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In vitro evaluation of immunomodulatory activities of goat milk Extracellular Vesicles (mEVs) in a model of gut inflammation

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ABSTRACT

Gut represents a major immunological defense barrier with mucosal immune system and intestinal epithelial cells (IECs). In all intestinal diseases, in particular inflammatory bowel disease (IBD), both the absorption and the local immune system are compromised and alternative effective therapies are sought after. Extracellular Vesicles (EVs) have the capability to regulate immune cells within the inflammatory microenvironment, by dampening inflammation and restoring intestinal barrier integrity. Recently, the immune-modulatory role of EVs has also been confirmed for milk EVs (mEVs), notable for their easy production, high sample volumes, cost-effective scalable production and non-toxic and non-immunogenic behavior. In this context, the aim of this study was to evaluate goat mEV anti-inflammatory and immuno-modulating effects on an *in vitro* model (IPEC-J2) of intestinal inflammation through gene expression evaluation with RT-qPCR and cytokine release dosage with ELISA test. After the establishment of a pro-inflammatory environment due to LPS stimuli, *IL6*, *CXCL8*, *IL12p35*, *IL12p40, IFNB, IL18, TLR7* and *NOS2* resulted significantly up-regulated in stimulated IPEC-J2 cells compared to those of the basal culture. After 48 h of mEV treatment in inflamed IPEC-J2 a partial restoration of initial conditions was detected, with the *IL18* and *IL12p40* significant down-regulation, and *IL12p35, EBI3, TLR7, BD1* and *BD3* up-regulation. IL-18 reduced protein production was also detected in supernatants. Moreover, a decrease of *MMP9* and *NOS2* together with a strong up-regulation of *MUC2* indicated a recovery of cellular homeostasis and, therefore, potential beneficial effects on the intestinal mucosa. Nevertheless, 48 h post-treatment, an increased gene expression and protein release of IL-8 was observed. This paper is one of the firsts to assess the effect of goat mEVs and the first one, in particular, of doing this on an *in vitro* model of gut inflammation. The obtained results show a potential capability of goat mEVs to modulate inflammation and to play beneficial effects on the intestinal mucosa.

1. Introduction

Extracellular Vesicles (EVs) are nano-dimensional spherical structures enwrapped by a lipid bilayer membrane, functioning as signaling mediators between cells with autocrine, paracrine, juxtacrine and endocrine activity (Kalluri and LeBleu, 2020). EVs are released in the extracellular environment by all cell types and found in any biological fluids (Kalluri and LeBleu, 2020; Andaloussi et al., 2013; Malkin and

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Bratman, 2020). In recent years, it was demonstrated their role in immunomodulation and modulation of inflammation to both immune and non-immune cells (Kalluri and LeBleu, 2020; Burrello et al., 2016; Marar et al., 2021; Mittal et al., 2020).

One of the most promising food sources of EVs in terms of quantity is milk, and milk-derived EVs (mEVs) have been investigated in humans and many mammals such as cows, buffaloes, pigs, camels and sheep (Hata et al., 2010; Chen et al., 2016; Badawy et al., 2018; Baddela et al., 2016; Quan et al., 2020). Among the possible sources, indeed, mEVs are notable for their easy production and high sample volumes compared with culture fluid or blood plasma and guarantees cost-effective scalable production of non-tumor EVs. In addition, the non-toxic and non-immunogenic behavior of mEVs in healthy models has been demonstrated, such as their application as carriers for chemotherapeutic agents (Santos-Coquillat et al., 2021). Recently, the described immunomodulatory role of EVs has also been confirmed for mEVs (Carr et al., 2021; Kalbermatter et al., 2021; van Herwijnen et al., 2016), probably due to their cargo rich in bioactive molecules, known to influence inflammation and immune system modulation, as also emerged from our previous studies (Mecocci et al., 2020; Mecocci et al., 2021).

Due to their characteristics as signaling mediators produced by all cell types, involved in immunity and inflammation, it is not surprising that EVs were found to be implicated in the pathogenesis of chronic inflammatory diseases such as diabetes, arthritis and inflammatory bowel disease (IBD) (Ocansey et al., 2020; Withrow et al., 2016; Xiao et al., 2019). On the other hand, recent studies in gut highlighted the EV capability to regulate immune cells and cytokines within the inflammatory microenvironment, dampening inflammation, restoring intestinal barrier integrity and gut microbiome composition and diversity (Ocansey et al., 2020). Prolonged gut inflammations, indeed, are often characterized by immune dysregulation of the gastrointestinal tract (Corridoni et al., 2014), barrier dysfunctions and dysbiosis (Nishida et al., 2018). The disruption of the intestinal barrier integrity can promote pathogen overgrowth of bacteria able to produce pro-inflammatory cytokines, enhancing the gut inflammatory condition. In addition, gut dysbiosis may further reduce some essential host-microbiome interactions, such as vitamin biosynthesis or immune system development (Nishida et al., 2018; Stolfi et al., 2022; Guan, 2019; Liew and Mohd-Redzwan, 2018; Franzoni et al., 2022; Breton et al., 2013; Guerre, 2020; Mavrommatis et al., 2021). When dysbiosis occurs, a significant increase in *E. coli* and a decrease of beneficial bacterial species (Iebba et al., 2016) is often observed, leading to gut innate immune response and health status alteration both in animals and humans. Therefore, the maintenance of intestinal homeostasis is essential to prevent the onset of new pathologies.

In this context, intestinal porcine epithelial cell line-J2 (IPEC-J2) represents a valuable model for investigating of host-pathogen interactions and could also be a promising model for *in vitro* studies of innate immune functions in response to dietary stimuli (Støy et al., 2013). IPEC-J2, derived from the jejunum of an unsuckled neonatal pig, is a cell line of animal origin similar to human intestinal epithelial cells (IECs) both for morphology and function (Schierack et al., 2006), characterized by the presence of microvilli and tight junctions and able to express and produce cytokines, defensins, toll-like receptors (TLRs), and mucins (Mariani et al., 2009). In particular, these cells spontaneously secrete IL-8, a pro-inflammatory chemokine essential for intestinal host-pathogen interactions (Razzuoli et al., 2013). For these properties, IPEC-J2 cells have already been applied to study bacterial and virus pathogenicity and the intestinal inflammatory and innate immune response both for animal and human intestinal pathologies (Razzuoli et al., 2013; Veldhuizen et al., 2009; Sonntag et al., 2005; Razzuoli et al., 2017a). Moreover, compared with animal models, cell line studies are less cost intensive, associated with no ethical concerns, and provide a highly-controlled simple model to investigate isolated factors.

Nevertheless, to the author's best knowledge, only few studies evaluated EVs derived from goat milk (Mecocci et al., 2022), since a great part of milk-derived EV investigations are focused on human and cow EVs. Indeed, this study was intended to evaluate goat mEV effect on inflammation, since the knowledge on the functionality of EVs derived from goat milk is very poor. Consistently, this study aimed to evaluate anti-inflammatory and immunomodulating effects of goat mEVs on an *in vitro* model of intestinal inflammation represented by the combined use of IPEC-J2 cells and the LPS stimulus.

2. Materials and methods

2.1. Milk collection

Milk was collected from one local goat farm, located in Umbria (Italy), previously selected and monitored by the Department of Veterinary Medicine (University of Perugia). The samples were obtained from bulk tank milk, in order to avoid interindividual variability, in late summer/autumn period. Before processing, milk was stored for <24 h at 4 °C, avoiding any intermediate cryo-preservation was performed in order to preserve vesicle morphology and reduce artifacts.

2.2. Extracellular Vescicles (EVs) isolation and size distribution assessment

Milk EVs (mEVs) were isolated as Mecocci et al. 2021 (Mecocci et al., 2021) where mEV isolation was verified through morphological characterization with transmission electron microscopy (TEM) (Mecocci et al., 2021), western blotting (Mecocci et al., 2021), Nanoparticle Tracking Assay (NTA) and the ExoView™ R100 technology (NanoView Biosciences, Brighton, MA, USA). Serial differential centrifugations (DC), alternated with a step in ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), and a final ultracentrifugation to recover mEVs in the pellet were carried out. In detail, two consecutive 3000 x g centrifugations for 10 min at room temperature (Eppendorf® Centrifuge 5810R with a F34–6-38 rotor) were applied to 300 ml of raw milk to eliminate fat globules in the upper layer and cells and cell debris in the pellet; then, 0.25 M EDTA (pH 7.4) was added to the supernatant in a 1:1 ratio, incubated for 15 min on ice, and centrifuged at 10,000 x*g* for 1 h at 4 °C. A centrifugation at 35,000 x*g* for 1 h at 4 °C was carried out for the supernatant in a Beckman Coulter Optima L-100 XP with an 45 Ti rotor, and a final ultracentrifugation at 200,000 x*g* for 90 min at 4 °C was applied collecting mEVs in the pellet. Pellets were resuspended in 1 X phosphate buffered saline (PBS) (Euroclone, Pero, Italy) sterile filtered (0.22 μm pore size) and conserved at −80 °C until use.

Isolated mEVs were observed at TEM and tested for the positivity to EV markers including cluster of differentiation 81 (CD81) and tumor susceptibility 101 (TSG101) at western blot and CD81, CD9 and CD63 at ExoView™. For the latter analysis, mEV pellets were resuspended in 400 μl of 1× PBS, filtered (0.22 μm pore size) and diluted 50× in the incubation solution buffer of the kit. The 50 μl of the solution were deposited on the chip for antibody-mediated capture of mEVs. After incubation and washing passages, mEVs have been made to react against fluorescently marked antibodies (CD81, CD9 and CD63). Moreover, mEVs were measured in terms of size distribution and concentration through the Malvern analytical Nano Sight NS300 NTA system (Malvern, Worcestershire, UK) (Fig. 1). Before measure, the mEV suspension was further diluted and filtered (0.22 μm pore size) in 1 X PBS to reach the most suitable concentration for the NTA system and five measurements were performed. Results are reported as mean +/− 1 standard error of the mean. The suspensions were utilized within a week from the isolation.

2.3. Cells cultures

IPEC-J2 cells (porcine jejunal epithelial cells, IZSLER Cell Bank code BS CL 205) were cultured in a mixture (1:1) of Dulbecco's Modified Eagle (DMEM) (Corning, Manassas, USA) and Nutrient Mixture F-12

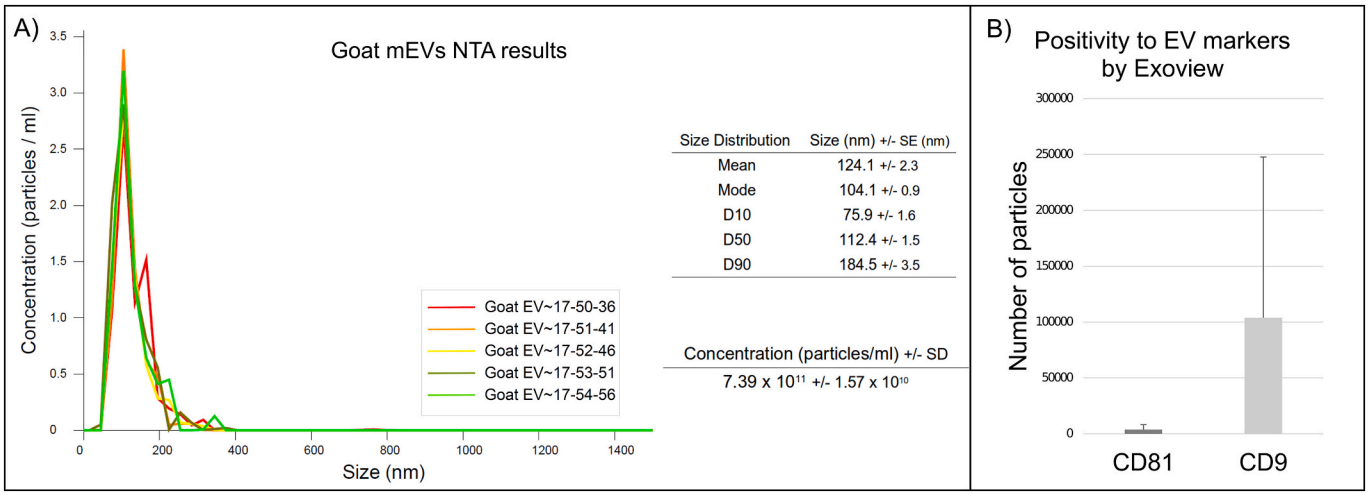

Fig. 1. Characterization of mEVs: A) Nanoparticle Tracking Assay (NTA) result of mEVs isolated from goat milk. The graph shows the particle amount based on size distribution with a peak at 105 nm (mode). The descriptive statistics for particle size distribution and concentration are reported in the table; B) Histogram of the number of particles resulted positive to CD9 and CD81 at the Exoview technology for mEV preparations. These results confirms the expression of CD9 and CD81, also highlighted by western blot results, thus indicating the presence of mEVs and the amount for different marker positivities.

(F12) (Biowest, Voden Medical, Meda, MB, Italy) enriched with 10% Fetal Bovine Serum (FBS, GIBCO™, Thermofisher scientific, Milan, Italy), 1% L-glutamine solution (Carlo Erba Reagents S.r.l., Milan, Italy) and 1% penicillin/streptomycin solution (Carlo Erba Reagents S.r.l., Milan, Italy). Cells were seeded into 12 well plates (1 ml per well, 3×10^5 cells/ml) and then incubated at 37 °C, 5%, CO_2 until confluence.

IPEC-J2 cells were exposed to a scalar amount of mEVs (10^{10}, 10^8 and 10^6) and incubated at 37 °C, 5% CO_2 48 h. For the determination of vesicle quantity to be used to treat cells, we started from few studies that uses EVs in *in vitro* models and tested different quantities in order to evaluate the most suitable amount to be used (Mecocci et al., 2022; Pacienza et al., 2018; Saari et al., 2015). Untreated cells were used as controls. Briefly, cells were seeded into 12 well plates (1 ml per well, 3×10^5 cells/ml) and then incubated at 37 °C, 5%, CO_2 until confluence. Controls cells were used for each experiment and incubated in the same condition of treated cells and exposed to medium only.

IPEC-J2 were treated with purified lipopolysaccharides (LPS) (1 μg/ml; from *Escherichia coli* 0111:B4, Merck KGaA, Darmstadt, Germany) for two hours. Then cells were washed and treated with 10^{10} concentrations of mEVs suspension and incubated at 37 °C, 5% CO_2 for 48 h. At the end of time point, cells were harvested and lysed with 400 μl of RLT buffer (Qiagen, Hilden, Germany) and, after incubation for 10 min at room temperature, were collected and stored at −80 °C until use. The experiment was repeated three times, with three technical replicates in each experiment, using mEV isolates derived from different milk samples. For each experiment we tested: 1) untreated cells (Control), 2) inflamed cells with LPS (in the following named "LPS") and 3) inflamed cells treated with mEVs (LPS + mEVs for 48 h). 48 h post-treatment, culture supernatants were also collected and their cytokine contents were investigated through multiplex ELISA (See 4.3.2).

2.3.1. Cell viability

Cell viability was determined using a trypan blue test after 24 h or 48 h with scalar quantity of mEVs (10^{10}; 10^8; 10^6) based on NTA measurement. Cells were diluted 1:1 in 0.4% trypan blue stain and loaded into the LUNA™ Reusable Slide (LogosBiosystems, FL 2 & 3 28 Simindaero 327 beon-gil, Dongan-gu Anyang-si, Gyeonggi-do South Korea). Cell samples in the LUNA™ Reusable Slide were counted with the LUNA-II™ Automated Cell Counter (LogosBiosystems, FL 2 & 3 28 Simindaero 327beon-gil, Dongan-gu Anyang-si, Gyeonggi-do South Korea). Data relative to the number of living cells were analyzed using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, USA). After checking for Gaussian distribution through the Kolmogorov-Smirnov test, data were analyzed using the Kruskal-Wallis test and the Dunn's Multiple Comparison post-doc Test. The significance threshold was set at $p < 0.05$, indicating differences between mEVs concentrations *vs.* relative control for each time point (24 h and 48 h).

2.4. RNA extraction and RT-qPCR

Total RNA was extracted from IPEC-J2 cells at previously indicated time points using RNeasy Mini Kit (Qiagen s.r.l., Milan, Italy) through the Qiacube System (Qiagen s.r.l., Milan, Italy) in accordance with the manufacturer's instructions. RNA extraction was assessed using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

The same amount of RNA for each sample (250 ng) was reverse-transcribed into cDNA, using iScript® cDNASyntesis Kit (Bio-Rad, Milan, Italy). Amplification was performed on CFX96™ Real-Time System (Bio-Rad, Milan, Italy) using SsoFast™ EvaGreen® Supermix (Bio-Rad, Milan, Italy) following a protocol previously described (Razzuoli et al., 2017b).

Primers of target and reference genes were derived from previous studies (Razzuoli et al., 2013; Razzuoli et al., 2017a; Franzoni et al., 2021; Carta et al., 2021) or designed in accordance with the sequences available on the Primer-BLAST online design platform (https://www.ncbi.nlm.nih.gov/tools/primerblast/, accessed on 31 July 2021). Primer pairs were placed in different exons or exon-exon junctions to avoid biases due to genomic DNA amplification. Specific primer pairs for the reference genome were verified *in silico* using In-Silico PCR software (https://genome.ucsc.edu/cgi-bin/hgPcr, accessed on 31 December 2021) to confirm their specificity for targeting.

Primer sequences of target genes, IL-8 coding gene C-X-C Motif Chemokine Ligand 8 (*CXCL8*), Interleukin 1 beta (*IL1B*), *IL6*, *IL12p35*, *IL12p40*, *IL18*, Toll like Receptor 1 (*TLR1*), *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9*, Beta defensin 1 (*BD1*), *BD2*, *BD3*, *BD4*, Interferon alpha-1 (*IFNA*), Interferon beta (*IFNB*), Cluster of Differentiation 14 (*CD14*), Tumor Necrosis Factor alpha (*TNFA*), Matrix metallopeptidase 9 (*MMP9*), Mucin 2 (*MUC2*), Nitric Oxide Synthase 2 (*NOS2*), Transforming Growth Factor Beta 1 (*TGFB*), Epstein-Barr virus induced gene 3 (*EBI3*) and Tight Junction Protein 1 (*TJP1*), and reference genes, glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* are reported in Table 1. A preliminary RT-qPCR reaction efficiency test was performed for each primer pair. Moreover, in order to verify the amplification of a specific products or primer dimer artifacts, the melt curves were

Table 1
Primer set sequences for target and reference genes; "*" indicates the subset of genes preliminarly tested at 24 h and 48 h after LPS stimulation.

	Gene	Primer sequences	Amplicon length	Source
Targets	*IL1B* *	For - 5′- AATTCGAGTCTGCCCTGTACCC – 3′ Rev. – 5′ – TGGTGAAGTCGGTTATATCTTGGC – 3′	110	(Razzuoli et al., 2017a)
	IL6 *	For - 5′- TGGCTACTGCCTTCCCTACC – 3′ Rev. – 5′ – CAGAGATTTTGCCGAGGATG – 3′	131	(Razzuoli et al., 2017a)
	CXCL8 *	For - 5′- TTCGATGCCAGTGCATAAATA – 3′ Rev. – 5′ – CTGTACAACCTTCTGCACCCA – 3′	175	(Razzuoli et al., 2017a)
	IL12p35 *	For - 5′- ATGCCTCAACCACTCCCAAA – 3′ Rev. – 5′ – TGTGCTGGTTTTATCTTTGGTGA– 3′	135	NM_213993.1
	IL12p40 *	For - 5′- TCAGGGACATCATCAAACCA – 3′ Rev. – 5′ – GAACACCAAACATCAGGGAAA – 3′	140	(Carta et al., 2021)
	IL18	For - 5′-CGTGTTTGAGGATATGCCTGATT-3′ Rev. – 5′-TGGTTACTGCCAGACCTCTAGTGA-3′	106	(Razzuoli et al., 2017a)
	EBI3 *	For - 5′- CAACGTCACAGCCATCCAC– 3′ Rev. – 5′ – GGTTTCCACTGCACCCAA – 3′	140	NM_001315682.1
	TLR1	For - 5′-AGA TTT CGT GCC ACC CTA TG– 3′ Rev. – 5′ – CCT GGG GGA TAA ACA ATG TG – 3′	276	(Franzoni et al., 2021)
	TLR2	For - 5′- CGG CTT CCA AGG ATG GAG AAA- 3′ Rev. – 5′- TCC AGA GAG TTG ACC TTG CAG –3′	71	(Franzoni et al., 2021)
	TLR3	For - 5′- TGAAGAACTTGATTTCCTTGGCA– 3′ Rev. – 5′ – GGCATGAAAACACCCTGGAG – 3′	93	(Franzoni et al., 2021)
	TLR4	For - 5′-TGGCAGTTTCTGAGGAGTCATG– 3′ Rev. – 5′ –CCGCAGCAGGGACTTCTC – 3′	71	(Razzuoli et al., 2017a)
	TLR5	For - 5′-TCAAAGATCCTGACCATCACA– 3′ Rev. – 5′ –CCAGCTGTATCAGGGAGCTT – 3′	59	(Razzuoli et al., 2017a)
	TLR7	For - 5′-GTGGAAATTGCCCTCGTTGT– 3′ Rev. – 5′ –GATGGATCTGTAGGGGAGCA– 3′	77	(Franzoni et al., 2021)
	TLR8	For - 5′- AAGACAACCAGTTACGTGAAATACC– 3′ Rev. –5′–GGGTGTTAAAAGATAATGACAGCAC–3′	236	(Yoo et al., 2019)
	TLR9	For - 5′- AGGACTTCATGCCAAACTGC– 3′ Rev. – 5′ – CGAGCAAACATCTCCGACTG – 3′	90	(Franzoni et al., 2021)
	BD1	For - 5′-CTGTTAGCTGCTTAAGGAATAAAGGC–3′ Rev. – 5′ – TGCCACAGGTGCCGATCT – 3′	80	(Razzuoli et al., 2017a)
	BD2	For - 5′-CCAGAGGTCCGACCACTA– 3′ Rev. – 5′ –GGTCCCTTCAATCCTGTT– 3′	87	(Razzuoli et al., 2017a)
	BD3	For - 5′- CTTCCTATCCAGTCTCAGTGTTCTGC– 3′ Rev. – 5′ –GGCTTCTGTAGACTTCAAGGAGACAT– 3′	308	(Razzuoli et al., 2017a)
	BD4	For - 5′-GTGGCTTGGATTTGAGGAGAGAGT– 3′ Rev. – 5′ –AGTGATACACAGGCCTGGAAGGAT–3′	232	(Razzuoli et al., 2017a)
	IFNA	For-5′-GGCTCTGGTGCATGAGATGC-3′ Rev-5′-GCCTTCTTCCTGAATCTGTCTCA-3′	*546*	(Cheng et al., *2006*)
	IFNB	For-5′-AGTTGCCTGGGACTCCTCAA-3′ Rev-5′-CCTCAGGGACCTCGAAGTTCAT-3′	59	(Razzuoli et al., 2011)
	CD14	For - 5′-TGCCAAATAGACGACGAAGA– 3′ Rev. – 5′ –ACGACACATTACGGAGTCTGA– 3′	364	(Razzuoli et al., 2017a)
	TNFA *	For - 5′- TGCCTACTGCACTTCGAGGTTATC – 3′ Rev. – 5′ – GTGGGCGACGGGCTTATCTG – 3′	125	(Razzuoli et al., 2013)
	NOS2 *	For - 5′- CGTTATGCCACCAACAATGG – 3′ Rev. – 5′ – AGACCCGGAAGTCGTGCTT – 3′	84	(Franzoni et al., 2021)
	TGFB *	For - 5′- CGC GTG CTA ATG GTG GAA AG – 3′ Rev. – 5′ – CCG ACG TGT TGA ACA GCA TA – 3′	87	XM_021093503.1
	MUC2 *	For - 5′- GCAAAGACCTCTAAGATGGCC – 3′ Rev. – 5′ – AGACCACACTCAGCCTCAG – 3′	132	NM_001113440.1
	MMP9 *	For - 5′- TGT GGA CCA GAT GTT CCC C – 3′ Rev. – 5′ – AGT CCA CCT GAT TCA CCT CG – 3′	130	NM_001038004.1
	TJP1 *	For - 5′- ACAGCAATTAAGGAAAAGTGGGA – 3′ Rev. – 5′ – CTTGGGTCGTGTGCTTCTTC – 3′	143	XM_021098883.1
Ref.	*GAPDH*	For - 5′- ATGGTGAAGGTCGGAGTGAA – 3′ Rev. – 5′ – AGTGGAGGTCAATGAAGGGG – 3′	61	NM_001206359.1

examined, and a single well-defined peak was observed in the negative first derivative plot.

A Normalization step was performed according to the expression levels of the reference gene after assessing its stability under different experimental conditions, using the Norm algorithm included in Bio-Rad CFX Maestro software (ver. 4.1 BioRad, Hercules, CA, USA). Relative normalized expression was assessed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), comparing different conditions at the relative time point ("Control" *vs* "LPS", "LPS+mEVs for 24h" *vs* "LPS" and "LPS+mEVs for 48h" *vs* "LPS") in the cell cultured model was assessed. The data were analyzed using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, USA). Gene expression data were submitted to a Kolmogorov-Smirnov test to check Gaussian distributions. Significant differences were checked by Kruskal-Wallis test and applying the post-doc Dunn's Multiple Comparison Test. The significance threshold was set at $p < 0.05$.

2.5. Multiplex assay and analyses

At 48 h post-treatment, culture supernatants were collected, centrifuged (at 2500 ×*g* for 3 min) and kept at −80 °C until use. Levels of IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α, IFN-γ and GM-CSF were determined using the Porcine Cytokine/Chemokine Magnetic Bead Panel Multiplex assay (Merck Millipore, Darmstadt, Germany) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, CA, USA), following the manufacturers' instructions (Sonntag et al., 2005).

Protein release data were submitted to a Kolmogorov-Smirnov test to check Gaussian distributions. Significant differences were evaluated through ANOVA followed by Dunnett's multiple comparison test or a Kruskal-Wallis test followed by Dunn's multiple comparison test. The significance threshold was set at $p < 0.05$.

3. Results

3.1. Morphological characterization of goat mEVs

From NTA assay, the goat mEV mean (± 1 standard error) diameter was 124.1 ± 2.3 nm, with a size distribution characterized by a unique peak and a narrow range (D10 = 75.9 +/− 1.6 nm and D90 = 184.5 +/− 3.5 nm), revealing a high homogeneity (Fig. 1A). Nanoparticle densities was reported as mean concentration (particles/ml) (± SD) of five measurements after one pellet (originated from about 15 ml of raw milk) resuspension in 400 µl of 1 X PBS that resulted in 7.39×10^{11}(± 1.57×10^{10}).

The NTA essay does not have the possibility to distinguish, morphologically and dimensionally, mEVs from other nanoparticles that may be present. For this reason, we have decided to evaluate mEVs at ExoView™instrument, using three EV markers (CD81, CD9 and CD63) to capture the vesicles on the support. After an incubation period and wash passages, the captured mEVs were incubated with the same fluorescently labeled antibodies, detecting the positivity to the three EV markers. The preparations showed a high positivity for CD9 and a milder positivity for CD81, while CD63 did not give acceptable results. Fig. 1B shows the number of particles that resulted positive for CD81 and CD9 in our preparations, confirming the isolation of mEVs as previously evaluated (Mecocci et al., 2021) by western blot that showed positivity also for TSG101, another known EV marker. Concerning TEM results, our preparations showed the presence of vesicles that kept the almost the round shape. Moreover, the positivity for CD81 was confirmed also at western blot that showed positivity also for TSG101, another known EV marker. TEM images and western blot results are reported in our previous study on mEV molecular characterization where the same isolation method of this work was adopted (Mecocci et al., 2021).

3.2. Cell viability

Following exposure of the IPEC-J2 to scalar concentration of mEVs (10^{10}, 10^8 and 10^6) for 24 h or 48 h, there are no statistically significant differences in the number of dead cells (Fig. 2) and total cells (data not shown).

3.3. mEV direct effect in IPEC-J2 gene expression

Following the viability assay results, we selected 10^{10} as the mEV concentration to be used for our *in vitro* test on IPEC-J2. First, the direct effect of mEV 10^{10} treatment for 24 h and 48 h on IPEC-J2 cells was monitored through RT-pPCR. A panel of 25 genes was analyzed (Table 1); after 24 h of mEV exposure, we observed a significant increase of *BD1* ($p < 0.0001$), *BD3* ($p < 0.0001$), *TLR7* ($p < 0.001$), *EBI3* ($p < 0.001$) and *CXCL8* ($p < 0.0001$) (Fig. 3). Other genes under study were not significantly modulated (Fig. 1S). After 48 h of mEV exposure up-regulation of *CXCL8* ($p < 0.0001$), *BD1* ($p < 0.0001$), *BD3* ($p < 0.05$), *TLR7* ($p < 0.0001$), *EBI3* ($p < 0.05$) and *NOS2* ($p < 0.05$) gene expression was observed (Fig. 3). Furthermore, the mEV treatment induced a significant decrease of *TGFB* ($p < 0.0001$), *BD4* ($p < 0.001$) and *TLR4* gene expression ($p < 0.05$) (Fig. 3). Other genes under study were not significantly modulated (Fig. 2S).

3.4. mEV effect in Inflammed IPEC-J2

To analyse the effect of mEVs in an inflammatory environment, we compared IPEC-J2 gene expression in cells stimulated with LPS (inflammation) to cell treated with LPS and mEVs 10^{10} at 24 and 48 h (Fig. 4 and Fig. S3). The mEVs effect on inflammation was analyzed with a subset of the panel (Tables 1, 12 genes depicted by *) after 24 h and 48 h of treatment. Related results showed a milder effect of 24 h treatment on IPEC-J2 compared to 48 h, with no significant gene expression modulation for the shorter time point (Fig. S3). For this reason, the complete panel was used only for 48 h of treatment (Fig. 4).

After 48 h of cells inflammatory stimulation, we saw a significant increase in *IL6* ($p = 0.0003$), *CXCL8* ($p < 0.0001$), *IL12p35* ($p = 0.013$), *IL12p40* ($p < 0.0114$), *NOS2* ($p < 0.0001$), *IFNB* ($p < 0.0001$), *IL18* ($p < 0.001$), *TLR7* ($p < 0.0001$) and a down-regulation of *EBI3* ($p = 0.02$) (Fig. 5). No significantly modulation was observed for the other genes under study *(IL1B, TNFA, TGFB, TJP1, MMP9, MUC2, IFNA, TLR4, BD1, BD2* and *BD4)*.

The co-treatment with mEV 10^{10} determined a significant decrease of *IL18* ($p < 0.0015$), *MMP9* ($p = 0.025$), *NOS2* ($p = 0.009$) and *IL12p40* ($p < 0.0465$) together with the up-regulation of *EBI3* ($p = 0.0096$), *CXCL8* ($p < 0.0092$), *IL12p35* ($p = 0,0011$), *TLR7* ($p < 0.0001$), *BD1* ($p = 0.0003$) and *BD3* ($p = 0.0031$). Moreover, the up-regulation of *MUC2* ($p < 0.0001$) gene expression was shown, indicating a recovery of cellular homeostasis and, therefore, potential beneficial effects on the intestinal mucosa (Fig. 4). No significantly modulation was observed for the other genes under study (*IL1B, TNFA, TGBβ, TJP1, IFNA, IFNB, TLR4*

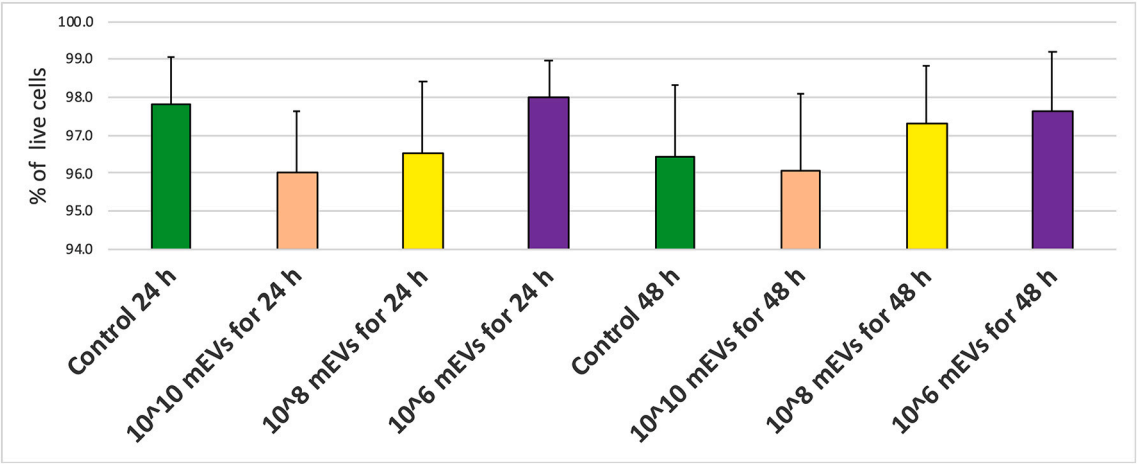

Fig. 2. Viability of IPEC-J2 after mEV exposure. Cell viability was determined using a trypan blue test. The different concentrations of mEVs did not determine a significant difference in vitality of IPEC-J2 neither after 24 h nor 48 h. Data are expressed as % of live cells ± SD. The suspensions were utilized within a week from the isolation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

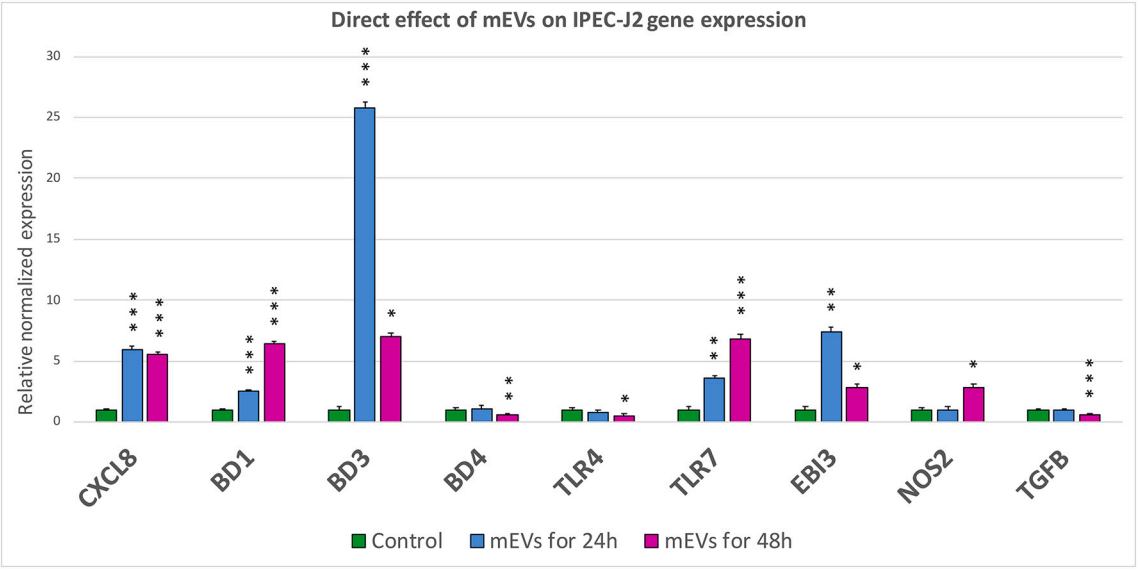

Fig. 3. Direct effect of mEV on IPEC-J2 cells after 24 h and 48 h of exposure. The modulation of innate immunity genes was evaluated in IPEC-J2 intestinal epithelial cells of swine. Cells were treated with mEVs (10^{10}/well) and incubated at 37 °C in 5% CO_2 for 24 h and 48 h. The mEV suspensions were utilized within a week from the isolation. Cells stimulated with medium only were used as untreated control. Total RNA was extracted from IPEC-J2 to evaluate gene expression. *Indicates significant differences between 24 h/48 h mEV treated cell gene expression and untreated control. *p < 0.05, **p < 0.001, ***p < 0.0001.

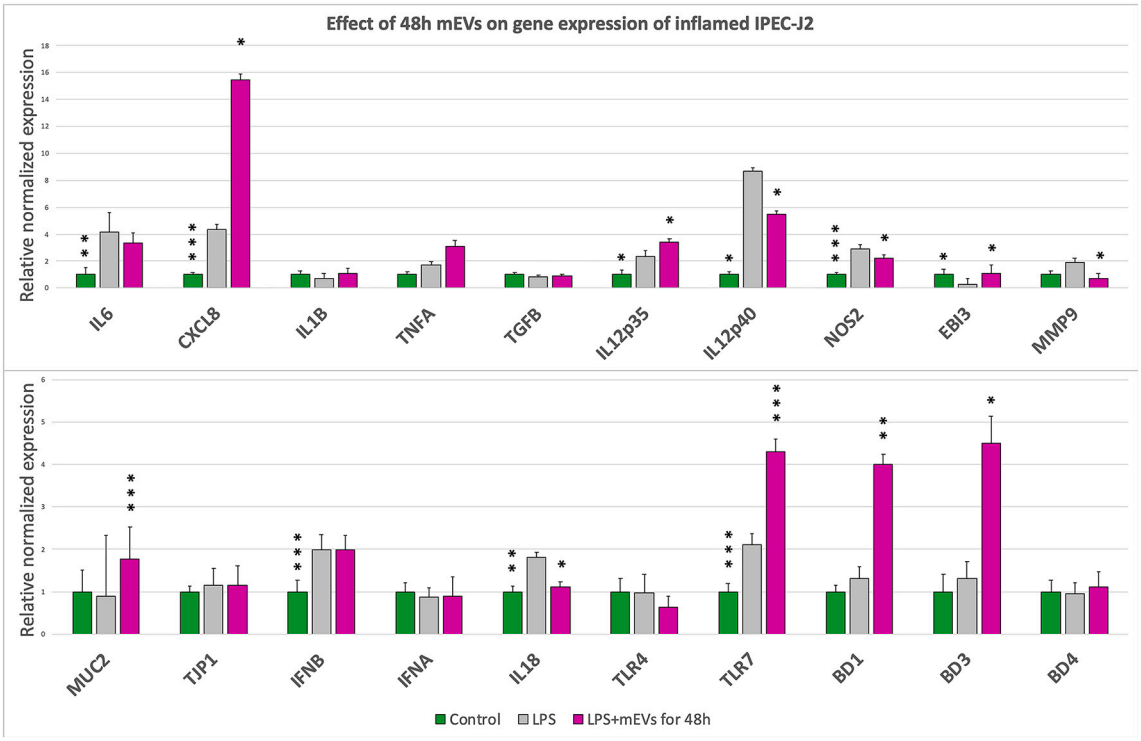

Fig. 4. Effect of 48 h 10^{10} mEVs on LPS inflamed IPEC-J2 cells. IPEC-J2 tested conditions were: untreated (control, green), inflamed (LPS, grey), 10^{10} mEV administering to JPEC-J2 in inflamed condition (LPS + mEVs, purple). Differences (control *vs* LPS; LPS + mEVs *vs* LPS) were evaluated through the Kruskal-Wallis test and applying the post-doc Dunn's Multiple Comparison Test. The asterisks indicate the statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The LPS treatment determined a significant increase of *IL6, CXCL8, IL12p35, IL12p40, NOS2, IFNB, IL18, TLR7* and a significant decrease of *EBI3*. The co-treatment LPS + mEV increase the gene expression of *CXCL8, IL12p35, EBI3, MUC2, TLR7, BD1, BD3* and decreased the expression of *IL18, IL12p40, NOS2,* and *MMP9*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and *BD4*).

3.5. Cytokine response to IPEC-J2 inflammation

To further understand the impact of mEVs in an inflammatory environment, we compared the cytokine contents in the supernatants of IPEC-J2 stimulated with LPS (inflammation) to cells treated with LPS and mEVs 10^{10} (Fig. 5).

LPS stimulation resulted in the release of pro-inflammatory cytokines: LPS-treated IPEC-J2 presented increased levels of IL-8, IL-18, IL-

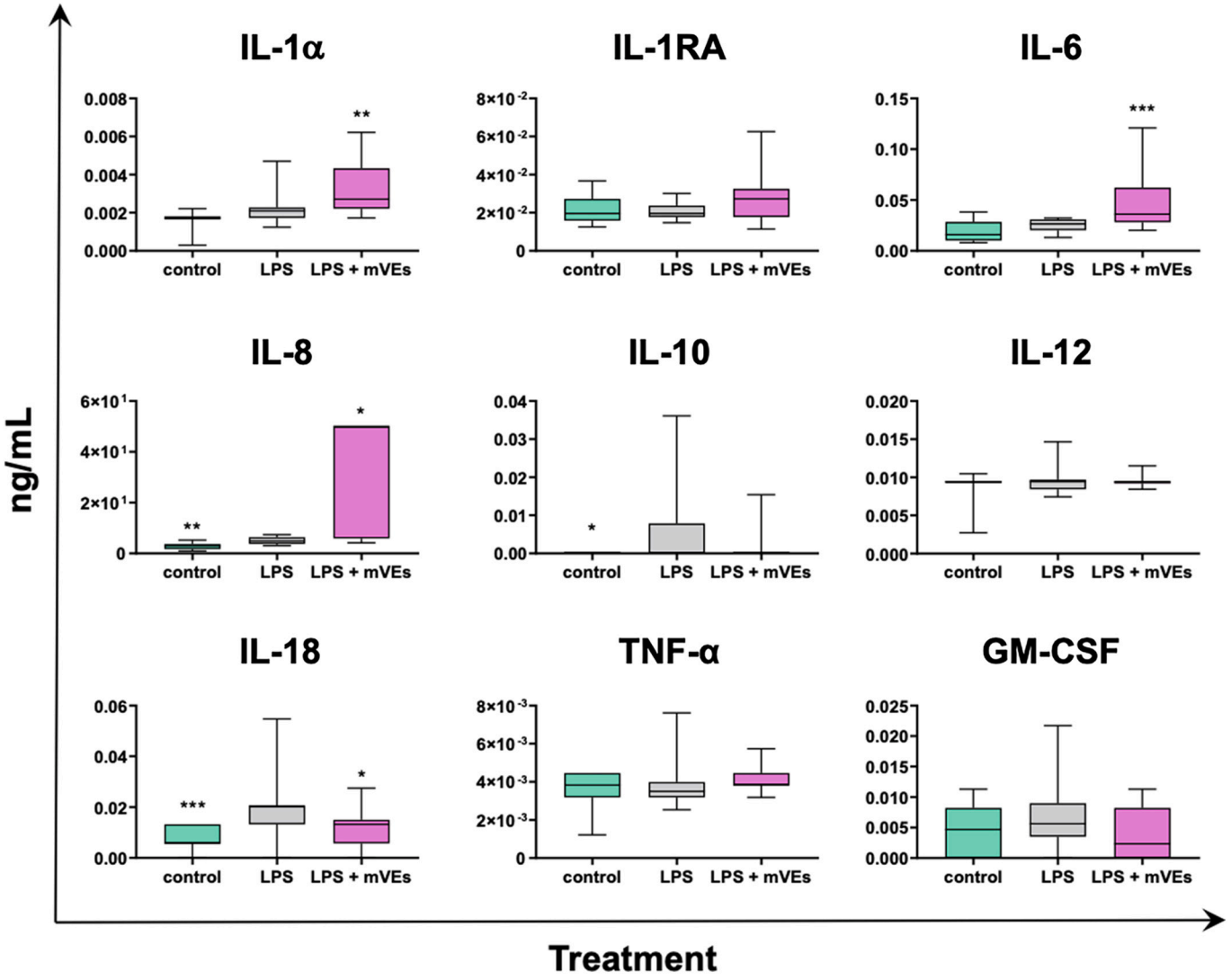

Fig. 5. mEVs impact on cytokine production by IPEC-J2. IPEC-J2 were left untreated (control, green), stimulated with LPS (inflamed condition, grey), or treated with LPS and 10^{10} mEVs suspension. Then, 48 h later, culture supernatants were collected, and levels of several cytokines were determined through multiplex ELISA. Data are presented as box-and-whisker plots displaying median and interquartile range (boxes) and minimum and maximum values (whiskers). Differences (control *vs* LPS; LPS *vs* LPS + mEVs) were evaluated through ANOVA followed by Dunnett's multiple comparison test or a Kruskal-Wallis test followed by Dunn's multiple comparison test; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

1α compared to untreated IPEC-J2, although the latter without statistically significance. LPS stimulation also triggered a modest, but, statistically significant increase of anti-inflammatory IL-10 (Fig. 5).

The impact of mEVs (10^{10}) was investigated and we observed that administration of these nano-sized structures in an inflammatory environment resulted in increased release of IL-1α, IL-6, IL-8. Nevertheless, lower levels of IL-18 were found in LPS + mEVs treated IPECJ2 compared to the LPS stimulated condition (Fig. 5). The other tested cytokines (IL-1RA, IL-12, TNF-α, GM-CSF) were not modulated by either LPS or mEVs (Fig. 5).

4. Discussion

The intestine is not only the responsible organ for the nutrient absorption, but it also represents one of the major immunological defenses against external antigens. In addition to the gut mucosal immune system, intestinal epithelial cells (IECs) are involved in the coordination of the intestinal immune response by the producing signaling molecules, including cytokines and chemokines (Støy et al., 2013).

When this organ is pathologically affected, such as in the case of chronic intestinal diseases, including inflammatory bowel disease (IBD), not only nutrients absorption is compromised, but also the local immune system is derailed (Saleri et al., 2021). Indeed, these disorders are characterized by a complex onset where different players (immune dysregulation, barrier dysfunction and dysbiosis) intervene, making hard to find effective therapies that primarly aim to restrain intestinal inflammation. Thus, new therapeutic targets and approaches are urgently required.

Molecules contained in the EVs could act on damaged enterocytes following chronic inflammation, improving the mucosal functionality, and restoring a situation of homeostasis. Our group has recently investigated these potentialities in EVs derived from milk, where bioactive molecules contained in the cargo, such as amino acids, nucleotides, nucleosides, mRNAs, and small RNAs have been fully characterized (Mecocci et al., 2020; Mecocci et al., 2021). Although these characteristics have been functionally evaluated in mEVs from different milk sources, to the authors' knowledge, goat mEVs have not yet been deeply investigated. Moreover, our previous metabolomic characterization (Mecocci et al., 2020) of goat mEVs had highlighted an enrichment in metabolites capable to influence the immune system, such as the

arginine and tryptophan amino acids (Hachimura et al., 2018) or the vitamin B9 (folate), essential for the maintenance of Regulatory T cells (Treg) (Kunisawa et al., 2012) whose dysfunction causes intestinal inflammation (Kinoshita et al., 2012). IPEC-J2 cell line is an *in vitro* model to investigate immune responses of the intestinal epithelium to diets (Støy et al., 2013; Qiu et al., 1800; Zhang et al., 2020). In the past years, it was used to study the influences of immunomodulators on intestinal cellular immune response, apoptosis and barrier function (Peterson and Artis, 2014). In this research, we used this model to investigate gene expression of epithelial and immune-related genes through RT-qPCR and cytokine release with the ELISA test, following goat mEV treatment alone or in association with a pro-inflammatory lipopolysaccharide (LPS) pre-stimulation.

Cytokines are central molecules of the inflammatory pathways that take part in different phases of gut inflammations, including in IBD, leading to both innate and adaptive immune pathways dysregulation. The pro-inflammatory cytokine increase is often associated with the gut inflammation severity, thus conferring to these molecules a major therapeutic role (Danese and Angelucci, 2009).

As first, the effect of mEVs on IPEC-J2 was investigated in basal conditions, treating cells for 24 h and 48 h without the previous induction of an inflammatory state (Fig. 3, Figs. S1-S2). mEVs had a marked effect after 48 h from administration with a greater number of significantly modulated genes compared to 24 h treatment. The mEV treatment modulated the expression of immune-related and oxidative-related genes, inducing the up-regulation of *BD1*, *BD3*, *TLR7*, *EBI3*, *NOS2*, *CXCL8* and the down-regulation of *BD4*, *TLR4* and *TGFB* (Fig. 3).

The second experiment consisted in mEV treatment of LPS pre-stimulated IPEC-J2 to evaluate vesicle impact on inflammation. To this aim, gene expression and cytokine release were tested after LPS stimulation (Fig. 4). A pro-inflammatory environment was established at 48 h of LPS stimulation, with a gene expression increase in most of the tested cytokines. *IL6*, *CXCL8, IL12p40*, *IL12p35*, *TLR7, IFNB* and *IL18* resulted significantly up-regulated in IPEC-J2 cells after 48 h of LPS stimulation accompanied by a decrease of *EBI3*. At the same time, a significant up-regulation of *NOS2* also revealed the induction of oxidative stress and cell damage (Fig. 4). These results were in agreement to cytokine protein levels detected in cell culture supernatants, where we observed that LPS stimulation triggered release of pro-inflammatory IL-8 and IL-18 (Fig. 5). After establishing an inflammatory state on IPEC-J2 cells, we wanted to test whether mEVs could attenuate the inflammation, modulate immune response and have beneficial effects on mucosa. Concerning results on mEV administration, 48 h treatment seems to be effective in homeostasis restoration (Fig. 4). Indeed, a decrease of tested cytokines *IL18* and *IL12p40* was found accompanied by an increase of *IL12p35*, *TLR7*, *CXCL8*, *BD1*, *BD3* and *EBI3*. Moreover, a gene expression decrease of *NOS2* and *MMP9* and an up-regulation of *MUC2* was shown, indicating a recovery of cellular homeostasis and, therefore, potential beneficial effects on the intestinal mucosa (Fig. 4).

4.1. Goat mEV effects on antimicrobial peptides (AMPs)

In detail, intestinal epithelial cells are selectively permeable, functioning as a nutrient-absorbing filter, but are also considered to be the first line of defense against foreign antigens, such as pathogens from the intestinal lumen. In this frame, our data highlighted the increased antimicrobial capacity of cells after exposure to mEVs. Indeed, mEVs were able to increase gene expression of antimicrobial peptides (AMPs) *BD1* and *BD3* in both inflamed and not-inflamed cells and to down-regulate *BD4* (Fig. 3) in the basal condition. All AMPs, produced as inactive pre-propeptides and cleaved for the activation, act in both extracellular or intracellular space (Liévin-Le Moal and Servin, 2006), representing an early response to pathogens. They exert microbicidal activity against Gram-negative, Gram-positive bacteria, fungi, enveloped viruses and protozoa (Ramasundara et al., 2009; Wehkamp et al., 2009). In the intestinal tract, AMPs and, in particular, β-defensins contribute to the balance between tolerance for microbiota and protection from pathogens. A decrease of defensins can compromise host immunity and can lead to dysmicrobism, a typical signature of IBD and for this reason, supposed to be a pathogenetic mechanism of these diseases (Mirsepasi-Lauridsen et al., 2018). In this context, the induction of defensin expression in enterocytes led by mEV treatment could be positive for host immunity regulation in IBD syndromes.

4.2. Goat mEV effects on Toll-like receptors (TLRs)

TLR family members are fundamental in pathogen recognition and innate immunity activation by binding with pathogen-associated molecular patterns (PAMPs). In the gut, PAMP recognition induces the activation of inflammatory pathways, making TLRs pivotal players in mediating the immune response through their action as linkers between the innate and adaptive immunity. TLRs exhibit different patterns of expression; in particular, TLR7 is highly expressed in plasmacytoid dendritic cells (pDCs) and its activation leads to the IFN expression (Sainathan et al., 2012). In fact, type I IFNs regulate innate immune system through their involvement in TLR signaling pathways and other stimulators of innate immunity, reported to be anti-inflammatory in IBD. Indeed a recent study showed that IFN-β and AMPs increase could produce remission in patients with UC and identifies the TLR7 ligand as a potent immunoregulator in the mucosa, thus promising for the treatment of IBD (Mannon et al., 2011). mEVs in our experiments led to a great stimulation of *TLR7* gene expression in both basal and inflamed IPEC-J2. Consequently, this could determine type I IFN production (although no modulation was observed in our experiment, probably due to the short time), making vesicles potentially protective for intestinal homeostasis.

TLR4 is the receptor for LPS and is related with inflammatory response MyD88-mediated. Several studies have demonstrated that the activation of the TLR4/NF-kappaB (NF-kB) signaling induces intestinal myeloid cell infiltration and inflammatory mediators secretion (Dejban et al., 2021). Moreover, growing evidence has currently indicated that the dysfunction of TLR4 signaling pathway plays a pivotal part in the pathogenesis of IBD, attracting the interest of research in developing many therapeutic approaches to silencing this pathway (Dejban et al., 2021). In our cellular model, there probably was a suppression of MyD88/NF-kB pathway since mEVs induced *TLR4* down-regulation in both the basal condition and after inflammation, although not significant for the latter. These results emphasized the helpful effect of mEVs on innate immunity and potentially in controlling the pathogenesis and progression of IBD.

4.3. Goat mEV effects on cytokines

TGF-β1-signaling is strongly implicated in gut homeostasis and regulates many mucosal cell types; its disruption is associated with the development of intestinal inflammation, indeed TGF-β1 levels are increased in IBD (Ihara et al., 2017). In particular, TGF-β1 with IL-6 drives the differentiation of Th17 cells, which promote inflammation and contribute to the progression of fibrosis inducing the production of collagen and matrix metalloproteinases (MMPs) (Burke et al., 2007). In our experiments, mEVs induced a significant down-regulation of *TGFB* in IPEC-J2 cultured in basal conditions, although no effects were detected after inflammation.

IL-12 is a member of the IL12 family, itself part of the IL-6 superfamily, together with IL-23, IL-27 and IL-35. These are dimeric cytokines consisting in two proteins encoded by different genes: IL-12 is composed of IL-12p35 and IL-12p40 subunits, while IL-35 in IL-12p35 and EBI3. So, *IL12p35* gene expression increase could be related to IL-12 and/or IL-35 protein production (Collison et al., 2010). mEV treatment induced the up-regulation of *IL12p35* in inflamed IPEC-J2, and *EBI3* in both inflamed and not inflamed cells (basal condition), together with the down-regulation of *IL12p40* gene expression after inflammation These

results, let us hypotize an increase in IL-35, an anti-inflammatory cytokine mainly produced by Treg, instead of IL-12. This thesis is corroborated by the IL-12 levels in supernatants that remained stable between LPS and mEV treatment conditions. The stimulation of *IL35* production by mEVs is a positive result because a recent study assessed the therapeutic potential of IL-35 recombinant protein in a mouse model of colitis, inducing the IL-10 production and the decrease of IL-6, TNF-α and IL-17A, alleviating the inflammation in acute colitis (Wang et al., 2018). Furthermore, *EBI3* is associated with the increase of Treg differentiation and Th17 decrease, which is a fundamental issue for IBD treatment. Indeed, an increased production of IL-17 by Th17 cells is often associated with IBD and, in turn, induces the MMPs expression and chemokine production involved in the recruitment and activation of granulocytes.

IL-8 is a chemokine that promotes monocyte recruitment. These cells, under gut-specific signals like, CSF1, TGF-β, IL-10 differentiate in macrophages. Moreover, on the basis of the immune-microenvironment, these cells can assume a pro-inflammatory (M1) or an anti-inflammatory (M2) state (Franzoni et al., 2021; Carta et al., 2021). M2 macrophages not only dampen Th1 and Th17 responses but they are also pivotal to re-establish the epithelial barrier functions (Carta et al., 2021). In this conceptual framework, gene expression increases of IL-8 coding-gene *CXCL8* in IPEC-J2 with and without inflammation, also confirmed in ELISA test on supernatants after LPS treatment and mEV administration, associated with up-regulation of *EBI3* and down-regulation of *TGFB* (in basal condition), could be helpful to the macrophage balance and IBD therapy.

Another statistically significant modulation induced by mEV treatment on inflamed IPEC-J2 cells is the down-regulation of *IL18* gene expression and protein release in supernatants after being increased by LPS. IL-18 is a cytokine involved in T cell (Th1, NK, Th2, IL17-producing γδ T cells) and macrophage activation and can have a role in various infectious, metabolic or inflammatory diseases including IBD (Nowarski et al., 2015). Indeed, in intestinal pathogenic conditions, the epithelial barrier disruption leads to bacteria massive entrance in the lamina propria and local macrophages production of IL-18 which is detrimental, inducing the leukocyte recruitment, mucus reduced production by goblet cells and favoring dysbiosis (Kaplanski, 2018). IL-18 is particularly able to inhibit goblet cell maturation by regulating the transcriptional program instructing goblet cell development and high level of IL-18 signaling in intestinal epithelial cells are associated with colitis severity (Nowarski et al., 2015).

4.4. Goat mEV effect on gut mucosa

Together with a decrease of pro-inflammatory cytokines, goat mEVs are able to reduce oxidative stress produced by inflammation, decreasing the transcription of *NOS2* and *MMP9* genes significantly up-regulated in LPS stimulated cells.

Another relevant result in administering goat mEVs was the up-regulation of *MUC2* gene, because its protein product is essential in preventing pathogen-induced epithelial injury, constituting the main component of the viscous mucus layer on the surface of the gastrointestinal tract that mediates innate immunity protective functions (Kissoon-Singh et al., 2013). A reduction of the colonic mucus was observed in IBD patients (van der Post et al., 2019), indeed, it seems to be essential for protecting the mucosal barrier and in maintaining the homeostatic cross-talk between microbiota and host intestinal cells (Kissoon-Singh et al., 2013). These results indicate a partial restoration of initial cell culture conditions when mEVs were administered for 48 h.

In conclusion, this work aimed to evaluate goat mEVs effect on an intestinal model of IECs, swine IPEC-J2, in basal conditions and after LPS stimulation.

In basal conditions, mEVs induced antimicrobial peptides *BD1* and *BD3* up-regulation and defensins expression, highlighting the increased antimicrobial capacity of cells and activity in host immunity regulation. Furthermore, the simultaneous great stimulation of TLR7 together with TLR4 down-regulation emphasizes the helpful effect of mEVs on innate immunity.

Indeed, after LPS stimulation mEVs were able to down-regulate *TGFB* and *IL12p40* and up-regulate *CXCL8* and *EBI3,* leading to preferential expression of anti-inflammatory IL-35 instead of pro-inflammatory IL-12, which is helpful to the macrophage balance and IBD therapy. Moreover, goat mEVs induces down-regulation of *NOS2, MMP9* and *IL18* gene expression and protein release, significantly up-regulated in LPS stimulated cells. Furthermore, *MUC2* up-regulation, can be important for preventing pathogen-induced epithelial injury because this protein constitutes the main component of the viscous mucus layer on the surface of the gastrointestinal tract that mediates innate immunity protective functions.

These results indicate a good restoration of initial cell culture conditions after goat mEV treatment.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.09.021.

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