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Probiomimetics—Novel *Lactobacillus*-Mimicking Microparticles Show Anti-Inflammatory and Barrier-Protecting Effects in Gastrointestinal Models

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There is a lack of efficient therapies to treat increasingly prevalent autoimmune diseases, such as inflammatory bowel disease and celiac disease. Membrane vesicles (MVs) isolated from probiotic bacteria have shown tremendous potential for treating intestinal inflammatory diseases. However, possible dilution effects and rapid elimination in the gastrointestinal tract may impair their application. A cell-free and anti-inflammatory therapeutic system—probiomimetics—based on MVs of probiotic bacteria (*Lactobacillus casei* and *Lactobacillus plantarum*) coupled to the surface of microparticles is developed. The MVs are isolated and characterized for size and protein content. MV morphology is determined using cryoelectron microscopy and is reported for the first time in this study. MVs are nontoxic against macrophage-like dTHP-1 and enterocyte-like Caco-2 cell lines. Subsequently, the MVs are coupled onto the surface of microparticles according to facile aldehyde-group functionalization to obtain probiomimetics. A significant reduction in proinflammatory TNF-α level (by 86%) is observed with probiomimetics but not with native MVs. Moreover, it is demonstrated that probiomimetics have the ability to ameliorate inflammation-induced loss of intestinal barrier function, indicating their potential for further development into an anti-inflammatory formulation. These engineered simple probiomimetics that elicit striking anti-inflammatory effects are a key step toward therapeutic MV translation.

1. Introduction

The increasing prevalence of intestinal inflammatory diseases poses a serious threat to global health.[1] These diseases include inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis, as well as autoimmune-associated celiac disease, and are characterized by an overshooting immune reaction in the gastrointestinal tract (GIT).[2–4] This leads to tissue damage and a variety of symptoms[2,5] such as diarrhea as well as extraintestinal symptoms related to malabsorption of nutrients and an increased risk of cancer.[6–8]

To date, there is no causal therapy available for inflammatory diseases of the intestine and long-lasting suppression and avoidance of triggers is usually required.[9] IBD is currently treated with immunosuppressive drugs. However, these drugs may not be effective for every patient because of individual pharmacogenetic differences.[10,11] Moreover, these drugs are also known to cause a number of side effects, including hepatic injuries and an increased susceptibility to infections.[12–14] In the case of celiac disease, the patients are required to maintain a lifelong gluten-free diet, which is difficult to achieve in the typical "western diet" as gluten is contained in a variety of food products, and even very small amounts can trigger an autoimmune reaction.[15]

Administration of probiotic bacteria has shown efficacy as an adjuvant therapy in ameliorating the symptoms related to IBDs.[16] According to the World Health Organization, probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host."[17] These bacteria, which include gram-positive *Lactobacilli* and *Bifidobacteria* as well as some *Escherichia coli* strains, have demonstrated multiple beneficial effects, such as modulation of the host immune system, improvement in the epithelial barrier function, and affecting the balance of the various bacterial strains in the gut.[18] Despite the promising potential of probiotics, they are still not always suitable for use as therapeutic agents because of their ability to proliferate. Especially, in the case of patients who take immunosuppressants for long-term control of IBD, the concomitant use of probiotics is not recommended as it can lead to bacteremia and sepsis.[19] A study reported that *Escherichia coli* Nissle 1917—a strain commonly used as probiotics—possessed the same genes responsible for pathogenicity as detected in other *E. coli* strains.[18] Once these silent genes are activated by unknown triggers, they may potentially cause pathogenic effects in patients, thus substantially limiting the safety of the probiotics.

In this study, we explored the approach of designing therapeutics based on bacterial membrane vesicles (MVs),

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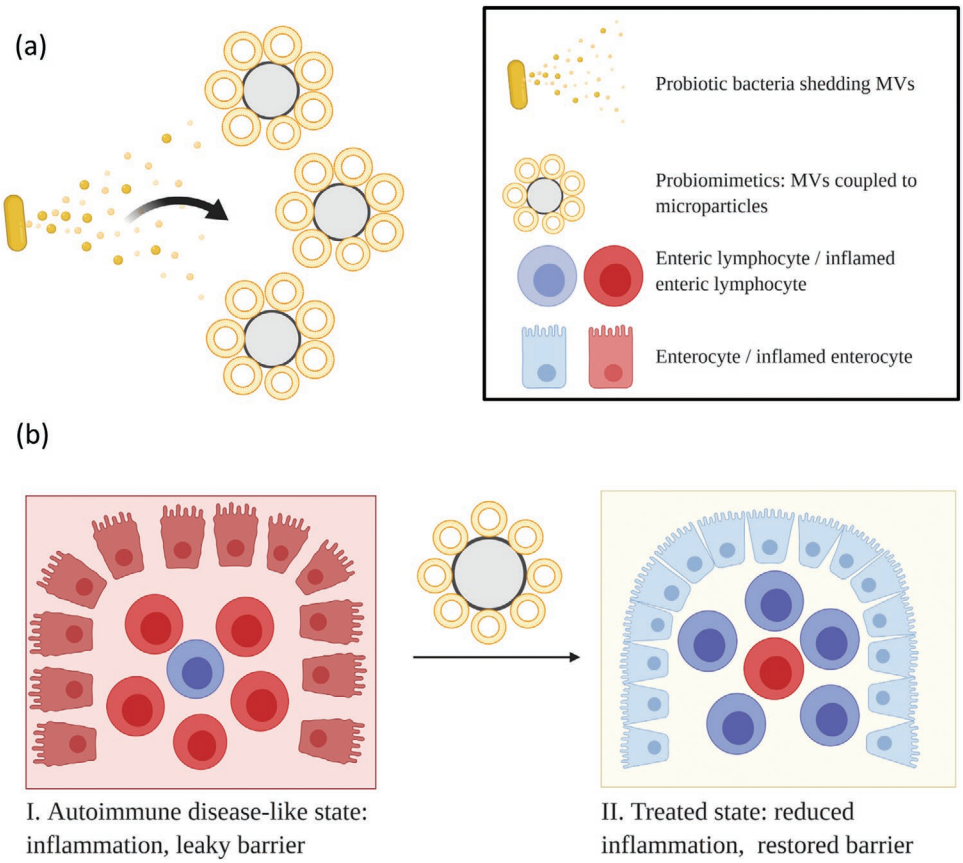

Figure 1. Concept of the study: a) Probiotic *Lactobacilli* shed membrane vesicles that are chemically coupled to the surface of microparticles. b) The obtained probiomimetics are characterized and assessed in suitable in vitro models. Using the immune-modulatory effects of the probiotic bacteria, they ameliorate overshooting tissue-inflammation and modulate immune response.

which might represent a viable and safer alternative to live bacteria.[20] MVs are phospholipid-based, naturally-produced vesicles that occur across all domains of life.[21–23] They possess various biological functions, including mediation of bacteria–bacteria communication and bacteria–host modulation.[24–28]

The use of eukaryotic vesicles (EVs) to treat autoinflammatory diseases has been reported earlier.[29–31] For example, the application of EVs, produced by human mesenchymal stem cells in inflammatory diseases, was shown to ameliorate autoimmune reactions.[32,33] However, it is difficult to produce large quantities of these mammalian vesicles for multipatient clinical applications.[29] In addition, the activity of eukaryotic EVs varies depending on the cell culture conditions and the passage of the cells.[34]

An alternative group of vesicles with inherent anti-inflammatory properties are MVs of probiotic bacteria.[35] These vesicles have shown promising anti-inflammatory effects in earlier studies.[36] They can be obtained in substantially higher amounts and can be easily standardized compared with mammalian EVs.[37] Nevertheless, they are still not optimal to be administered as such as they are easily eliminated after administration owing to their small size and are prone to quick dilution in the GIT upon oral administration.[38]

Here, we utilized probiotic MVs for the first time to mimic the promising therapeutic properties of living probiotic bacteria, while avoiding the aforementioned therapeutic disadvantages by combining them with biomaterials. In doing so, we present a novel therapeutic system, probiomimetics (PBMs), comprising MVs from probiotics coupled onto the surface of microparticles to allow controlled and high concentration application of MVs (**Figure 1**). Using the MVs coupled to microparticles will be advantageous because we anticipate they will be enriched on the apical membrane of the intestinal mucosa rather than being quickly eliminated, as was the case for similarly sized particles.[39] This may lead to an increased concentration of MVs on the mucosal cells where inflammation takes place. In addition, the developed nonproliferating system has been proposed to have substantial safety advantages and can be used in a broad variety of patients, including immunocompromised patients. Our "probiomimetics" represent a unique and novel strategy of combining functional biomaterials with inherently active MVs to target autoimmune inflammatory dispositions.

2. Results and Discussion

2.1. Characterization of *Lactobacillus* MVs

The MVs were isolated and purified, and their size and particle concentration were determined by nanoparticle tracking

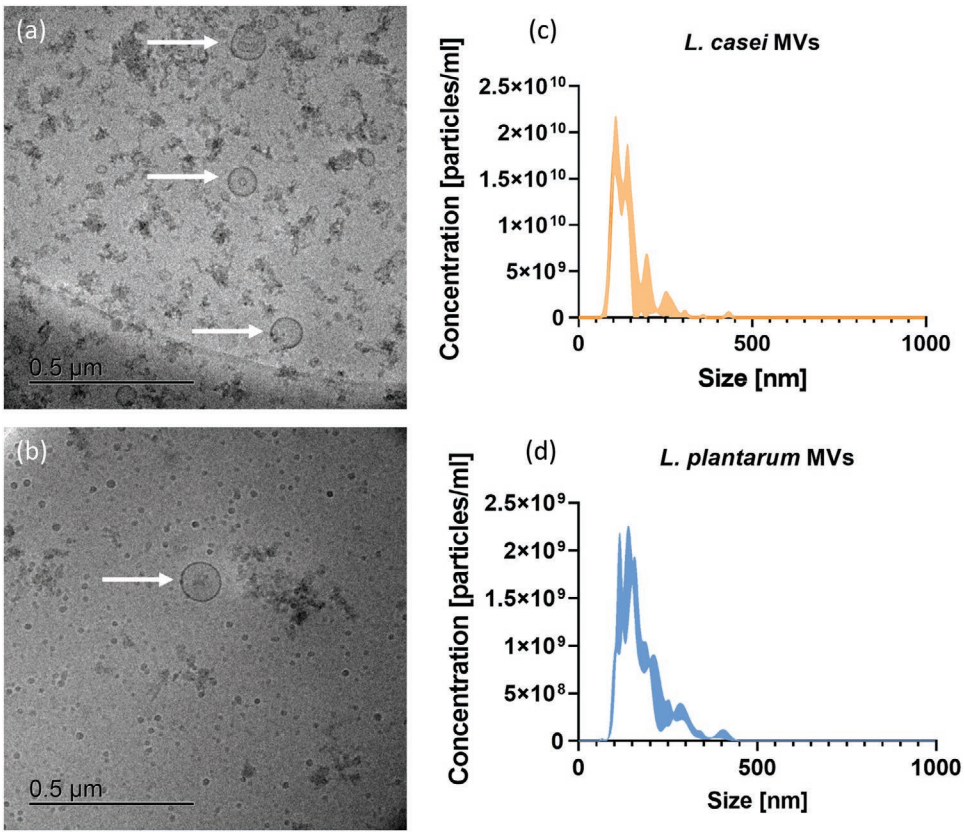

Figure 2. Characterization of the *Lactobacillus casei* and *L. plantarum* MVs used in this study. Representative cryo-transmission electron microscopy image of a) *L. casei* MVs and b) *L. plantarum* MVs. Images were acquired of MVs from the ultracentrifugation pellet before further purification by size-exclusion chromatography to ensure sufficient MV concentrations for imaging. MVs are marked with arrows. Furthermore, copelleted proteins are visible. Typical size distribution of c) *L. casei* MVs and d) *L. plantarum* MVs measured via nanoparticle tracking analysis.

analysis (NTA). The mode sizes of *Lactobacillus casei* and *Lactobacillus plantarum* MVs were 113 ± 12 and 117 ± 24 nm, respectively. These results are consistent with previous studies on MVs from other *Lactobacillus* species, such as *Lactobacillus acidophilus,* while MVs from *Lactobacillus reuteri* were found to be larger in size.[40,41] The particle concentrations ranged from ≈6 × 10^{11} for *L. plantarum* to 2 × 10^{12} for *L. casei*, which was 300 to 2000 times higher than for mammalian cells.[40] Additionally, the protein content in the MV-containing fractions 12–16 and the later fractions until fraction 48 of the size-exclusion chromatography was determined by the bicinchoninic acid (BCA) assay. A successful baseline separation of the MV peak was observed compared to the peak containing free proteins (Figure S3, Supporting Information).

Similar to the particle concentration, the protein content in *L. casei* (120.8 ± 7.6 µg mL^{-1}) was higher than that in *L. plantarum* (20.3 ± 5.9 µg mL^{-1}).

To the best of our knowledge, this study reported the first cryo-transmission electron microscopy images of the MVs of both *Lactobacillus* strains. The images were acquired from the pellet before purification via size-exclusion chromatography (SEC), which allowed the detection of protein aggregates within the samples. The size of the MVs appeared to be smaller in the images than the size obtained in bulk (**Figure 2**). This could be explained by the fact that NTA measures the hydrodynamic diameter while electron microscopy showed the MV morphology. The MVs appeared to be round-shaped particles, with an electron-dense membrane. In accordance with the NTA-data, the concentration of *L. casei* MVs was found to be higher than the concentration of *L. plantarum* MVs.

Next, we measured the viability of macrophage-like dTHP-1 cell line and intestinal Caco-2 cells, incubated with different concentrations of MVs, to screen for toxic effects of MVs and exclude damage to cells as a result of high MV concentration (**Figure 3**). Two different assays were used, the PrestoBlue assay, which measures the metabolic activity of the cells,[42] and the lactate dehydrogenase (LDH) assay, which assesses the presence of the intracellular enzyme LDH. This enzyme can only be detected in the presence of cytotoxic agents which lead to cell lysis. In the PrestoBlue viability assay, no significant difference was detected between MV samples and the negative controls, containing only medium and phosphate-buffered saline (PBS), despite treatment with a very high concentration of MVs. Concentrations used were as high as 300 000 MVs per cell for Caco-2 and 100 000 MVs per cell for dTHP-1, with no toxic effects observed.

For further evaluation of cytotoxicity, an LDH-assay was performed. LDH is an intracellular enzyme that is released during cell lysis, after the cell dies, thus indicating the cytotoxic

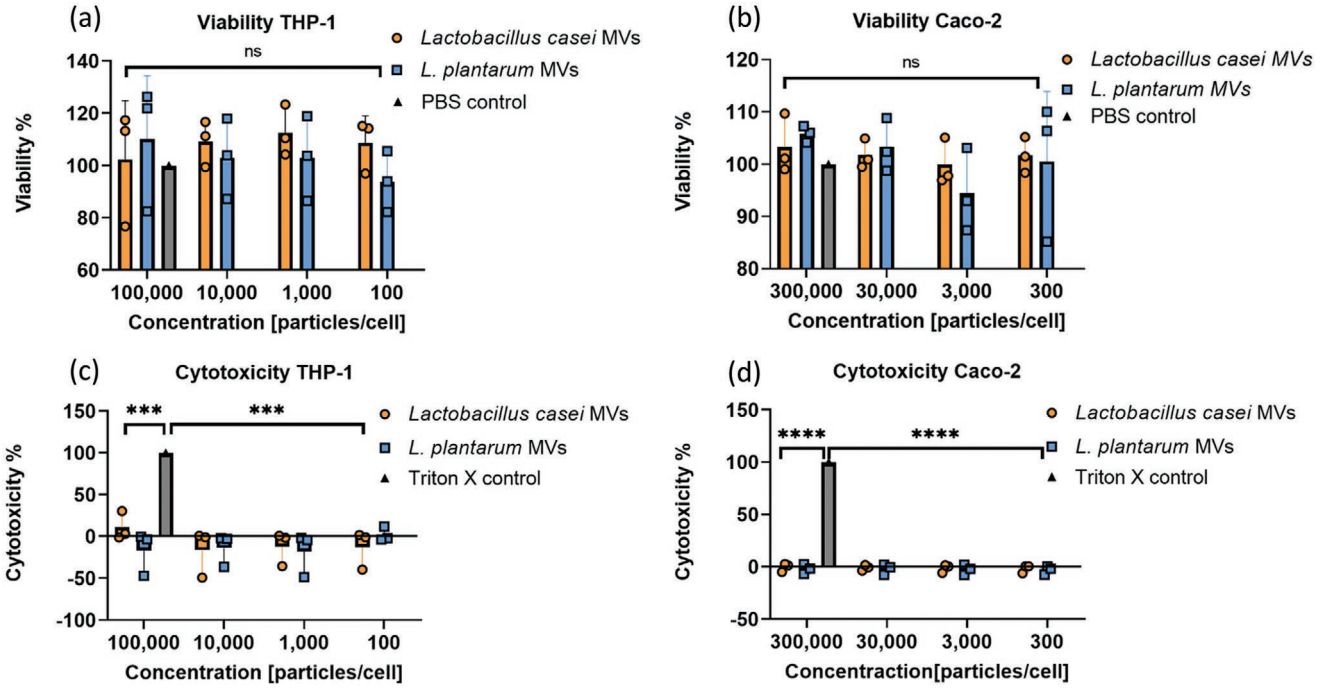

Figure 3. Cytotoxicity testing of the Lactobacillus MVs. a,b) PrestoBlue assay, measuring the metabolic activity of the two tested cell lines Caco-2 and THP-1 treated with MVs. c,d) Lactate dehydrogenase assay for assessing the intracellular enzyme lactate dehydrogenase released in case of cell lysis, thus indicating the presence of cytotoxic agents. No cytotoxic effect was detected in either cell line. Triton X (1%) was used as the death control, whereas phosphate-buffered saline was used as the live control. All experiments were conducted in three biological replicates; error bars indicate the standard deviation of the mean values

effects of the treatment. No difference in the concentration of LDH in the cells treated with MVs and the live control was observed, which was consistent with the results from the cell viability analysis. These findings are in accordance to what was observed for MVs of other Lactobacillus species.[3] Overall, our findings indicate a low risk for cytotoxicity-related side effects, thus suggesting that the probiotic MVs are suitable for further development as therapeutic agents.

2.2. Assembly and Characterization of Probiomimetics

To design the probiomimetics, we coupled the MVs from *L. casei* and *L. plantarum* onto the surface of microparticles. For proof-of-principle, we used aldehyde-group bearing polystyrene FACS beads that could react with the lysine residues of vesicular proteins.[43] The beads are commercially available in the required low-micrometer size range, resembling bacterial dimensions. In order to find the optimal conditions for the loading of the MVs onto the microparticles, the effect of different pH-values (pH 3,5,7, and 9) during the reaction of the particles with the MVs was assessed. As seen in the scanning electron microscopy (SEM) images in **Figure 4**, visible coverage of the particles was observed at pH 5 and 7, but almost no MVs could be observed on the particle surface at pH 3 and 9. As shown by SEM images, more MVs from *L. plantarum* were found on the surface of microparticles than on the surface of *L. casei* MVs. Noticeably, the MVs of the two different strains showed different behaviors with respect to the arrangement on the particle surface. While *L. casei* MVs seemed to be arranged in a flat layer, *L. plantarum* MVs were arranged in thicker clusters which were clearly visible in the SEM images.

The presence of MVs on the particle surface was also confirmed by measuring the protein content of all the coated particle formulations as well as the native MVs. The protein content in *L. casei* MVs was six times higher than in *L. plantarum* MVs. Interestingly, this difference could not be observed for the microparticles coated with both vesicle types with similar protein concentrations. This hinted to a possible saturation effect of the particle loading, which may have prevented the surface from being quantitatively covered by the MVs. Possible reasons for this observation might be steric hindrance or charge repulsions of the proteins on MV surface.

2.3. Testing of the Biological Effect of MVs and Probiomimetics

A hallmark of the IBD pathogenesis is a disrupted barrier function of the intestinal epithelium.[44] Based on these findings, we studied the barrier-protective effects of the probiomimetics. For this, we induced inflammation in a monolayer of Caco-2 cells, seeded on transwell inserts using lipopolysaccharide (LPS), and measured the trans-epithelial electric resistance (TEER) after 3 and 6 h. The TEER values are a marker of barrier integrity, which is important in the case of IBD and celiac disease, as a decreased barrier function is a common indication of pathogenesis (**Figure 5**).[15,45]

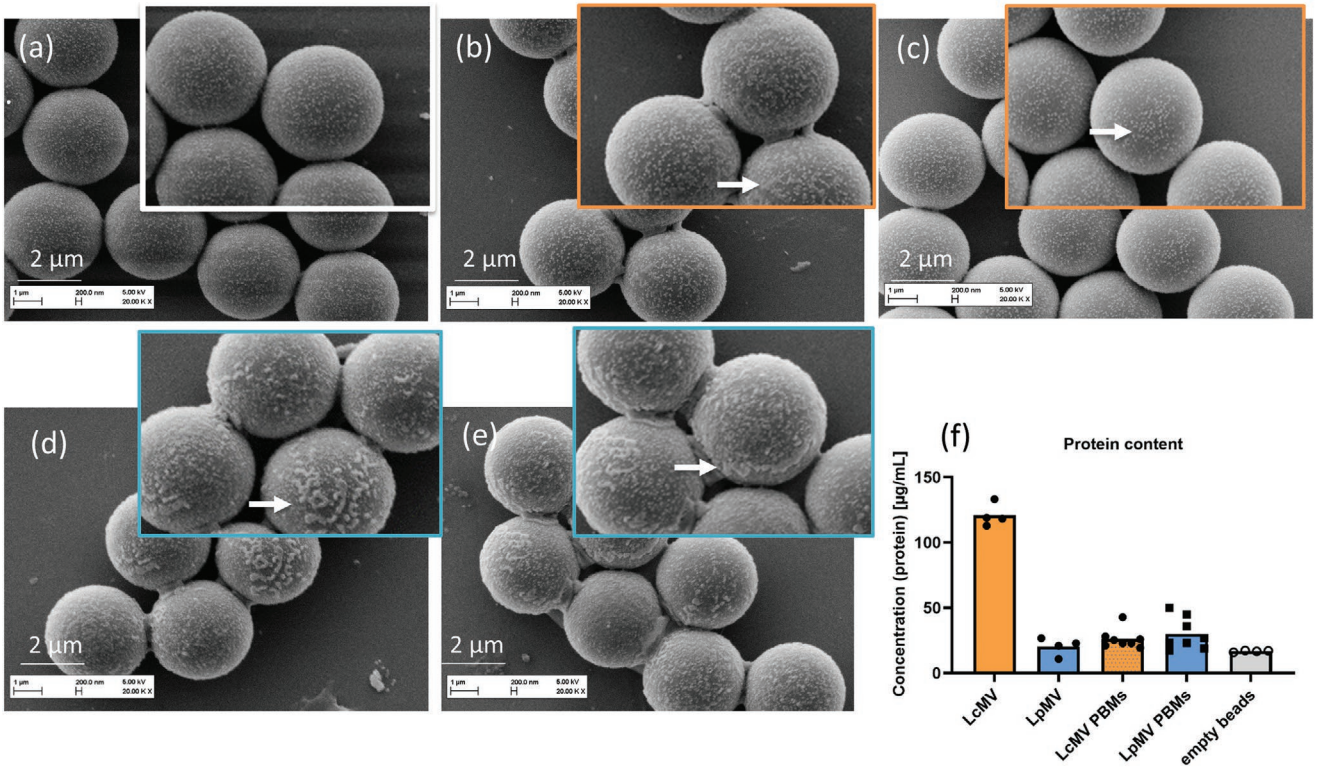

Figure 4. Morphological characterization of the probiomimetics via scanning electron microscopy. a) Untreated beads as control; b) probiomimetics coated with *L. casei* MVs c) at pH 5 and at pH 7. d) Probiomimetics coated with *L. plantarum* MVs at pH 5 and e) at pH 7. *L. plantarum* MVs are arranged in clusters on the microparticle surface, whereas *L. casei* MVs appear flatter. f) Comparison of protein content in MVs and probiomimetics measured via bicinchoninic acid assay as a surrogate for the dose of MVs. $n = 4$–9. Results report means values including standard deviation of all experiments.

After 3 h, the TEER values decreased by approximately 20 ± 5% in all conditions. TEER values were lowered by 22% after 6 h, when the cells were treated with LPS, while almost no decrease in TEER was observed for untreated control cells. Importantly, treatment of inflamed cells with probiomimetics led to a substantial recovery of the TEER values and reverted the effect of the LPS stimulation. All probiomimetics restored the epithelial barriers almost to the original levels, while treatment with MVs alone led to a continued increase of the barrier disruption. These results demonstrated the advantage of using the probiomimetics over MVs alone.

To further verify that the beneficial effects of the probiotics were conserved in their respective MVs and the probiomimetics, an in vitro assay was conducted to quantify the anti-inflammatory effects of MVs and probiomimetics. Macrophage-like dTHP-1 cells and enterocyte-like Caco-2 cells were stimulated with LPS (10 µg mL^{-1}) to mimic inflammatory processes present in the GIT environment. Cells were co-treated with the native MVs or coated microparticles for 6 and 24 h, respectively and the concentration of released cytokines was measured using enzyme-linked immunoassays (**Figure 6**). The cytokine release of the cells was normalized to the protein concentration of each sample to account for the MV to microparticle loading ratio that resulted in a different MV dose for the respective samples. We additionally tested vesicles of the common intestinal pathogen *Shigella flexneri*, as well as phosphatidylserine-containing liposomes with proven anti-inflammatory effect as a synthetic comparator to our natural probiomimetics.[46] Data were then normalized to the protein concentration to correct for the yields obtained for the different MVs. Data on the liposome control and the *Shigella flexneri* MVs can be found in Figures S2 and S3 (Supporting Information), as this normalization could not be applied there.

For all probiomimetics, a remarkably strong reduction in the TNF-α concentrations compared with LPS control was observed at both time points. Interestingly, no differences were observed for probiomimetics prepared from MVs from both *Lactobacillus* strains. The uncoated microparticles did not induce any proinflammatory effects, indicating that the reduction in TNF-α was induced by the MVs. For the native vesicles, those from *L. casei* showed a less pronounced downregulation of TNF-α compared to the probiomimetics. Overall, the highest reduction in pro-inflammatory cytokine release was observed for *L. plantarum* MVs alone and the corresponding probiomimetics.

We also investigated the effects of probiomimetics on the release of anti-inflammatory factor, IL-10, as it was previously shown that some probiotic MVs can increase the release of this cytokine.[47] IL-10 concentrations in the samples treated with the MVs as well as in the probiomimetics were higher than with LPS alone, confirming an anti-inflammatory effect. Interestingly, in all the tested conditions, *L. plantarum* MVs and *L. plantarum* MV-coated microparticles showed a higher anti-inflammatory effect than *L. casei* MVs and *L. casei* MV-coated microparticles, even though their concentrations were consistently fivefold lower. This effect seemed to be of

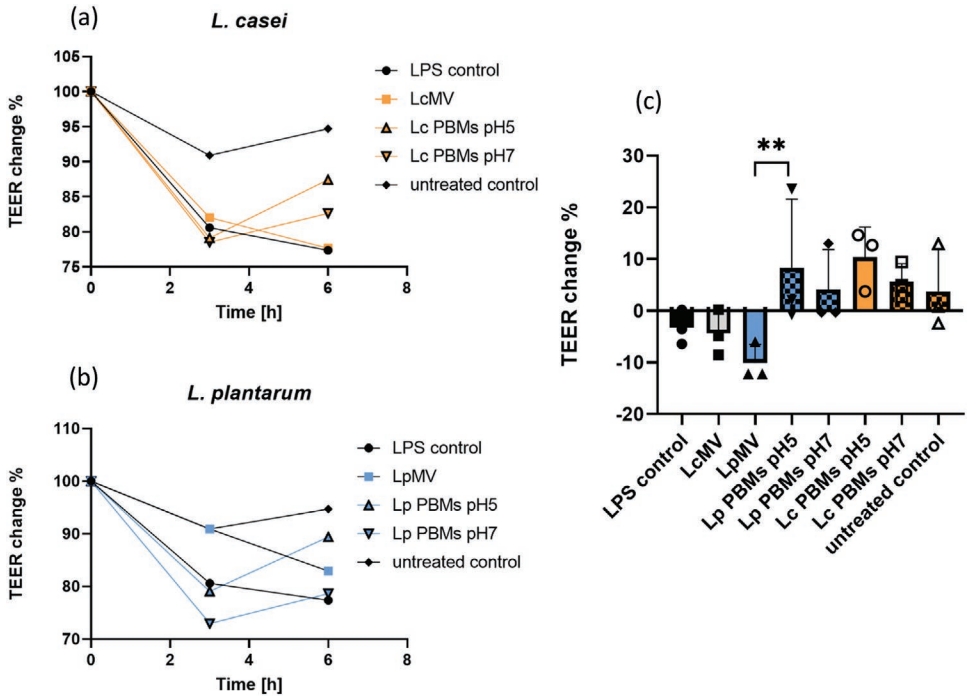

Figure 5. Trans-epithelial electric resistance values of enterocyte-like Caco-2 cells as a surrogate for the intestinal barrier function. a,b) Change in the TEER values over the course of the experiment, as an average of three individual experiments. When treated with probiomimetics for 3 h, the cells recover their barrier function. In contrast, cells treated with MVs show continued decline in TEER values. c) Total difference in the TEER values from 3 to 6 h. Probiomimetics helped recover the barrier, unlike free MVs. TEER, trans-epithelial electric resistance.

a shorter duration than the reduction of TNF-alpha concentrations, which could be concluded by the fact that the differences were less pronounced after 24 h. In contrast, treating the cells with LPS and liposomes led to a lower level of IL-10 than control treatment with LPS. These findings suggest that the probiomimetics induce a specific anti-inflammatory response. Further evaluations of the probiomimetics should include testing their effect in more complex in vitro models as well as in vivo.

We additionally studied the effect of MVs and probiomimetics on the release of proinflammatory interleukin-8 (IL-8) by Caco-2 cells. Here, a similar pattern to what was shown for the TNF-alpha-release of dTHP-1 cells could be observed. The probiomimetics as well as *Lactobacillus plantarum* MVs showed a strong, ≈12-fold, inhibition of IL-8 release, while the effect observed from *Lactobacillus casei* MVs was twofold.

Similar effects were seen with MVs from different *Lactobacillus strains,* such as *Lactobacillus rhamnosus* and *L. reuteri*.[48] This effect has been proven to be linked to the innate immune system, which is also consistent with the effects seen on the dTHP-1 cells.[49]

Other *Lactobacillus strains,* such as *Lactobacillus kefir,* have anti-inflammatory effects on Caco-2 cells and they ameliorated colitis in a mouse model.[50] According to a recent study by Choi et al. these effects seem to be related to multiple mechanisms, such as the reduction of NO-production and the amelioration of endoplasmic reticulum stress.[51]

Our results indicate a pronounced inflammation-regulatory effect of the novel probiomimetics. Particularly, in the case of probiomimetics prepared from *L. plantarum* MVs, the new mimetic system induced a substantial release of the regulatory cytokine IL-10, and had a significant inhibitory effect on the release of the pro-inflammatory factors, TNF-α and IL-8.

3. Conclusion

In this study, it was shown for the first time that *Lactobacillus* MVs can be coupled onto the surface of microparticles to create a novel bacteriomimetic system. In addition, the ability of *Lactobacilli* to produce high amounts of MVs in liquid culture was demonstrated, which could be easily scaled up. Bacteria grown in suspension may be cultured at higher volumes, for example in fermenters, which would increase the MV yield. Industry-level production is conceivable, similar to the production of therapeutic antibodies by *Escherichia coli*, which are also grown in suspension.[52] This is especially true in comparison to extracellular vesicles obtained from adherent mammalian cells, which produce MV concentrations 100–1000 times smaller than the *Lactobacilli* used here.[53] A pronounced inflammation modulatory effect of both the MVs, free as well as the corresponding probiomimetics, was observed. In contrast to the native MVs, probiomimetics demonstrated the potential to ameliorate LPS-induced loss of barrier function in a transwell model of intestinal cell line Caco-2. *Lactobacillus* MVs alone did not show this ability, thus indicating the importance of a controlled and high concentration delivery system. Our findings highlight the valuable potential of probiomimetics that can be further

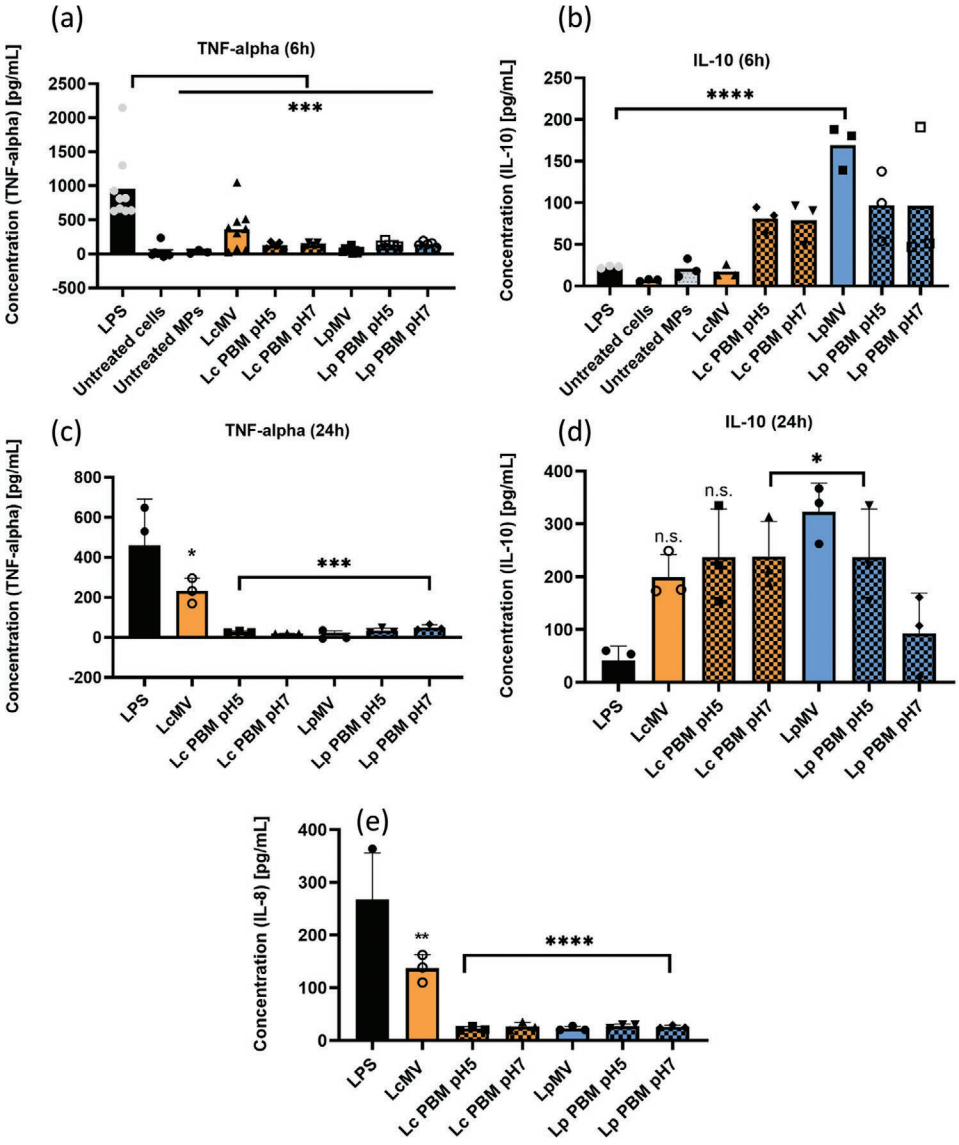

Figure 6. Cytokine production in inflamed macrophage-like dTHP-1 cells as well as in enterocyte-like Caco-2 cells. Cells were stimulated with lipopolysaccharides (10 μg mL^{-1}) and cotreated with probiomimetics or MVs. Supernatants were harvested after 6 or 24 h, and the protein content was analyzed using ELISA. To exclude the effects of different MV concentrations during the assembly of the probiomimetics, all results were normalized to the protein content. a) ELISA measurement of proinflammatory TNF-α released in dTHP-1 cells after 6 h. b) Release of anti-inflammatory IL-10 after 6 h. c) ELISA measurement of proinflammatory TNF-α released in dTHP-1 cells after 24 h. d) Release of anti-inflammatory IL-10 after 24 h. e) Release of proinflammatory IL-8 in enterocyte-like Caco-2 cells after 24 h. Values represent the mean of 3–9 biological replicates with standard deviations.

developed as novel therapeutic agents for patients with IBD. Future studies should incorporate scaling-up of the probiomimetics' production as well as testing of their effects in suitable in vivo models.

4. Experimental Section

Cell Culture: CaCo-2 HTB-37 (ATCC, Manassas, VA) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Waltham, MA), supplemented with 10% fetal calf serum (FCS) (Gibco, Waltham, MA) and 1% nonessential amino acid mix (Gibco, Waltham, MA). Cells were supplemented with fresh medium every 2–3 d. Cells were split after one week, when they were 80–90% confluent.

THP-1 (DSMZ, Braunschweig, Germany) cells were grown in suspension in RPMI-1640 (Gibco, Waltham, MA) medium. After every 3–4 d, 2.5 mL of cell suspension was transferred to 10 mL of medium. For assays, THP-1 cells were centrifuged and redispersed in a medium containing phorbol-12-myristate-13-acetate (7.5 ng mL^{-1}) and seeded into 96-well plates, at a density of 1×10^5 cells per well. Cells were then allowed to differentiate for 24 h for viability testing and 48 h for cytokine assays.

Measurement of TEER: Caco-2 HTB-37 cells in passage 30 ±10 were seeded on Corning Transwell inserts, at a density of 2×10^4 cells per well. Cells were then allowed to grow for 11 d at 37 °C and 5% CO_2. The medium was changed every 2–3 d (500 μL in the apical compartment and 1500 μL in the basolateral compartment). After 11–12 d, TEER ($t = 0$) was measured in every well. Subsequently, the medium was aspirated in the apical compartment, and 250 μL of fresh medium supplemented

of LPS (10 µg mL^{-1}) from *E. coli* (0111:B4, gamma-irradiated, BioXtra, suitable for cell culture, Sigma-Aldrich, St. Louis, MO) and 250 µL of sample (Microparticle or EV suspension) was added. TEER values were measured using EVOM^2 (World Precision instruments, Sarasota, FL) after 3 and 6 h. Then, the supernatant was collected for the quantification of cytokines.

Bacterial Culture: *L. casei* (DSMZ, Braunschweig, Germany) was cultivated on deMan-Rogosa-Sharpe agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 5 d at 37 °C under microaerophilic conditions in an Ecotron HT incubator (infors, Basel., Switzerland). From this stock (stored at 4 °C), liquid culture was inoculated, transferring one single colony into 100 mL of deMan-Rogosa-Sharpe liquid medium. The culture was allowed to grow for 48 h at 37 °C. *L. plantarum* (NCIMB, Aberdeen, UK) was cultivated on deMan-Rogosa-Sharpe agar for 5 d at 30 °C in an Ecotron HT incubator (infors, Basel., Switzerland). From this stock plate (stored at 4 °C), liquid cultures were inoculated using one single colony into 100 mL of deMan-Rogosa-Sharpe liquid medium. The culture was allowed to grow for 48 h at 30 °C. For both strains, culture conditions were chosen according to the instructions given by the suppliers. Microscopy images of the bacteria can be found in Figure S1 (Supporting Information).

MV Isolation: After 48 h of growth, bacterial cultures were centrifuged for 5 min at 9500 × *g* to remove residual bacteria. Next, the EV-containing supernatant was filtered through a 0.45 µm polyvinylidene difluoride membrane (Stericup-HV 150 mL Durapore PVDF 0.45 filter bottles, Merck, Darmstadt, Germany). Supernatants were then transferred to ultracentrifuge tubes and centrifuged for 2 h at 100 000 × *g* at 4 °C. Then, the supernatant was discarded, and the pellet was redispersed in filtered PBS (400 µL). The resuspended pellet was purified via a SEC column filled with 35 mL Sepharose CL-2B (GE Healthcare, Braunschweig, Germany). Fractions of 1 mL were collected. The particle concentration of the fraction used for the experiment was measured by nanoparticle-tracking analysis.

Vesicle Characterization: Nanoparticle Tracking Analysis (NTA): Sample size and particle concentration were measured using NanoSight (Malvern Panalytical, Malvern, UK) and analyzed using the NTA 3.3 software. The camera level used on the instrument was 15 and the detection threshold value was set to 5.

Vesicle Characterization: Determination of Protein Content: Protein content of EVs and EV-containing microparticles was assessed using QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's specifications.

Vesicle Characterization: Preparation of MV-Coated Microparticles: A 500 µL volume of aldehyde/sulfate latex beads 4 µm (Invitrogen, Waltham, MA) suspension was washed thrice with 1 mL of highly purified water (MilliQ quality) and resuspended in 500 µL of highly purified water. Next, 300 µL of MV suspension, an amount that was found via method optimization, was mixed with 10 µL of the purified beads and 690 µL of PBS. This mixture was then adjusted to the desired pH-values pH = 5 and pH = 7 using sodium hydroxide solution and hydrochloric acid and incubated for 16 h at room temperature (RT), under shaking (300 rpm), allowing the lysine groups of the MVs' surface proteins to react with the aldehyde groups on the microparticle surface. The microparticles were then purified by centrifugation at 2500 × *g* for 5 min at 4 °C and subsequent exchange of the supernatant by 1 mL of PBS. The washing procedure was repeated twice. After the washing steps, the PBMs were resuspended in 1 mL of sterile filtered PBS.

Viability Assays: Cells were seeded into the inner 60 wells of 96-well plates. Approximately 2 × 10^4 CaCo-2 HTB-37 cells suspended in 200 µL of DMEM, supplemented with 10% FCS and 1% nonessential amino acid (Invitrogen, Waltham, MA). After allowing the cells to grow for 48 h, the medium was aspirated, and 100 µL of fresh medium (without FCS) was added, followed by the addition of 100 µL of the sample. The controls used were death-control (medium supplemented with 2% TritonX) and live-control (PBS).

Cells were incubated with EVs of the highest-concentrated SEC fraction (≈5 × 10^{11} – 5 × 10^{12} EVs mL^{-1}) and three serial 1:10 dilutions for 24 h. PrestoBlue (ThermoFisher Scientific, Waltham, MA) reagent was diluted 1 in 10 in the respective medium of the cells. After incubation for 24 h, 100 µL of the medium was sampled for analysis by the LDH-assay. The remaining medium was aspirated and cells were supplemented with 100 µL of the diluted PrestoBlue reagent. After 20 min of incubation at 37 °C, fluorescence of the emerging fluorescent dye was measured.

A 100 µL volume of the supernatant was mixed with 100 µL of LDH-reagent (Roche), prepared according to the supplier's protocol. After an incubation time of 5 min at RT, absorbance of the solution was measured at $\lambda = 492$ nm.

Cytokine Assay and Enzyme-Linked Immunosorbent Assay: THP-1 cells were differentiated with 7.5 ng mL^{-1} phorbol-12-myristate-13-acetate for 48 h. Twenty thousand Caco-2 cells per well were seeded and cultured for 48 h until 90% confluence was reached. Next, the medium was aspirated and 100 µL of fresh medium, supplemented with 10 µg mL^{-1} lipopolysaccharides from *E. coli* 0111:B4, was added. The cells were then supplemented with 100 µL of either medium, EV suspension or microparticle suspension. Additionally, cells without LPS were used as an untreated control. Every condition was applied in three replicates. Cells were then incubated for 6 or 24 h, respectively at 37 °C in 5% CO_2. Three samples for every condition were pooled and stored at -80 °C.

The supernatants were thawed and the concentrations of IL-10 and TNF-α were analyzed using Human IL-10 ELISA Set (Diaclone, Besançon, France), Human TNF-α ELISA Set (Diaclone, Besançon, France), and Human IL-8 ELISA Set (Diaclone, Besançon, France), respectively, according to the supplier's protocols.

SEM: All microparticle samples were centrifuged 5 min at 2500 × *g*. The supernatant was discarded, and the pellet was resuspended in 1 mL of highly purified water to dissolve any excess buffer salts. This washing step was repeated once. A 2 µL volume of each sample was transferred to a silica wafer and allowed to dry at RT for ≈2 h. Samples were then sputtered with a 10 nm layer (Quorum Q150R ES) of gold and imaged under high vacuum using an accelerating voltage of 5 kV and a beam current of 1.978 pA (Zeiss EVO MA15 LaB_6)

Cryo-Transmission Electron Microscopy: The EV sample (3 µL) was transferred to a copper grid, blotted for 2 s, and then plunged into undercooled liquid ethane at –165 °C (Gatan Cryoplunge3). The grid was then transferred under liquid nitrogen to a cryo-TEM sample holder (Gatan model 914). Low-dose bright-field images were acquired at –170 °C, using a JEOL JEM-2100 LaB_6 Transmission Electron Microscope and a Gatan Orius SC1000 CCD camera.

Data Analysis: Data were represented as mean values of 3–9 individual experiments, with error bars indicating the standard deviation. Statistical analyses were conducted using one-way analysis of variance followed by Tukey's multiple comparisons test to assess differences between individual groups.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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