and indicate that both EV-based interventions enhance immune maturation in early life without triggering detrimental inflammatory responses.

In terms of intestinal barrier development, the neonatal intestine is characterized by high permeability, facilitated by lower expression of tight junction proteins and higher expression of the neonatal Fc receptor (FcRn), allowing the passive transfer of maternal IgGs and microbial molecules [343]. In our study, interventions with microbiota EVs shifted this profile toward a more mature phenotype. On day 8, EcoR12 EVs significantly upregulated the expression of the tight junction protein occludin (OCLN), whereas EcN EVs significantly reduced FcRn mRNA expression. On day 16, as the intestinal tract matured in the untreated control animals, the expression of these genes became similar in all groups. These early changes, however, suggest that EV administration may accelerate the natural maturation process of the gut barrier.

Moreover, both interventions promoted an intestinal trophic effect by increasing villus area. EcN EVs primarily increased villus width, while EcoR12 EVs enhanced villus length.

This trophic effect may contribute to improved nutrient absorption capacity, further supporting the role of bacterial EVs in promoting gut development during early life.

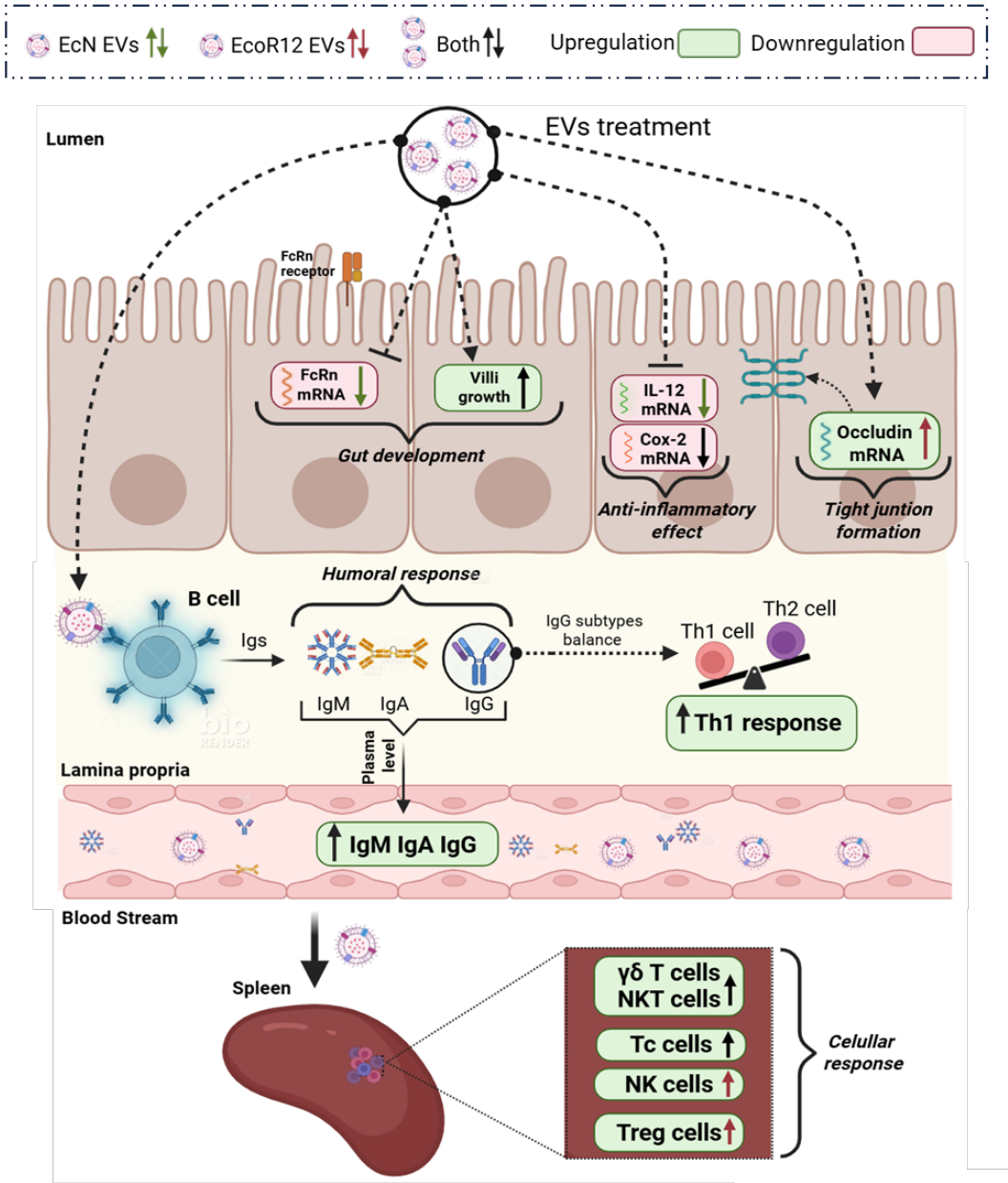


Figure 15. Graphical summary of the key findings presented in **Article 1** of this thesis.

The neonatal rat model resembles the gut immaturity of human preterm newborns, a condition that negatively influences digestion, absorption of nutrients and the pattern of the gut microbiota. In turn, this condition contributes to disturbances in neural, functional and immune development of the intestinal system, which predispose to intestinal complications and infections, such as necrotizing enterocolitis [344–347]. Overall, the results of chapter 1 suggest that interventions involving probiotic/microbiota EVs could be examined as a postbiotic strategy to prevent intestinal infections and help intestinal maturation to meet adequate nutrient absorption in preterm newborns. Also, such interventions could potentially be beneficial for enhancing the immune system and ameliorate allergies in infants or later in life.

EcN and EcoR12 derived-EVs improve intestinal architecture, modulate antiviral immune responses, and reduce diarrhea in a neonatal rat model of rotavirus infection.

RV infection remains one of the leading causes of acute gastroenteritis in children, often resulting in severe diarrhea and significant fluid loss. In recent years, the role of the gut microbiota in preventing and modulating viral infections has gained increasing recognition [348]. Accordingly, beyond standard oral rehydration and nutritional support, the use of probiotics has been evaluated in clinical trials as a strategy to reduce diarrheal severity and other gastrointestinal symptoms [52–56]. Moreover, oral RV vaccines have been incorporated into childhood immunization programs, particularly in low-income countries, to reduce disease burden and prevent mortality. However, their efficacy remains limited, especially in children suffering malnutrition and in poor health and hygiene conditions [349,350].

In this context, probiotic-based interventions offer a promising complementary approach to boost immunity and improve vaccine responses, as demonstrated by *E. coli* EcN in a gnotobiotic piglet model [351]. Similarly, bacterium-like particles derived from immunobiotic *Lactobacillus* strains have been shown to enhance both intestinal and systemic immune responses elicited by attenuated RV vaccines [352]. Given the

potential risks associated with probiotic use in neonates and immunocompromised individuals, current biotic strategies are increasingly shifting toward non-viable alternatives such as postbiotics [211,212].

Therefore, in the study presented in **Article 2**, EVs derived from the probiotic strain EcN and the gut commensal EcoR12 were evaluated as a postbiotic intervention aimed at alleviating clinical symptoms and enhancing immune responses in a preclinical rat model of rotavirus infection during the neonatal period (Results summarized in Figure 16). The EV dosage was established based on previous findings demonstrating the safety and tolerability of oral EV administration from these strains during the neonatal period [237].

In this model, infection with the simian RV strain SA-11 on day 5 of life induced moderate diarrhea without affecting growth parameters, consistent with previous reports using this model [353–355]. Daily administration of EcN or EcoR12 EVs did not alter weight, length, or BMI but significantly reduced the severity and incidence of RV-associated diarrhea. Moreover, EcoR12 EVs shortened the duration of diarrhea. To date, no studies have assessed the *in vivo* impact of microbiota-derived EVs on RV infection. In this thesis, we therefore sought to explore the mechanisms through which these vesicles alleviate diarrheal symptoms.

One key mediator of RV-induced diarrhea is serotonin. RV stimulates serotonin release from enterochromaffin cells via its non-structural protein NSP4, which acts in a calcium-dependent manner. Released serotonin activates vagal afferent fibers linked to vomiting centers and contributes to enhanced intestinal motility and diarrhea, primarily through 5-HT₃ receptors (HTR3) [55,166,172]. In mice, RV infection downregulates the serotonin transporter (SERT) gene in the ileum, without affecting the rate-limiting synthesis enzyme THP1 [172]. Similarly, in our neonatal rat model, RV infection increased intestinal serotonin levels, though it did not alter SERT mRNA expression. This discrepancy may stem from differences in sampling timepoints or intestinal regions analyzed.

Although neither EV intervention reduced serotonin levels, both EcN and EcoR12 EVs upregulated SERT mRNA expression, particularly in the EcN-treated group. SERT is responsible for serotonin reuptake and degradation in enterocytes. Its dysfunction is implicated in several intestinal disorders, including enteric infections and inflammatory bowel disease [52,356]. Notably, beneficial gut bacteria such as *Lactobacillus rhamnosus* GG, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii* have been shown to increase SERT expression [357,358], with EVs from *A. muciniphila* displaying a more potent effect than the live bacterium [357].

Additionally, both EV interventions downregulated HTR3, the gene encoding the HTR3 receptor, in RV-infected animals to levels even lower than those of controls. Since HTR3 is directly involved in serotonin-driven diarrhea, its suppression may be a key mechanism by which these EVs alleviate symptoms. This is consistent with the antidiarrheal effects of HTR3 antagonists [52]. In contrast, no significant differences were found in HTR4 expression, although levels in the EcN-EV group were comparable to controls. HTR4, when activated by serotonin, promotes MUC2 and TFF3 secretion from goblet cells, aiding epithelial repair [359]. Thus, preserving HTR4 expression may support mucosal healing in RV-infected neonates.

The clinical improvement observed in EV-treated pups was accompanied by a reduction in fecal viral shedding on day 6 post-infection, which corresponds to the peak of viral elimination [355,360]. As EVs do not contain live bacteria, these results provide new insights into the protective effects of postbiotic components. Previous *in vitro* studies demonstrated that EcN supernatants could reduce RV replication in colonic epithelial cells, but the specific bioactive factors were not identified [361]. Our results show that EVs act as vehicles for these antiviral factors, extending the findings to both EcN and EcoR12.

Regarding the humoral immune response, the RV group showed no significant increase in anti-RV antibodies compared to controls, consistent with previous reports in neonatal rats [360]. However, both EV-treated groups exhibited higher plasma titers of

anti-RV antibodies at days 8 and 16. These values correlated with reduced viral shedding and improved clinical outcomes. Moreover, EcN and EcoR12 EVs enhanced overall humoral immunity by significantly increasing plasma IgA, IgM, and IgG levels. IgM and IgG responses were evident from day 8, while IgA increases emerged by day 16. These results confirm that viral challenge accelerates vesicle-induced immunogenicity, which is in line with the effects previously observed in healthy neonatal rats, as described in **Article 1** of this thesis.

Analysis of IgG subclass distribution revealed that RV infection shifted the immune response toward a Th1 profile at day 8, a change that normalized by day 16. However, in EV-treated groups, the Th1/Th2 ratio remained elevated on both days, suggesting enhanced and sustained maturation of the immune system. High post-infection titers of anti-RV IgA and IgG are known good correlates of protection [362,363], supporting the hypothesis that microbiota EVs may contribute to long-term immunity. EVs also stimulated cellular innate immunity. RV infection alone increased splenic cytotoxic T cells (TCR $\alpha\beta$ ⁺CD8⁺). EV interventions further increased the frequency of NK⁺CD8⁺ and TCR $\gamma\delta$ ⁺CD8⁺ cells, the latter representing a primitive T cell lineage known for its capacity to rapidly respond to pathogenic challenges [342]. These findings suggest that EVs contribute to the broad immune-enhancing effects attributed to the parent probiotic EcN in various infection models [364–366].

Taken together, the enhancement of humoral and cellular responses supports the potential use of EcN- and EcoR12-derived EVs as vaccine adjuvants in oral immunization strategies against RV. This is particularly relevant given the limited efficacy of existing vaccines in developing regions [350].

Finally, we assessed the expression of immune-related genes in the intestinal mucosa during the diarrheal phase (day 8). EcoR12 EVs promoted the upregulation of TLR2, IL-12, and CD68, which are key markers of innate immune activation and antiviral defense [367–369]. In contrast, EcN EVs did not induce these inflammatory markers but significantly reduced FcRn expression, a receptor involved in maternal IgG uptake

whose expression declines with intestinal maturation [343]. RV infection tended to increase FcRn expression, suggesting a delay in mucosal development. The ability of EcN EVs to normalize FcRn expression indicates their role in supporting intestinal maturation even under pathogenic stress, as previously demonstrated in healthy neonates [237].

Moreover, EcN EVs preserved villus length, a structural marker of absorptive capacity, in RV-infected pups. This effect was not observed in the RV + EV-EcoR12 group. Villus atrophy is a hallmark of RV-induced mucosal damage [370], and protection against this alteration highlights the strain-specific benefits of EcN EVs in maintaining epithelial integrity and promoting functional recovery.

Goblet cells and the secreted mucins also play an active role in host defense during RV infection [370,371]. In response to viral challenge, goblet cells secrete MUC2 and other glycoproteins that enhance the mucus barrier, facilitating the trapping and neutralization of viral particles. However, this defense mechanism must remain balanced, as goblet cell depletion or dysfunction can contribute to local inflammation [372]. In our study, during the acute phase of diarrhea (3 days post-infection), RV-infected animals exhibited reduced MUC2 gene expression and fewer goblet cells per villus in jejunal samples compared to the control group, in line with previous findings in RV-infected mice [175]. The intervention with EcoR12 EVs did not prevent this early loss of goblet cells, whereas treatment with EcN-derived EVs preserved both MUC2 expression and goblet cell density to levels indistinguishable from those of non-infected controls. After diarrhea resolution (day 16), mucin expression and goblet cell numbers in the RV and RV + EV-EcoR12 groups returned to baseline. Notably, animals treated with EcN EVs exhibited significantly elevated MUC2 mRNA levels and goblet cell numbers in the jejunum even after infection clearance, suggesting a prolonged stimulatory effect. The upregulation of MUC2 at day 16 paralleled the increased goblet cell numbers, supporting the notion that EcN-derived EVs not only protect mucus

production during acute infection but may also enhance long-term mucosal barrier function.

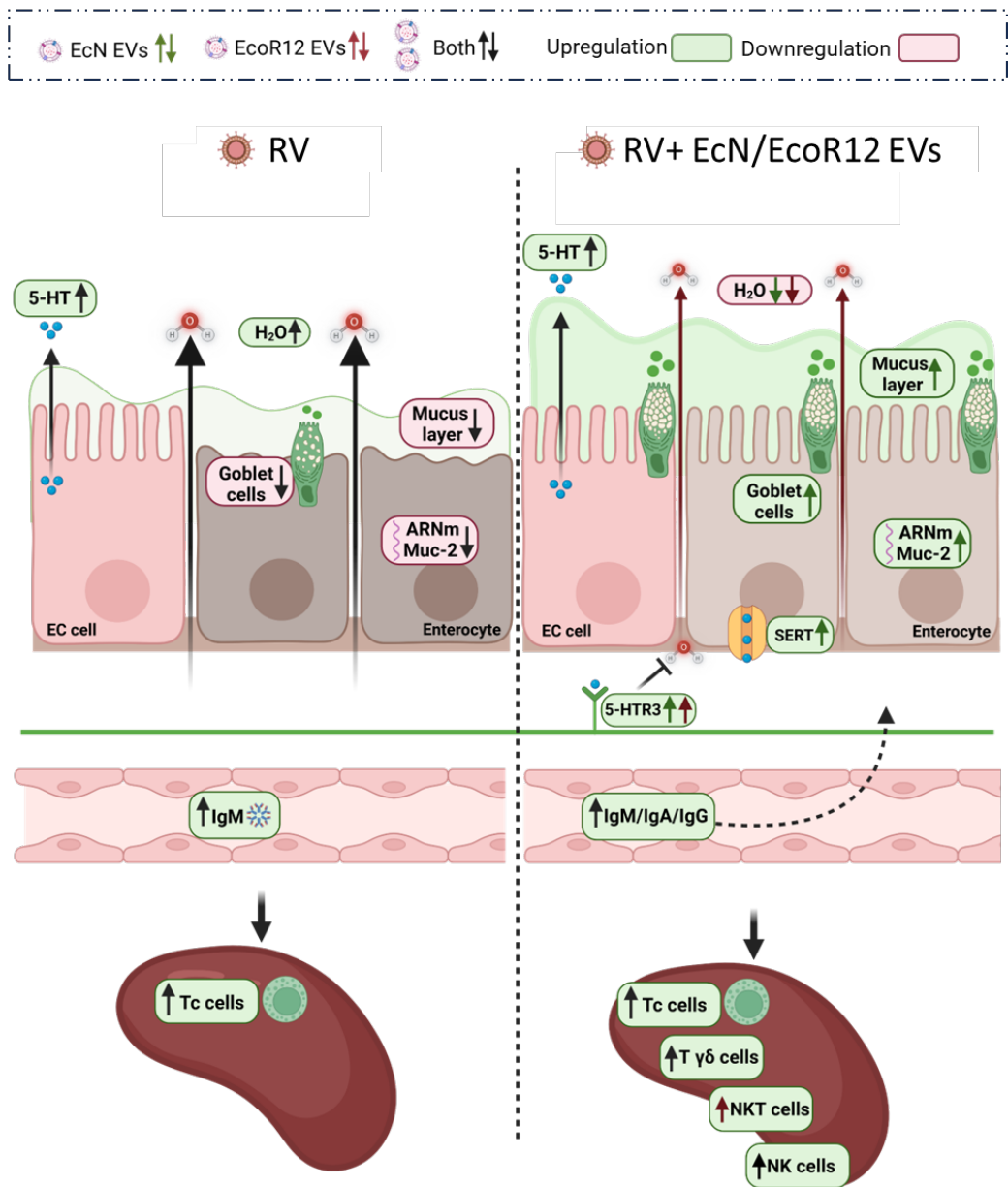


Figure 16. Graphical summary of the key findings presented in **Article 2** of this thesis.

Taken together, the findings presented in **Articles 1 and 2** provide evidence that microbiota-derived EVs represent a safe postbiotic alternative to the use of live probiotic bacteria for alleviating clinical symptoms of rotavirus infection and enhancing both innate and adaptive immune responses during the neonatal period. Importantly, the beneficial effects of interventions involving microbiota or probiotic EVs were found to be strain-specific. Additionally, the results highlight the therapeutic potential of EcN- or EcoR12-derived EVs in protecting newborns against other enteric infections. This is particularly relevant in the context of preterm neonates, who are highly vulnerable to conditions such as necrotizing enterocolitis due to the functional immaturity of their intestinal barrier and immune system [79]. Clinical trials will be necessary to confirm the safety and efficacy of microbiota EV-based interventions in human infants. Furthermore, the ability of EcN and EcoR12 EVs to stimulate humoral and cellular immune responses supports their potential use as adjuvants to improve the immunogenicity and protective efficacy of anti-rotavirus vaccines. In contrast to previous studies that evaluated the probiotic EcN as a vaccine adjuvant in gnotobiotic piglets [351], the present model demonstrates the effectiveness of isolated EVs in ameliorating infection and enhancing immunity in neonatal animals colonized with an indigenous microbiota.

Microbiota-derived EVs as modulators of liver function: insights into the gut–liver axis under healthy conditions

As mentioned earlier, microbiota derived EVs circulate systemically and carry bioactive molecules that facilitate intercellular communication. Under physiological conditions, these vesicles play a pivotal role in maintaining systemic homeostasis. However, under dysbiosis and increased intestinal permeability, they may contribute to disease progression. While numerous studies have explored the local effects of EVs in the gut, particularly their role in modulating barrier integrity and immune responses, their systemic impact remains poorly understood, especially in physiologically healthy models. Among the interorgan axes influenced by gut derived signals, the gut liver axis

is particularly significant, as the liver is continuously exposed to microbial products and metabolites translocated from the intestine via the portal vein.

Although several studies have evaluated the effects of beneficial bacteria and their EVs in the context of liver diseases such as metabolic dysfunction-associated fatty liver disease (MAFLD) and alcohol associated liver disease (ALD) [255,303,373,374], their role in healthy physiological conditions remains largely unexplored. Our study addresses this knowledge gap by investigating the hepatic effects of EVs derived from *Escherichia coli* strains in neonatal rats, a model characterized by an immature intestinal barrier that allows enhanced translocation of luminal contents [341].

While previous chapters focused on the impact of EVs on gut maturation and antiviral immunity during early life, the study presented in **Article 3** shifts the attention toward their influence on liver physiology under homeostatic conditions. To this end, EVs derived from the probiotic strain EcN and the gut commensal EcoR12 were evaluated as a postbiotic intervention to modulate hepatic lipid metabolism, oxidative stress, and inflammatory responses (Results summarized in Figure 17). Two complementary experimental models were employed: (i) a preclinical neonatal rat model and (ii) an *in-vitro* hepatocellular model using HepG2 cells. These models enable the study of physiological EV trafficking and function in the absence of pathology.

As presented in the previous chapters, oral administration of EVs from EcN and the commensal EcoR12 is safe and promotes intestinal and immune development in neonates [237,238]. Here, we prove that these orally administered EVs reach the liver, as evidenced by increased levels of LPS and OmpA in hepatic tissue, natural components of Gram-negative bacterial vesicles. While control animals exhibited basal levels of these markers, likely originating from EV released by endogenous microbiota, the elevated levels in treated animals confirm EV trafficking via the gut liver axis. Our results were consistent with previous findings in mice [375–377]. Moreover, we demonstrated that administered EVs reach the liver through the portal vein as part of the normal gut-liver communication network, rather than as a consequence of

increased paracellular passage induced by the treatment. Notably, after seven days of treatment (day 8 of life), plasma zonulin levels were reduced by EVs from either the probiotic EcN or the commensal EcoR12. This finding confirms the efficacy of administered EVs in counteracting the increased intestinal permeability associated with intestinal immaturity in early life, and suggests their potential applicability in other conditions characterized by a leaky gut. Despite reducing intestinal permeability, the administered EVs reach the liver, confirming the natural trafficking of EVs released by gut bacteria through the gut-liver axis in healthy individuals.

To evaluate the immunological impact of hepatic EV accumulation, we assessed markers of inflammation. EcoR12 EVs did not trigger adverse inflammatory responses, as the expression levels of pro-inflammatory cytokines (IL-12 and TNF- α) and inflammation-associated enzymes (iNOS, COX2) remained comparable to those in control animals at both day 8 and day 16. In contrast, EcN EVs transiently suppressed IL-12 and TNF- α mRNA levels on day 8, suggesting anti-inflammatory effects in a time frame corresponding to the phase of intestinal immaturity and enhanced permeability. This finding aligned with earlier reports describing the anti-inflammatory potential of EcN EVs in other models of increased intestinal permeability [240,334]. By day 16, differences in cytokine expression had resolved, though COX2 and iNOS showed a declining trend in the EcN group. These enzymes, which contribute to inflammation and ROS production, are tightly linked to oxidative stress. Accordingly, we observed that both EcN and EcoR12 EVs downregulated hepatic CYP2E1 expression, a key enzyme associated with ROS generation and disease progression in chronic liver conditions [378]. This regulation was accompanied by a robust induction of key enzymes involved in the cellular antioxidant response, specifically SOD1, CAT, and GPX. By reducing hepatic ROS production and enhancing the antioxidant enzyme arsenal, EVs released by microbiota beneficial *E. coli* strains can protect liver from oxidative stress and subsequent tissue damage. In addition, EVs derived from the probiotic EcN may contribute to the attenuation of liver inflammation.

Regarding hepatic lipid metabolism, both EcN and EcoR12 EVs modulated key pathways known to be altered in fatty liver diseases. Specifically, treatment with these EVs led to the downregulation of genes involved in fatty acid synthesis, including ACC1, FASN, and SREBP1c, while upregulating CPT1 and PPARA, which are associated with fatty acid oxidation. These changes were accompanied by lower expression levels of the cannabinoid receptor CNR1, a regulator of lipogenesis that is elevated in fatty liver diseases [378,379]. Together, these findings indicate that microbiota derived EVs can promote a metabolic profile favoring lipid degradation over accumulation.

To support the physiological relevance of these findings, we used HepG2 hepatocytes exposed to low EV concentrations (5 µg/mL). Both EcN and EcoR12 EVs enhanced antioxidant responses and shifted metabolic gene expression towards an anti-lipogenic profile similarly to *in-vivo* results. Notably, they showed greater efficacy at lower doses than EVs from *A. muciniphila*, a widely studied next generation probiotic [380,381]. This suggests that beneficial *E. coli*-derived EVs may efficiently influence liver function under physiological conditions, supporting their role in systemic regulation.

In summary, our data show that microbiota derived EVs exert effects at both ends of the gut-liver axis. In the intestine, EVs help reduce epithelial permeability, limiting the translocation of luminal antigens and harmful compounds to the liver. Upon reaching the liver via the portal circulation, they modulate gene expression patterns associated with antioxidant responses and lipid metabolism, protecting against oxidative stress and metabolic injury. Notably, EVs derived from EcN also exhibit anti-inflammatory effects in hepatic tissue.

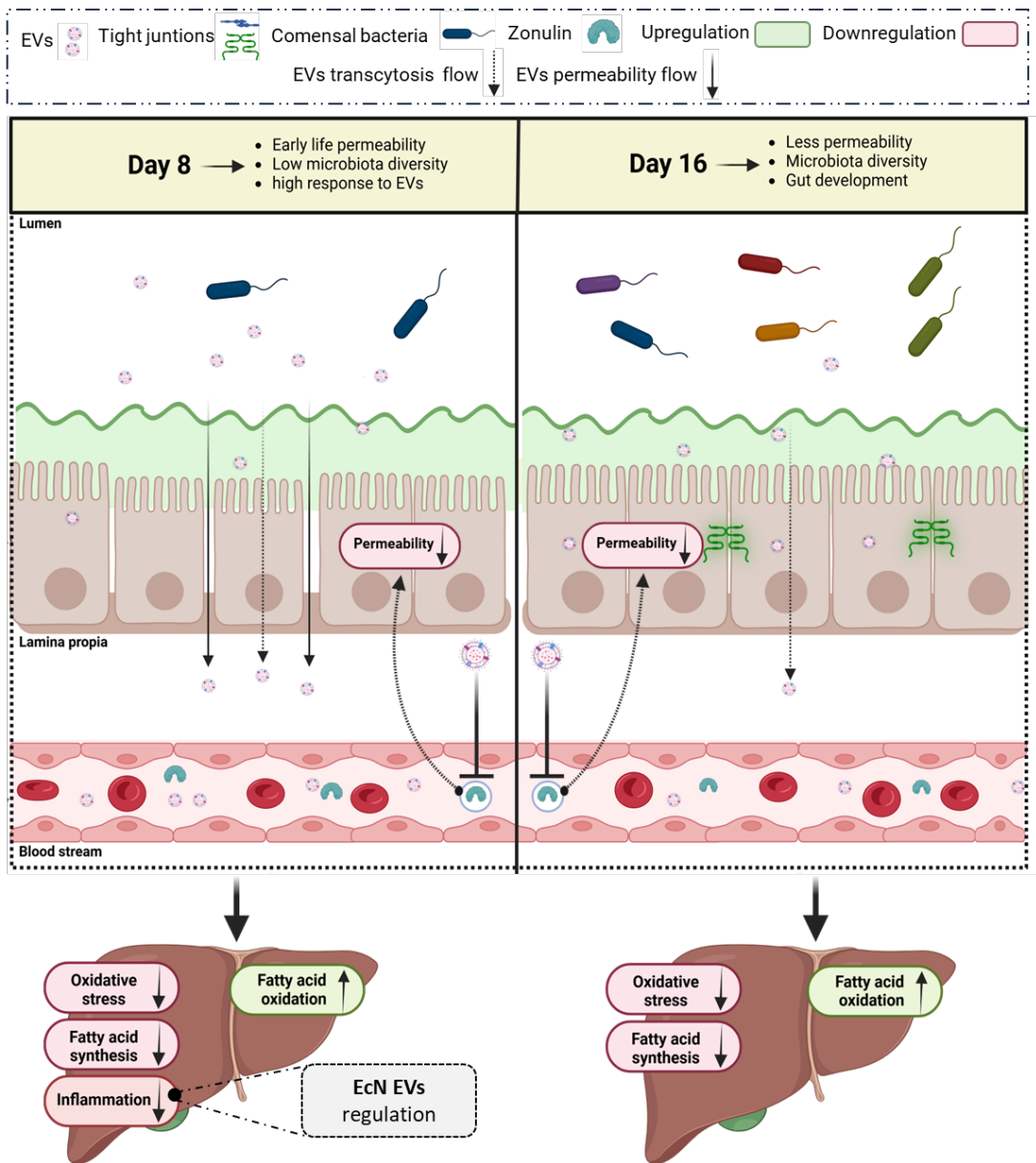


Figure 17. Graphical summary of the key findings presented in **Article 3** of this thesis.

The continuous trafficking of EVs along the gut-liver axis highlights their role in sustaining systemic equilibrium, which could be especially relevant during the neonatal period to ensure long-lasting physiological benefits. These effects highlight their function as natural agents of gut liver communication and suggest their therapeutic potential as postbiotics. Given the rising incidence of MAFLD and the lack of effective

treatments for early-stage disease [382–384], EVs based interventions represent a promising avenue. Their stability, safety, and capacity for targeted signaling support their inclusion among the most promising postbiotic candidates and represent a novel strategy to modulate gut-organ communication and maintain liver health [209,211,212,327,385].

EVs from *E. coli* strains promote intestinal barrier integrity and neuronal maturation: evidence from human iPSC-derived organoids and neurones

EVs released by gut-resident bacteria are emerging as central players in the communication between the intestinal microbiota and distant organs. Among the various intercommunication pathways modulated by microbial signals, the gut–brain axis has garnered particular attention due to its integrative role in immune, endocrine, metabolic, and neural regulation. Owing to their nanoscale size and molecular complexity, bacterial EVs can cross biological barriers and deliver bioactive cargo to host cells, thereby modulating physiological functions beyond the gut. Emerging evidence indicates that these vesicles may affect neurodevelopmental processes by regulating intestinal permeability and immune signaling pathways, supporting their potential role in shaping behavior, synaptic function, and neurological health [386,387].

Therefore, the work presented in **Article 4** of this thesis investigates the effects of EVs derived from the probiotic strain EcN and the commensal strain EcoR12 on two essential components of the gut-brain axis: intestinal epithelial barrier regulation and neuronal development. For this purpose, two independent experimental models were generated from human induced pluripotent stem cells (iPSCs): intestinal organoids, designed to evaluate epithelial barrier modulation, and iPSC-derived neuronal cultures, employed to assess structural parameters related to neuronal maturation (Results summarized in Figure 18). These complementary systems provide a physiologically relevant platform for dissecting the dual actions of microbial EVs on gut and brain compartments under defined conditions.

In intestinal organoids, treatment with EcN- or EcoR12-derived EVs significantly upregulated the expression of epithelial markers ZO-1, Villin-1, and E-cadherin at low concentrations. These molecules are central to the structural organization of tight and adherens junctions, and to the functional maturation of the epithelial barrier [388,389]. Claudin-3 expression was also enhanced, although only at higher doses of EVs, indicating a dose-dependent regulatory effect. Claudin-3 is a sealing component of tight junctions, whose loss has been associated with barrier disruption and increased permeability [390]. These findings support the hypothesis that microbiota-derived EVs can directly modulate epithelial plasticity and contribute to the maintenance of barrier integrity, which is essential for limiting the translocation of microbial-derived molecules and preserving homeostasis within the gut–brain axis. Notably, the expression of ZO-1 is known to be reduced in preclinical models of irritable bowel syndrome and chronic stress [391], reinforcing its role as a key junctional protein disrupted in gut–brain axis pathologies. The coordinated upregulation of multiple barrier-related genes observed in our study suggests that microbial EVs may trigger epithelial remodeling programs associated with tissue stability and defense, and could thus serve as protective modulators against environmental or host-derived stressors that enhance intestinal permeability.

To assess the potential impact of microbiota-derived EVs on the neuronal compartment, we treated iPSC-derived human neurons with EVs from EcoR12 and evaluated parameters related to neuronal maturation. This model mimics the access of circulating EVs to the brain through the blood–brain barrier. Despite a reduction in the number of neurons, cells exposed to EVs displayed increased dendritic branching and larger nuclear size, features commonly associated with neuronal development and transcriptional activation [392,393]. Dendritic arborization is a hallmark of functional maturation, enabling neurons to establish synaptic connections and integrate inputs from surrounding networks. Similarly, nuclear enlargement often correlates with metabolic activation and gene expression changes during differentiation.

These data suggest that microbial EVs may influence early neurodevelopmental processes by modulating pathways related to cytoskeletal remodeling, transcriptional activity, and cellular growth. Although the specific mediators within the EV cargo responsible for these effects remain to be identified, previous studies have shown that microbial proteins, small RNAs, and metabolites carried by EVs can interact with host receptors and modulate intracellular signaling cascades [387]. Our findings therefore raise the possibility that bacterial EVs act as postbiotic regulators of neuronal maturation, supporting their proposed role as effectors within the gut–brain axis.

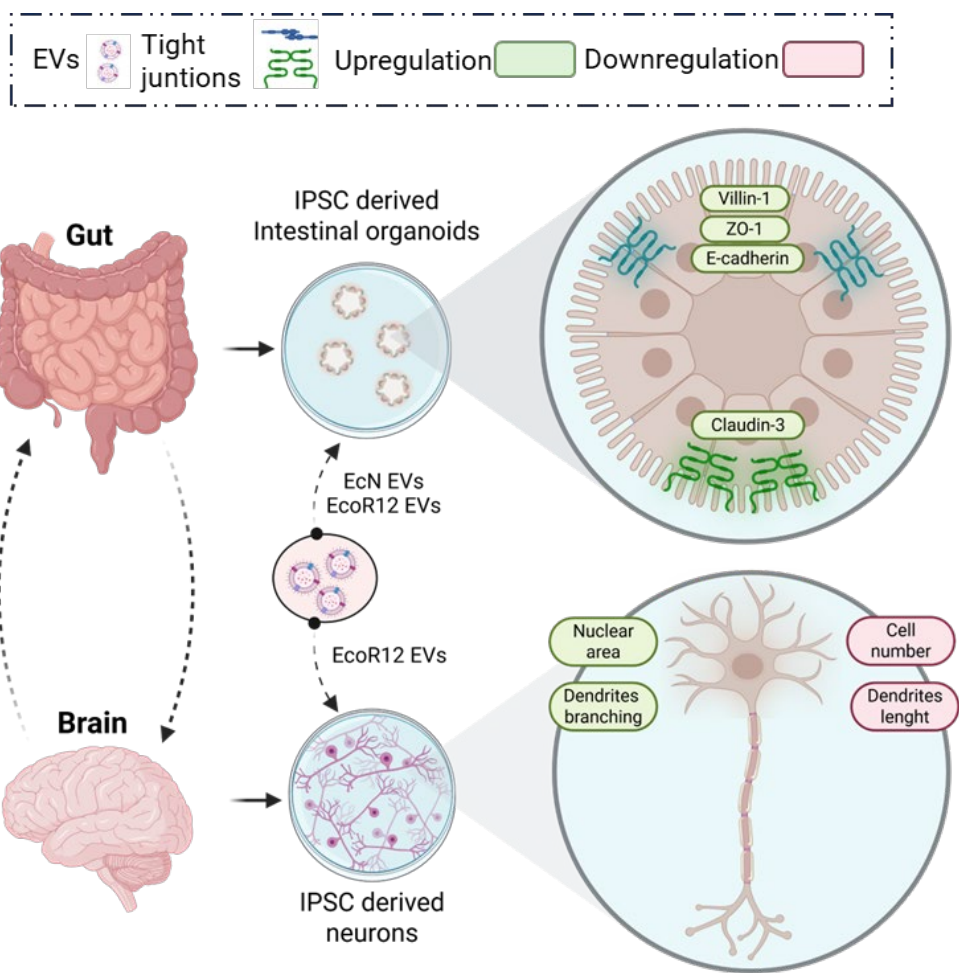


Figure 18. Graphical summary of the key findings presented in **Article 4** of this thesis.

Altogether, the results presented in this article highlight the capacity of EVs derived from *E. coli* strains to simultaneously reinforce epithelial barrier integrity and promote structural features associated with neuronal differentiation. These dual actions underscore the functional relevance of microbiota-derived EVs as postbiotic modulators of gut–brain communication. Given the central roles of epithelial permeability in chronic disorders involving inflammation, dysbiosis, or neuroinflammation, our findings add to the growing body of evidence that positions microbial EVs as promising tools for modulating host physiology beyond the gut.

Final remarks and future perspectives

This thesis provides new insights into the molecular mechanisms by which EVs modulate intestinal responses under both healthy and infectious conditions, as well as their capacity to influence distal organ function through the gut–liver and gut–brain axes. In this context, we demonstrated that EVs derived from *Escherichia coli* strains EcN and EcoR12 are capable of inducing a controlled activation of the immune system, both humoral and cellular responses, while simultaneously enhancing intestinal maturation and regulating permeability during early stages of development. These beneficial effects were further confirmed in a rotavirus infection model, where improvements in epithelial barrier integrity and immune activation were associated with a clinically meaningful attenuation of disease severity.

In addition, we observed that EVs modulated hepatic gene expression signatures involved in lipid metabolism, promoting an ant-lipogenic profile under physiological conditions. This was accompanied by anti-inflammatory and antioxidant effects, suggesting that the induced preventive molecular pattern may be relevant for conferring protection against chronic liver diseases. Regarding the gut–brain axis, the upregulation of tight junction proteins such as ZO-1 and claudin-3 supports their role in reinforcing intestinal barrier integrity and limiting paracellular permeability. Finally, EVs derived from the EcoR12 strain promoted neuronal maturation, reinforcing their potential neuromodulatory effects. These findings must be interpreted within the

broader framework of microbiota–host interactions, where dysbiosis and impaired barrier function are increasingly linked not only to local intestinal disorders, but also to the development of systemic conditions via the gut–liver and gut–brain axes. In this context, the ability of bacterial EVs to modulate both intestinal and extraintestinal targets highlights their relevance as emerging molecular mediators with postbiotic potential.

The results obtained in this thesis support the idea that EVs derived from *Escherichia coli* EcN and EcoR12 strains could be valuable candidates for future therapeutic strategies. Their capacity to regulate mucosal immunity, restore epithelial integrity, modulate hepatic metabolic pathways, and influence neuronal structure opens new avenues for their application in the prevention or management of chronic diseases in which intestinal dysfunction plays a central role. Future research should focus on elucidating the specific molecular components responsible for these effects, optimizing their delivery, and validating their efficacy in disease models that more closely resemble human pathophysiology.



CONCLUSIONS

This work provides strong evidence on the role of EVs from the probiotic EcN and the commensal EcoR12 as important mediators of both intestinal and systemic homeostasis.

Article 1 – Healthy Rat Neonatal Model

1. **Immune system maturation:** EVs from *E. coli* EcN and EcoR12 promote immune development by increasing IgA, IgM, and IgG levels, enhancing Th1 responses, and expanding cytotoxic T cells, NK cells, $\gamma\delta$ T cells, and regulatory T cells.
2. **Intestinal barrier development:** EVs upregulate tight junction proteins (Occludin), reduce FcRn expression, and increase villus size, accelerating gut maturation without inducing inflammation.

Article 2 – Rotavirus Infection Model in Neonatal Rats

3. **Clinical improvement during infection:** EcN and EcoR12 EVs reduce diarrhea severity and duration, preserve intestinal villi, and enhance mucosal protection during rotavirus infection in early life.
4. **Modulation of serotonin signaling:** EVs increase SERT expression and downregulate HTR3, suggesting a mechanism to reduce serotonin-mediated diarrhea.
5. **Enhanced antiviral humoral and cellular immunity:** EVs increase anti-rotavirus antibodies titers (IgA, IgG), maintain a higher Th1/Th2 ratio, and stimulate NK and cytotoxic T cell responses, supporting their use as oral vaccine adjuvants.

Article 3 – Gut–Liver Axis in Healthy Neonatal Rats and HepG2 cells

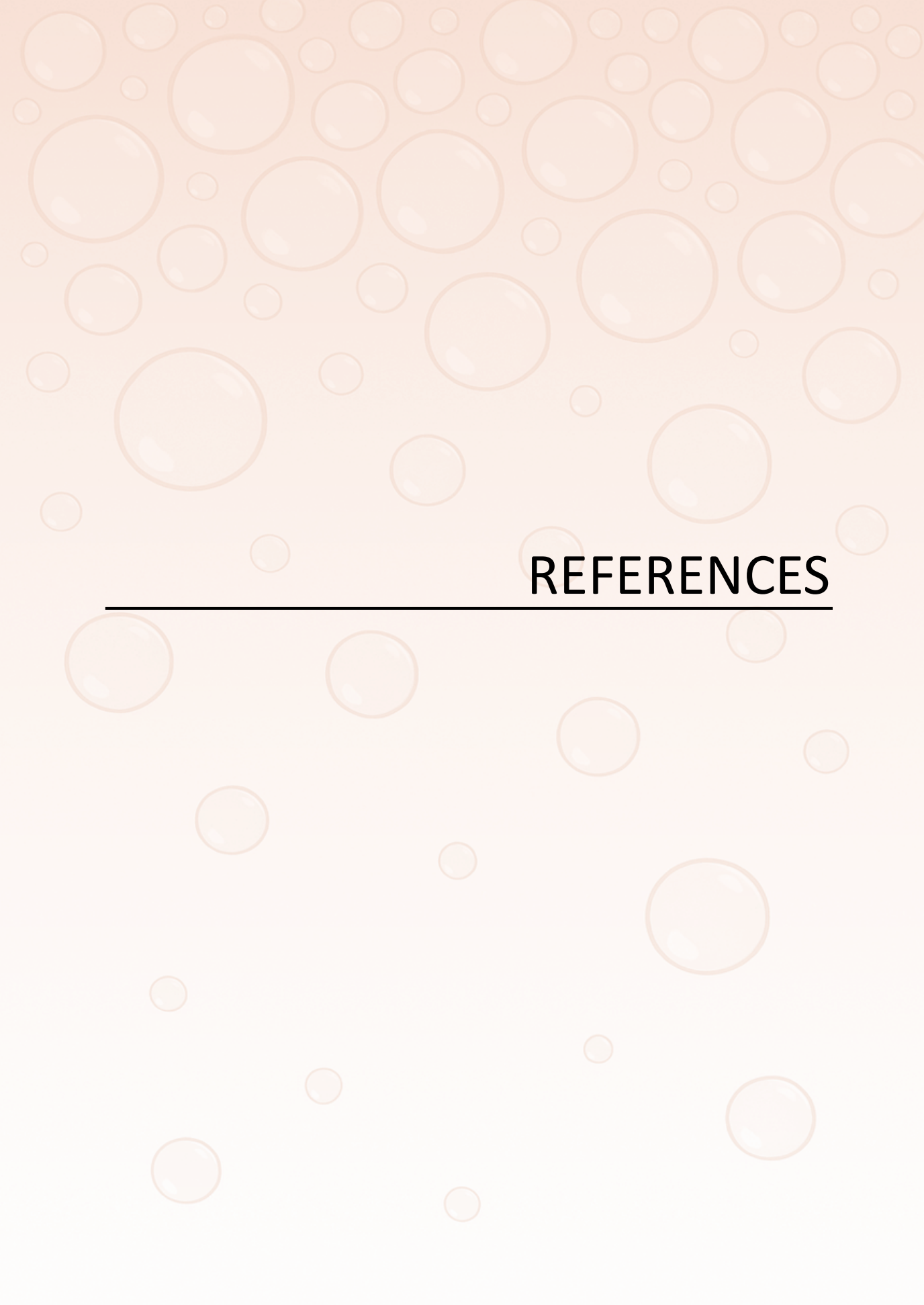
6. **EV biodistribution and hepatic effects:** EVs reach the liver via the gut–liver axis and modulate genes involved in inflammation, oxidative stress, and metabolism, confirming their systemic impact.
7. **Improve hepatic redox homeostasis:** Both EcN and EcoR12 EVs downregulate CYP2E1 while upregulating antioxidant enzymes (SOD1, CAT, GPX), protecting liver tissue from damage.

8. **Strain-specific modulation of hepatic inflammatory genes:** EcoR12 EVs did not cause liver inflammation, while EVs from the probiotic EcN exert specific anti-inflammatory effects by downregulating *IL12* and *TNFA* expression.
 9. **Metabolic modulation in the liver:** EVs reduce the expression of lipogenic genes (*ACC1*, *FASN*, *SREBP1c*, *CNR1*) and increase fatty acid oxidation genes (*CPT1*, *PPARA*), promoting an anti-lipogenic metabolic profile.
-

Article 4 –iPSC-Derived Models to Assess the EV impact on the Gut–Brain Axis

9. **Reinforcement of epithelial barrier integrity in iPSC-derived intestinal organoids:** EVs upregulate ZO-1, Claudin-3, E-cadherin, and Villin-1, enhancing epithelial structure and gut–brain barrier protection.
 10. **Maturation of iPSC-derived neurons:** EcoR12 EVs increase dendritic branching and nuclear size, indicating their potential neuromodulatory role in brain development.
-

Global conclusion: The capacity of EVs from the probiotic EcN and the commensal EcoR12 to regulate mucosal immunity, restore epithelial integrity, modulate hepatic metabolic pathways, and influence neuronal structure opens new avenues for their application as postbiotics in the prevention or management of chronic diseases in which intestinal barrier dysfunction plays a central role.



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