Anti-inflammatory microparticles for the treatment of autoimmune diseases

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I. Summary

Inflammatory processes are a crucial part of the body's response to damage by injuries and pathogens. However, it plays an important role in pathophysiology of, e.g. autoimmune diseases, and poorly healing wounds. Probiotic bacteria possess anti-inflammatory properties, making them candidates for treatment of inflammation. However, their origin as living organisms makes them unsuitable for therapeutic use in e.g. immunocompromised or immunosuppressed patients.

Here, a novel bacteriomimetic therapeutic was developed by coupling the membrane vesicles (MVs) produced by two probiotic *Lactobacillus casei* and *Lactobacillus plantarum* to the surface of synthetic microparticles (MPs). The MVs were harvested from different bacterial culture conditions and characterized regarding their physicochemical properties and biological effect. The MVs showed no cytotoxic effects and suppressed the release of pro-inflammatory cytokines *in vitro.* Properties of the MVs depended on bacterial strain and culture conditions. MV-coated MPs showed anti-inflammatory effects *in vitro* and improved the restoration of barrier in an *in vitro* enterocyte model. Anti-inflammatory properties were also observed in a wound healing model, where the MV-coated MPs in a pharmacopoeial hydrogel improved healing of a tail wound in mice. This underlines the potential of probiotic bacteria MVs in antiinflammatory therapy and the advantages of coupling the MVs to synthetic MPs to create a bacteriomimetic system.

II. Zusammenfassung

Entzündungen sind Bestandteil der Antwort des Körpers auf Verletzungen und Pathogene. Wird die Entzündung nicht adäquat reguliert, ist sie Teil der Pathophysiologie z.B. bei Autoimmunerkrankungen oder schlecht heilender Wunden. Der anti-inflammatorische Effekt probiotischer Bakterien macht sie zu Kandidaten für die Behandlung von Entzündungen, wobei ihre Eigenschaft als lebende Organismen sie für den Einsatz bei z.B. immunsupprimierten Patienten ungeeignet macht.

Hier wurde ein bakteriomimetisches System entwickelt, in dem die Membranvesikel (MVs) von *Lactobacillus casei* und *Lactobacillus plantarum* auf synthetische Mikropartikel (MPs) gekoppelt wurden. Die MVs wurden aus verschiedenen Kulturbedingungen isoliert und ihre physikochemischen Eigenschaften und biologischen Effekte charakterisiert. Sie zeigten keine Zytotoxizität und unterdrückten *in vitro* die Ausschüttung pro-inflammatorischer Zytokine. Die Eigenschaften der MVs waren abhängig vom Stamm und den Kulturbedingungen. Die MVbeschichteten MPs waren anti-inflammatorisch *in vitro* und führten zu einer verbesserten Wiederherstellung der Barriere *in vitro*. Die anti-inflammatorischen Eigenschaften wurden *in vivo* gezeigt, indem die MV-beschichteten MPs, in einer Hydrogel-Matrix, die Wundheilung am Schwanz von Mäusen verbesserten. Diese Arbeit zeigt das Potential von MVs probiotischer Bakterien als anti-inflammatorische Therapeutika, sowie die Vorteile des bakteriomimetischen Ansatzes, die MVs auf synthetische MPs zu laden.

III. Abkürzungsverzeichnis / List of abbrevations

CD	Crohn's disease
DAMP	danger-associated molecular pattern
ELISA	enzyme-linked immunosorbent assay
EVs	extracellular vesicles
GC	glucocorticoids
GIT	gastrointestinal tract
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IL	interleukin
JAK	janus kinase
MVs	membrane vesicles
NK-cells	natural killer cells
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PRR	pattern recognition receptor
SEC	size exclusion chromatography
SEM	scanning electron microscopy
STAT	Signal Transducers and Activators of Transcription
TEM	transmission electron microscopy
TGF-β	transforming growth-factor β
TNF	Tumor necrosis factor
UC	ulcerative colitis

Novel anti-inflammatory microparticles for the treatment of autoimmune diseases

1. Introduction:

1.1. The physiology of inflammation and inflammatory diseases

Inflammation is a physiological response to any type of tissue damage, e.g. infection or injury. It is characterized by an activation of the immune system at the site of tissue damage.[1] In cases where the inflammatory response is not adequately resolved, various diseases are caused by the persistent inflammation. These include two main overlapping main classes, namely autoinflammatory diseases and autoimmune diseases.[2] The commonly applied definition is that in the pathogenesis of autoimmune diseases, the adaptive immunity plays a major role, while autoinflammatory diseases are the result of malfunctions of innate immunity. However, as the innate and adaptive immune response are interconnected through a variety of mechanisms, the transition between the two groups of diseases is not clear.[3] Both autoimmune and autoinflammatory diseases share the common thread, that the immune system attacks the body's own tissue.[4] These groups of diseases both are characterized by increasing prevalence rates in many parts of the world.[5,6]This highlights the need for novel therapeutic options for overshooting inflammation.

Inflammation is characterized by five cardinal symptoms: *calor* (fever), *rubor* (redness), *tumor* (swelling and edema), *dolor* (pain) and *functio laesa* (loss of function).[7] These macroscopic symptoms are the result of an increased permeability of the vascular endothelium at the site of the tissue damage (Figure 1), as well as adaptions of the tissue to the purpose of eliminating pathogens or restoring tissue damage.[8]

This increased endothelial permeability ensures that cells of the innate and adaptive immune system can pass through the endothelium to promote removal of cellular debris and pathogens that may have invaded the tissue. The inflammatory reaction can be triggered by a variety of factors that are recognized by pattern recognition receptors (PRR). These are part of the innate immune system and include receptor types, such as toll-like receptors and NOD-like receptors. Such factors can be categorized as DAMPs (danger-associated molecular patterns, e.g. components released from dead or damaged cells such as ATP or urate crystals) and PAMPs (pathogen-associated molecular patterns, e.g surface structures or toxins of pathogenic organisms that invaded the site of tissue damage).[8–10] During the inflammation, tissue hormones, such as eicosanoids (most notably leukotrienes and prostaglandins) are released, which further promote and sustain the inflammatory reaction. Further involved in sustaining the inflammation are cells of the innate immune response (e.g. macrophages, antigen-presenting cells, NK-cells) and the adaptive immune response (T-cells and B-cells) as well as a variety of pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF) and different interleukins.[11,12]

After the initial tissue damage has been repaired, it is physiologically necessary to end the inflammation. Under physiological circumstances, this is achieved via different mechanisms.14 Notably, apoptosis of neutrophils is a key player in the resolution of inflammation. It induces macrophages to a phenotype switch to resolution promoting macrophages, which produce anti-inflammatory cytokines, such as IL-10 and transforming growth-factor β (TGF-□).15

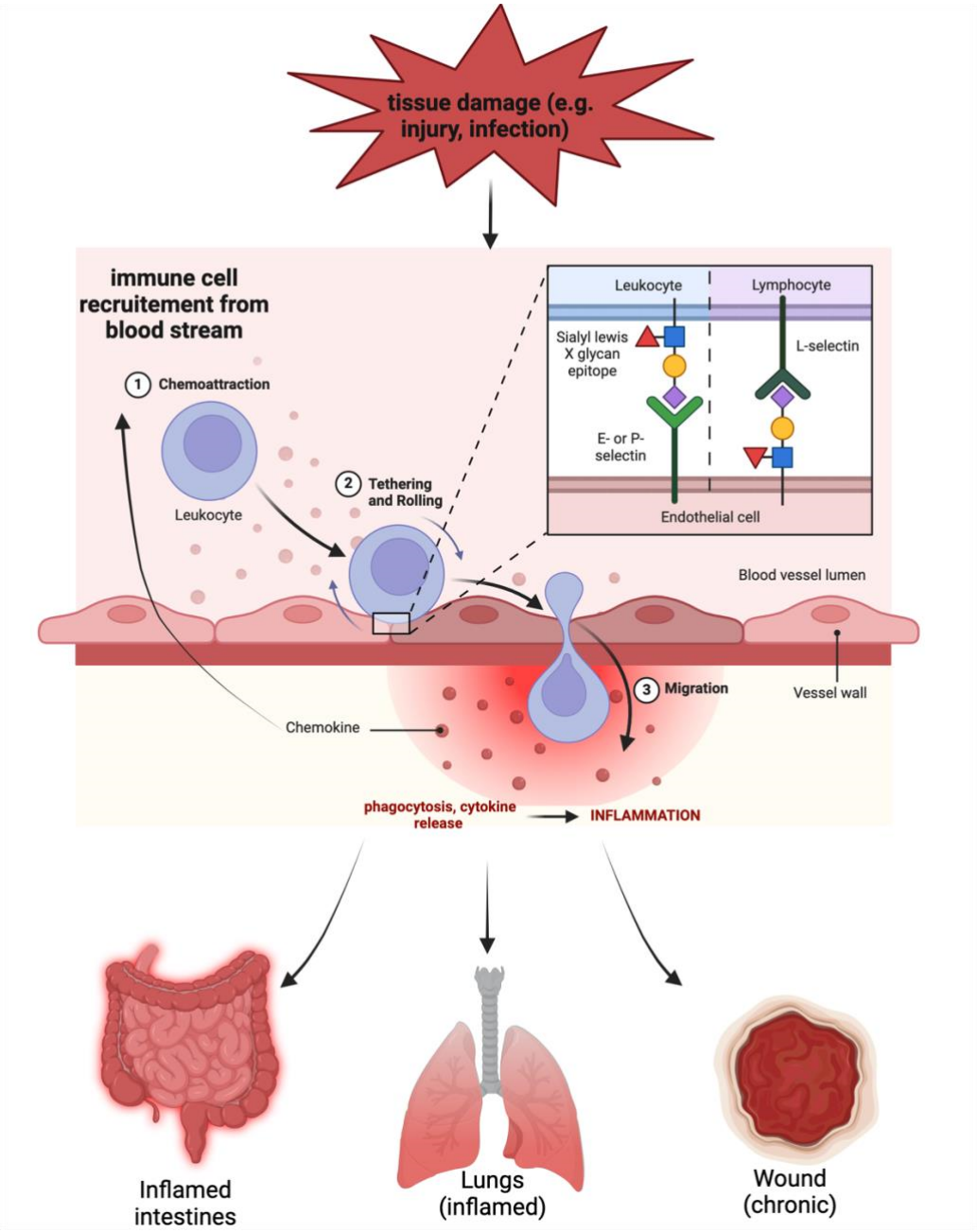

Figure 1 General overview on inflammatory processes: Tissue damage causes the attraction of leukocytes and triggers the inflammation. This leads to attraction lymphocytes to the site of the tissue damage. The vascular endothelium becomes leaky, which allows the immune cells to pass through, while simultaneously causing the five cardinal symptoms of inflammation, namely calor (fever), rubor (redness), tumor (swelling and edema), dolor (pain) and functio laesa (loss of function).[15] *Chemokines are released, which promote and sustain the inflammation.*

Autoimmune and autoinflammatory diseases can occur in a variety of organs and tissues causing a widespread amount of diseases, with more than 80 different diseases known today.[16] The most well-known examples for this group of illnesses include inflammatory diseases of the intestine e. g. Chron's Disease or Celiac Disease, the endocrinological system, e. g. Diabetes mellitus or Addison's Disease, the skin e. g. atopic dermatitis or psoriasis as well as manifestations in the nervous system such as multiple sclerosis or myasthenia gravis.[17–20] Another subgroup of autoinflammatory diseases occurs without the involvement of autoreactive T-cells. This group of diseases includes more rare diseases, such as familiar Mediterranean fever or Majeed's disease.[2] They all are characterized by rising prevalence rates worldwide, especially in industrialized countries.[21] This group of diseases poses a great burden on healthcare systems worldwide. Their effect has been shown to be comparable to strokes with approximately 10 million patients in the US alone.[22] Prevalence rates differ depending on the location, with Europe and North America being the most affected regions, where a prevalence of more than 0.3 % was reported for inflammatory bowel diseases.[23] While a substantial amount of treatment options for autoimmune diseases are available, the vast majority of these can only suppress the symptoms, as the tissue damage is in most cases irreversible by the time the disease is diagnosed. Therefore, further treatment options are urgently needed.[24]

This work focuses on two groups of these diseases namely the manifestations in the gastrointestinal tract as well as the skin. These therapeutic targets were chosen, as they are the most accessible to local therapy, which the therapeutic system we developed is most suitable for.

1.1.1 Pathophysiology and symptoms of inflammatory diseases of the intestine

Inflammatory diseases of the intestine pose a great burden on global health systems worldwide. According to recent estimates, they the intestine affect around 1 million people in the US and approximately 3 million patients in Europe.[25] The pathogenesis and symptoms of those diseases will be discussed in the following paragraphs. Three different autoimmune diseases of the intestine are discussed, namely ulcerative colitis (UC), Crohn's Disease and Celiac Disease.

All three diseases share a variety of symptoms linked to the damage to the intestinal barrier, caused by the autoimmune reaction. The most well-known symptoms are those of a typical gastrointestinal nature. They include abdominal pain and cramping, nausea and diarrhea-like symptoms as well as bloating but also constipation.[26 27]

The destruction of the intestinal epithelium leads to severe impairment of digestion and the absorption of micronutrients, resulting in a deficiency of especially vitamin D.[28] This has major implications for the prevalence of secondary diseases associated with celiac Disease. These include osteoporosis, mainly linked to the lack of vitamin D, but also a variety of other autoimmune diseases, such as type-1 diabetes, or autoimmune-induced hypothyroidism.[29] Due to the malabsorption of micronutrients and the generally increased inflammatory response, a variety of extraintestinal manifestations were described.[30,31] This group of symptoms can be directly related to the inflammation of the gastro-intestinal tract (GIT), such as episcleritis and aphtous mouth-ulcers but also linked to the micronutrient deficiency, such as osteoporosis and peripheral neuropathy.[32]

Patients of IBDs and celiac disease share a greater susceptibility for other autoimmune diseases. One example for co-occurrence of IBDs/celiac disease and extraintestinal autoimmune diseases is the known correlation of celiac disease with type 1 diabetes mellitus.[18]

Lastly, the ongoing inflammatory process also facilitates the onset of colorectal cancers and seemingly unrelated symptoms, such as depression and fatigue, which was found to be correlated with highly active inflammatory bowel disease. [33 34]

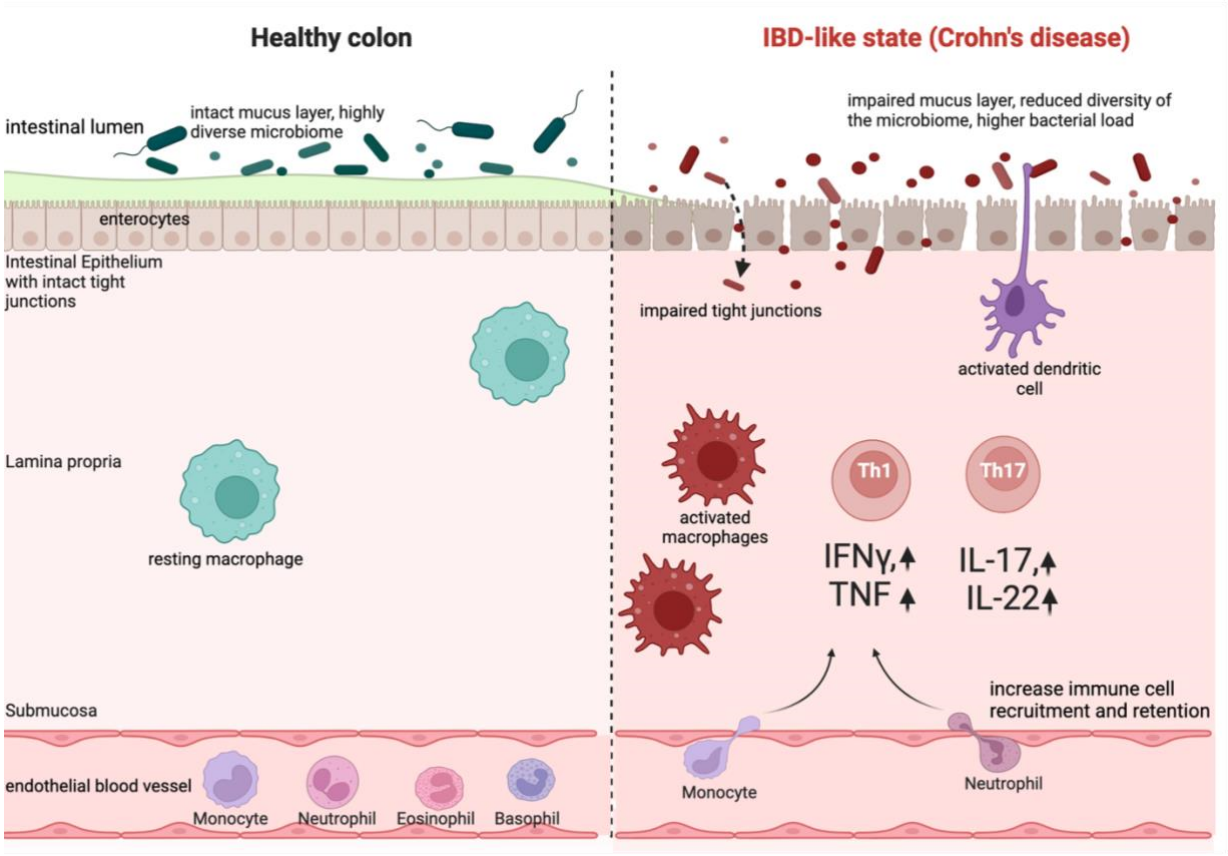

Figure 2: Pathophysiology of Crohn's disease as an example for IBDs. Leaky epithelium leads to bacteria from the intestinal reaching the lamina propria, where cells of the innate immune system are activated. These activate T-cells, stimulating them to produce pro-inflammatory cytokines, thus further promoting the inflammation.[35] *Created with BioRender.com*

While clinical symptoms are similar, distinct differences are known for the pathophysiology, which will be explained in the following paragraph. Pathogenesis is complex for the three diseases Crohn's disease (Figure 2), ulcerative colitis, and celiac disease, and current hypotheses indicate an involvement of environmental, genetic, and microbial factors as root causes for IBD pathogenesis.[36]

Several studies also at the hypothesis, that a leaky epithelial barrier might be a contributor to the pathophysiological state of the gut in both, UC and Crohn's disease, as it allows pathogenassociated molecular patterns to reach immunocompetent cells inside the lamina propria, where dendritic cells seem to play a major role.[37–39] This is backed by studies that indicate an increased abundance of distinct bacterial strains such as *Enterococcus faecalis* is linked to undesirable clinical outcomes in Crohn's disease patients.[40] It is unclear however, whether the decreased barrier functionality of the mucosa is the causal for UC and Crohn's or a symptom of the autoimmune reaction.
While pathophysiology is similar for both IBDs, differences between UC and Crohn's disease were described regarding the cytokines and cell types involved and the areas of the GIT affected by the respective diseases.

Crohn's disease is characterized by multiple inflammation loci, interrupted by unaffected areas, occurring in every part of the GIT, from the mouth to the colon, whereas the distal ileum is the most frequently affected part.[41] In Crohn's disease patients, all layers of the mucosa are affected, from the epithelium to the *lamina propria*.[36] The disease is maintained by the influx of immune cells, both of the adaptive immune response, such as B-cells, CD4+ as well as CD8+ T-cells, but also cells related to the innate immune response, such as monocytes and natural killer (NK) cells.

UC, while sharing common features regarding pathophysiology, shows inflammation mainly in the rectum and colon with no unaffected areas in between. Here, mainly the mucosa and submucosa are affected, but not deeper layers of the intestine.[42] The differences in pathophysiology of UC and Crohn's disease have not been fully understood, however, differences in JAK/STAT signaling have been found to play a role.[43]

In celiac disease, contrary to the IBDs, symptoms are triggered by the intake of gluten.[44,45] Here, gluten intake can act as a "switch" triggering the onset of symptoms, which then can disappear, after gluten intake is stopped.[46] The onset of the disease is facilitated by a variety of genetic, and environmental factors as well as the microbiome.[44,47] One special risk factor

known for celiac disease is known to be two special HLA haplotypes, namely HLA-DQ2 and DQ8. Involvement of autoantibodies against the enzyme transglutaminase-2 is characteristic for celiac disease.[45] The autoimmune reaction after gluten intake leads to a degradation of the intestinal epithelium in the shape of villous atrophy, where the ratio of villous height and crypt depth is decreased to a value lower than 2.[48]

1.1.2 Current therapeutic options for IBDs and celiac disease

There is no curative therapy available for neither IBDs nor celiac disease, which makes lifelong therapy a necessity for IBD and celiac disease patients.[49,50]

For celiac disease, there is no symptomatic therapeutic available on the market. Experimental therapies are shown in Figure 3. The only possibility to ameliorate their symptoms is the lifelong adherence to a gluten-free diet.[45] This diet is hard to achieve, especially in industrialized countries used to a “western” style of diet. It is extremely challenging for patients to achieve an entirely gluten-free diet because even small traces of gluten can be sufficient to trigger symptoms.[51] Another shortcoming of this approach is its price, with gluten-free diet being up to 124% more costly for the patients. Lastly, even a gluten-free diet fails to ameliorate the symptoms for up to 30% of the patients. To this day, no FDA or EMA-approved therapeutic has reached the market. However, two approaches have been examined in phase-III clinical studies. One route involves the endopeptidase latiglutenase, an enzyme which can degrade gluten in the intestine.[52] Another therapeutic route, which has been examined, involves the tight junctions that are affected by the autoimmune reaction. Here, the peptide larazotide acetate has shown promising results *in vitro* and *in vivo.*[53] An These two therapeutics are the only ones, which have reached phase III of clinical studies until the writing of this thesis, however, lazarotide failed to achieve sufficient clinical efficacy in the mentioned clinical study.[54]
Novel, experimental therapeutic options for Celiac disease were examined, which intervene in the pathophysiology of the intestinal inflammation. Here, several targets further downstream in the inflammatory cascade come into question. One, after the gluten crossed the epithelial barrier, it reaches antigen-presenting cells, which can be addressed with immunotolerability therapy or by inhibiting antigen presentation.[26] Cytokine responses, involved in the pathophysiology can also be addressed, with a known example being interleukin-15, which can be inhibited using kinase inhibitors, such as tofacitinib.[55]

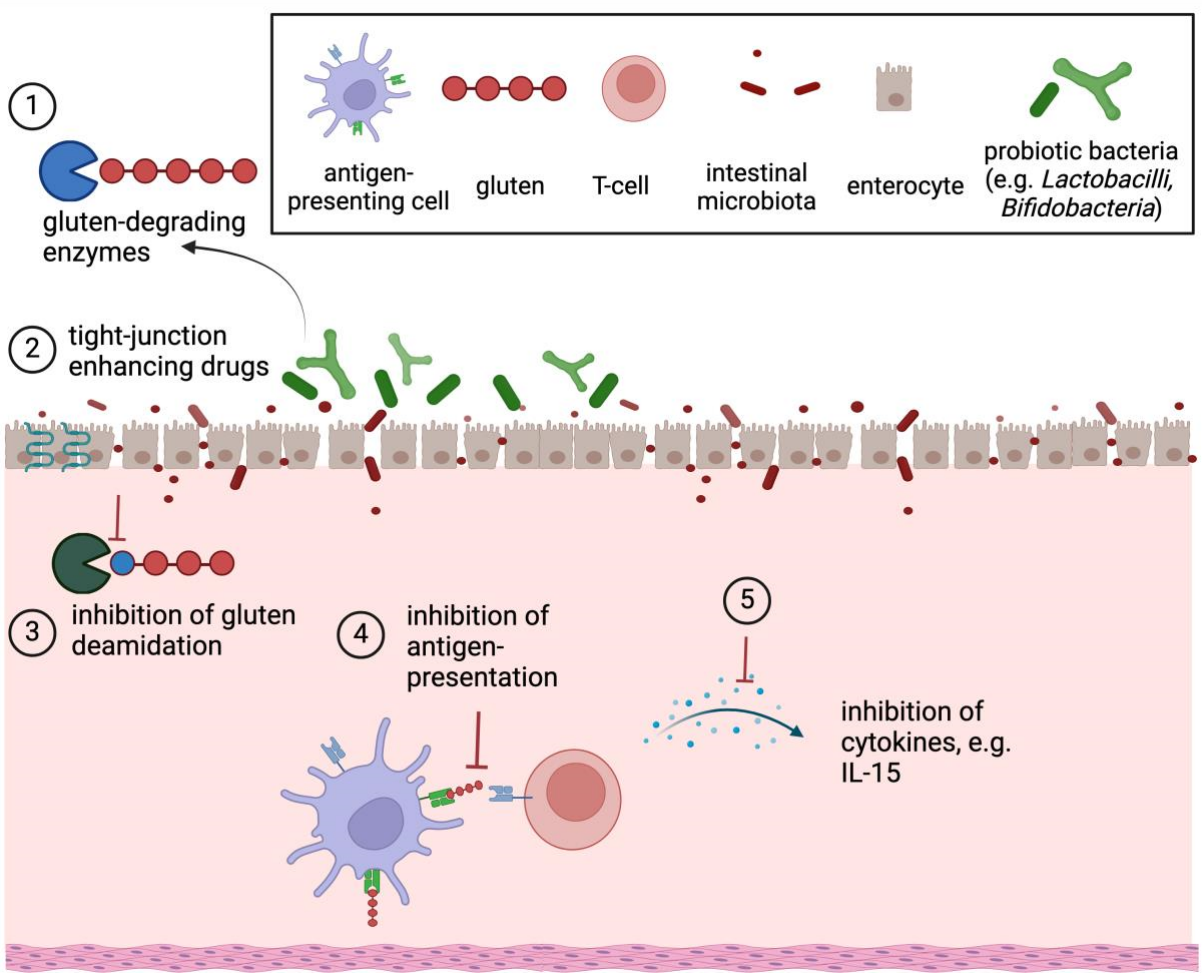

Figure 3: Experimental targets for the therapy of celiac disease. Latiglutenase (a glutendegrading enzyme) and lazarotide (a tight-junction enhancing drug) have reached phase III in clinical studies but failed to display sufficient therapeutic benefits[53] Inhibition of proinflammatory cytokine production was shown for kinase-inhibitors, such as tofacitinib as well as EVs derived from hookworms. Some Probiotic bacteria can digest gluten and ameliorate symptoms by improving barrier function of the enterocytes[56]

While both IBDs have been described as early as 1859 in the case of UC and 1932 in the case of Crohn's disease, no curative therapy has been developed until now.[57–59]

Therapies available today are symptomatic and aim at suppressing the inflammation, which is present in the pathogenesis of the IBDs. Currently, several classes of anti-inflammatory drugs are used to achieve remission.[60] These include the 5-aminosalicylic acid compounds mesalazine and sulfasalazine, which are used to maintain remission, glucocorticoids, and TNF-blocking biologics as a second line therapy.[61–63] Glucocorticoids (GCs) and TNF blockers share a common downside. They both are prone to inducing side effects, which can have a severe impact on the patients' quality of life. An example for this is the widespread use of GCs. Even though the clinical standard therapy is the use of local GCs, such as budesonide, which have a lower rate of side effects, compared to systemically active GCs, their prolonged use still retains the danger of the typical GC side effects.[62] These include hypertension and

hyperglycemia, as well as the typical Cushing's syndrome.[64] Anti-TNF agents such as Infliximab may even, if not carefully monitored, facilitate the occurrence of opportunistic infections and the development of T-cell lymphomas.[65]

In recent years, several novel therapeutic approaches have emerged, including the use of immunosuppressants, such as cyclosporine or methotrexate.[66] These can also be combined with biologicals, such as anti-TNF agents and antibodies suppressing cell-adhesion, e.g. Ustekinumab. Such combination therapies are mostly used for therapy refractory IBD. It needs to be noted, however, that evidence regarding the safety and effectivity of such combination therapies is still limited.[67]

Despite the availability of symptomatic treatment for IBDs, up to 33% of IBD patients cannot reach a state of remission under the currently available therapeutics.[68] These numbers show that – similarly to the 30% of celiac disease patients unresponsive to a gluten-free diet – the development of novel therapeutic options for IBDs and celiac disease is crucial to lower the global disease burden of inflammatory diseases of the GIT.

1.1.3 Inflammatory diseases of the skin and wound healing disorders

Autoimmunity and overshooting inflammatory responses also play a role in multiple diseases of the skin as well as wound healing disorders.[69] Skin is the largest organ of the human body. Its primary purpose is the defense against any external noxae, such as trauma and pathogens.[70] As such, it needs strong mechanisms to recover from such noxae, in the shape of wound healing.[71] In recent years, however, the incidence of wound healing disorders has increased severely, with chronic wounds affecting 6.5 million patients and treatment costs of 20 million dollars annually in the US alone.[72,73] Here, the immune system and overshooting immune reactions at the site of the wound play a major role in the pathogenesis, and a connection between wound healing disorders and autoimmune diseases has been described.[74] This supports the hypothesis, that overshooting inflammation is a major contributor to the development of chronic wounds and fibrosis and excessive scar formation.[75] This is also underlined by the fact, that among patients treated for chronic leg ulcers, a highly significant portion of the patient suffered from a comorbidity of autoimmune diseases.[76]

1.1.4 Wound healing and the role of overshooting inflammation in wound healing disorders

Wound healing is complex process, the body uses to repair any physical damage to the skin. It is organized in a distinct sequence of four overlapping physiological processes (Figure 4). The first of these is hemostasis, the process, during which a blood clot is formed via platelet aggregation and an enzyme cascade, the coagulation cascade leading to a solid fibrin aggregate, which prevents any further blood loss.[77] The next step, triggered by the platelet aggregation, is inflammation, even though the role of inflammation in the wound healing process is not fully understood. This phase has been shown to be of importance to clear the wound site of contaminations and necrotic tissue. Here, the wound site is infiltrated with immune cells, such as monocytes and macrophages as well as neutrophil granulocytes and other lymphocytes.[70] However, conflicting results indicate, wound healing might be possible without any inflammation. This has been shown to be the case for fetal wound healing, which results in a scarless closure of the wound.[78] It has also been demonstrated, that the wounds of CD-26 deficient mice heal without scar formation.[79]

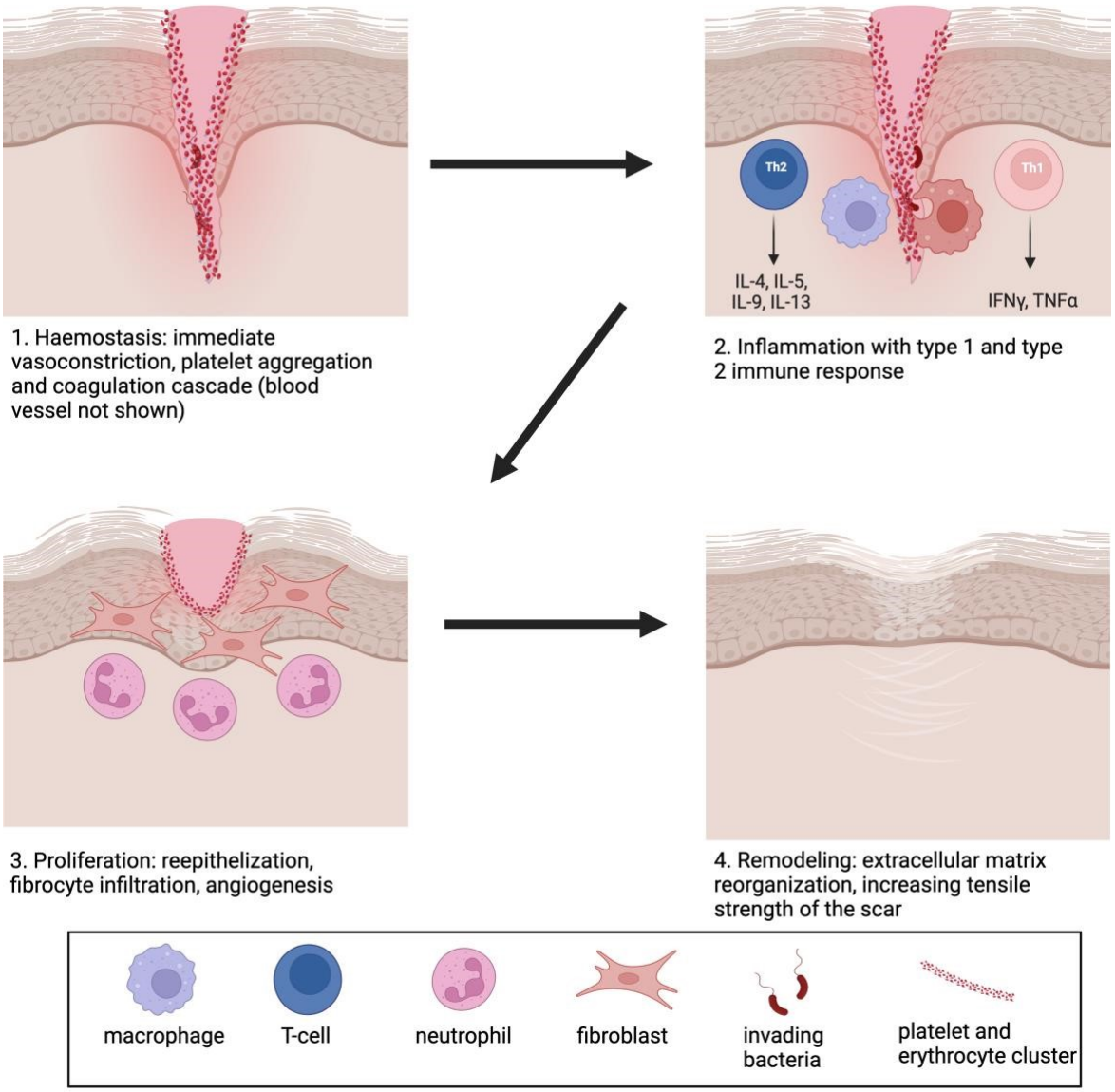

Figure 4: Schematic representation of the wound healing process. After the bleeding is stopped by vasoconstriction, platelet aggregation and coagulation (1), immune responses occur, to fight any invading pathogens (2). Following, keratinocytes proliferate and migrate into the wound (3). Lastly, wound remodeling results in a mature scar (4). Created with BioRender.com

There have been reports of the importance of the type of immune response present during the wound healing process. The immune response can be distinguished between type 1 and type 2 immune response.[80]

Type 1 immune response is targeted towards the clearance of the wound from invading, rapidly replicating microorganisms, such as bacteria and fungi and viruses. Thus, it involves the recruitment of classically activated, M1 macrophages, T_H1 T-helper cells and a cytokine response involving Interferon-γ and interleukin-12. Type 1 immune response is activated by pathogen-associated molecular patterns.[81] Notably, an excessive type 1 immune response is linked to chronic, non-healing wounds. This is highlighted by the fact that a lack of interleukin10, a cytokine known to regulate type 1 immune responses, leads to chronic, non-healing

wounds.[82] This cytokine is present in fetal wounds, which are known to heal without scar formation.[78] IL-10 is one of the body's major anti-inflammatory mediators, resulting in a switch from a type 1 immune reaction to a type 2 immune reaction.[83] There have been indications to the hypothesis, that a type 1 immune response is only essential to the wound healing process, if the wound is invaded by microorganisms.[84] Type 1 immune reactions are generally directed towards intracellular pathogens, such as bacteria and viruses.[85]

After the type 1 immune reaction has been concluded and pathogens have been eradicated, the wound healing process continues via the infiltration of the wound area with fibroblasts. These can be transformed into collgagen-producing myofibroblasts, which leads to wound contraction.[86] This step has been found to be influenced by TGF□.[87] Finally, the previously released macrophage cytokines TNF and IL-1 as well as growth factors trigger keratinocyte migration into the wound bed.[88]

Type 2 immunity, contrarily, is characterized by the recruitment of alternatively activated, M2 macrophages, T_H2 T-helper cells, eosinophils, basophils, mast cells and the production of interleukin-4, interleukin-5, interleukin-9, and interleukin-13. It serves two different evolutionary purposes.[82] First, it generally acts as a regulator to type one immune responses, thereby exerting protective effects against type-1 immunity-driven inflammation in a variety of autoimmune diseases, such as Crohn's disease or psoriasis.[82,89] Second, type 2 immune responses are involved in the defense against parasitic pathogens, such as helminths, but also venoms.[81,85] In wound healing, the purpose of type 2 immunity is to promote angiogenesis and restoration of the epithelia. [80,90]

Similar to type 1 immune responses, an excessive activation of type 2 immune reactions can facilitate the development of fibrosis , which in the case of type 2 immunity occurs via the upregulation of transforming growth-factor β (TGF-β).[91] Afterwards, the keratinocytes proliferate and differentiate to close the wound, resulting in a scar.[92,93]

In summary, under normal conditions, with environmental pathogens and bacteria from the skin microbiome present in the wound, both types of immunity are necessary parts of the wound healing process, but the right balance is required. While overshooting type 1 immune reaction can lead to chronic non-healing wounds, overshooting type 2 immune reaction leads to strong fibrosis and excessive scar-formation.[82]

1.1.5 Current therapeutic and experimental options for wound healing disorders

High-level therapy guidelines valid at the time of writing this thesis recommend strategies of mostly physical measures to increase wound healing in non-infected patients. These include mainly covering of the wounds as a physical barrier against environmental impurities and pathogens. Another recommended measure is the use of hydrogels to maintain a moist wound environment. For other, frequently used therapeutic options, such as iodine-containing polymers or colloidal silver, several randomized clinical trials demonstrate a beneficial effect on wound healing outcome in infected wounds. However, the current standard of care does not include any therapeutics specifically targeting the overshooting inflammation that contributes to poorly healing wounds.[94]

So far, only one FDA-approved drug exists, that is specifically targeted at improving wound healing, namely becaplermin, a formulation of platelet derived growth factor (PDGF) in a hydrogel matrix, which however can only be used in specific diabetic patient groups. Use of the drug has further been restricted in recent years due to concerns that beclapermin can facilitate cancer development.[95] Beclapermin showed an increased percentage of patients with complete wound closure (50% compared to 35% in the placebo group) in a phase-III study.[96] The active ingredient of beclapermin, PDGF, is physiologically a part of wound healing. In the body, it is secreted by different cells involved in wound healing, such as vascular endothelial cells, platelets and fibroblasts. It serves multiple purposes in wound healing, namely increasing the production of extracellular matrix, as well as inducing mitosis. This also explains the possible site effect, of PDGF, as a contributor to cancer.[97–99]

Different experimental therapies have been explored to combat poorly healing wounds. These include tissue engineering and bioengineered skin substitutes as well as nanotherapeutics and stem cell-based therapeutics. Nanoparticulate drug delivery systems can be applied to deliver drugs locally to the wound, thus helping to achieve locally higher concentrations of e.g. antibiotics in the case of wound infections.[100] Nanoparticulate formulations have also been used to deliver growth factors to the wound.[101]

The recently examined therapies focus mainly on later phases of the wound, mainly via growth factors and stem cells, while the earlier phase of wound healing, namely inflammation recieves fewer attention. However, Qian et al. found that wound healing can be impaired and scarring increased by increased inflammation. They hypothesize, this effect might be caused by neutrophils, which by degranulation counteract the wound healing by inducing further damage to the tissue. The authors found that counteracting the inflammation in a rabbit model, lead to lower scar elevation, however at the compromise of a longer time period until complete wound

closure.[102] These findings, combined with the high cost and disease burden of poorly healing wounds, mentioned above, show the potential of anti-inflammatory therapeutics for the treatment of such poorly healing wounds.

Here, we develop a novel anti-inflammatory therapeutic system that is both suitable for the treatment of poorly healing wounds as well as gastrointestinal diseases.

1.2. Definition of Probiotic bacteria

According to the WHO, probiotic bacteria are bacteria that can confer health benefits, when applied in adequate amounts.[103,104] These bacteria include both, gram-positive species, such as *Lactobacilli* and *Bifidobacteria* and gram-negative species, such as specific strains of *Escherichia coli.*[105] The term probiotic bacteria is used for bacteria, which can exhibit a variety of therapeutic effects when applied, such as ameliorating infectious and inflammatory gastrointestinal diseases.[106]

The most common use case for probiotic bacteria is the treatment of gastrointestinal diseases, ranging from lighter symptoms, e.g. irritable bowel syndrome to more severe intestinal autoimmune diseases, such as ulcerative colitis, Crohn's disease, and celiac disease. In this indication, live bacteria have already been used as commercially available adjuvant therapeutic options.[107,108] Examples for the use of probiotic bacteria in inflammatory diseases of the intestine will be discussed in the following paragraphs.

1.2.1 Clinical use of probiotic bacteria

A well-known example is *E. coli* Nissle 1917, a strain which has been on the market as a drug product since 1917, where several mechanisms of action were discussed. There is evidence for direct antimicrobial effects, by preventing the growth of pathogenic *E. coli* strains, as well as anti-inflammatory effects by decreasing the release of pro-inflammatory cytokines via tolllike receptor signal pathways.[109,110]

Clinical outcomes for the treatments of IBDs has been mixed, and seems to greatly depend on various factors, such as the exact disease to be treated. In clinical studies, several probiotic strains were tested against both, Crohn's disease and ulcerative colitis.[111] While Van Gossum et al. found, that for Crohn's disease, *Lactobacillus johnsonii* treatment did not prevent relapses after ileo-caecal resections, Tursi et al described that a mixture of L. paracasei, L. plantarum, L. acidophilus, and L. delbrueckii subsp bulgaricus), three strains of bifidobacteria (B. longum, B. breve, and B. infantis), and one strain of Streptococcus thermophilus (VSL Pharmaceuticals,

MD) significantly improved symptom scores in patients with ulcerative colitis. Here, the authors observed a synergistic effect between the probiotics and 5-amino salicylates, which are the standard therapy to maintain remission in UC. It needs to be noted, however, that the mechanism of this synergism has not yet been understood.[112,113]

Similar results were also found for the strain E. coli Nissle 1917, which showed similar potential to retain remission in UC as the currently used drug mesalazine and showed prolonged remission in Crohn's disease patients.[114,115]

Although gastrointestinal diseases have been the primary research focus for the use of probiotic bacteria in the past, their immune regulatory properties have gained interest for other applications, such as allergic diseases, as well as inflammatory diseases of the skin. Examples for such applications of probiotic bacteria will be described in the following.

One known example is the effect of probiotic treatment in allergy patients, as described by Taniuchi et al. who used the strain *Bifidobacterium breve* M16-V to treat allergic reactions to cow-milk. In this study, treatment with the probiotic bacteria reduced symptoms of atopic dermatitis triggered by milk intake significantly when compared to an untreated control group. Importantly, the effects seemed to be person-specific and not correlated with the amount of the bacteria detected in the patients' stool.[116] Similarly Montassier et al. investigated the effect of a commercially available preparation containing 11 probiotic bacteria strains on the occurrence of antibiotic resistance plasmids in the intestines of patients. They found effects to be person specific and concluded, that administration of living probiotic bacteria after the patients were treated with antibiotics may even contribute to the spread of antibiotic resistance genes.[117] This highlights the fact that the administration of live bacteria can be associated with a range of unpredictable effects and ways need to be found to separate the beneficial effects of the probiotic bacteria from the adverse effects. It further highlights that applying live probiotic bacteria can be helpful to some patients while for other patients, it may not be effective at all.

This is supported by the findings of Zmora et al. The authors assessed whether administered probiotic bacteria were able to colonize the intestine of treated individuals. They found via multi-omics analyses that probiotic colonization of patients' guts was highly dependent on individual features of the treated person.[118] This further highlights the need to find ways to harness the beneficial effects of probiotic from their administration as live bacteria if they are to be used in a therapeutic setting.

1.2.2. Mechanisms of action of therapeutically used probiotic bacteria in GIT and skin diseases

Various mechanisms of actions have been described for the probiotic bacteria. One such mechanism is direct communication with the host immune system. The effects can, depending on the species and the culture conditions, vary between mild pro-inflammatory effects and antiinflammatory effects.[119,120] Other mechanisms that have been described range from concurrence for biological niches to direct antimicrobial effects. The following paragraphs highlight examples of the effect and mechanisms of action of the probiotic bacteria in GIT and skin diseases.

1.2.3. Mechanisms of action of probiotic bacteria in GIT diseases

Several strains of probiotic bacteria, such as *Lactobacillus casei* were also shown to possess promising properties in cancer prevention [121–124] *Lactobacilli* are a genus of rod-shaped, nonspore-forming gram-positive bacteria, with more than 260 known species. They belong to the class of *Bacilli* in the phylum of *Firmicutes.*[125] They can be, depending on the strain, homofermentative or heterofermentative with the common characteristic that one of the end products of their metabolism is lactate.[126] They have shown a wide range of biological effects that are highly depended on the exact strain.[127]

In a study conducted by Jacouton et al, it was observed in an IBD mouse model, that the probability of colorectal cancer decreased upon treatment with the strain *Lactobacillus casei* BL23. The authors found that treatment with *Lactobacillus casei* BL23 displayed two separate mechanisms contributing to the cancer preventive effect. On one hand, the treatment lead to increased apoptosis via caspase pathways, on the other hand, the authors could also detect 1an antiproliferative effect via downregulation of cytokines involved in tumorigenesis, mainly IL-22.[121]

Another possible mechanism of action for probiotic bacteria is concurrence for the biological niche with pathogenic bacteria, which is known for various *Lactobacillus* strains. These were shown to produce a class of ribosomally synthesized proteins named bacteriocins.[129] This heterogenous class of peptides confers their bacteriostatic effects by intercalation into the bacterial membrane.[130] It has been shown that *Lactobacilli* can be useful in the treatment of uropathogenic infection via this mechanism of action.[129] Anti-infective properties of probiotic bacteria have also been shown in the treatment and prevention of bacterial GIT infections, such as *Helicobacter pylori*. These anti-infective properties can in part be explained by interference with the immune system, as the probiotic bacteria can interfere with the innate and

adaptive immune response i.e. via modulation of TLR4 pathways, resulting in the inhibition of IL-8 production.[128] The example of *Helicobacter pylori* infections can help to illustrate the variety of biological effects that can be observed for probiotic bacteria.[131]

Various meta-analyses describe adjuvant effects of probiotic bacteria in *Helicobacter pylori* eradication therapy. These are caused by various mechanisms. Besides general gastrointestinal benefits and increased therapy adherence to the antibiotic therapy via the reduction of antibiotic induced diarrhea, direct effects on *Helicobacter pylori* have also been described.[124] A possible contributor to anti *Helicobacter pylori* efficacy has been reported by Chen et al, who showed in an *in vitro* study, that *Lactobacillus gasseri* Chen, and L. plantarum 18 inhibited adherence of *H. pylori* to gastric epithelial cells, an effect which could also be shown for the cell-free supernatants of the *Lactobacilli*, which contain their membrane vesicles.[132] The authors demonstrated that the strains *L. acidophilus* and *L. bulgaricus* and their cell-free supernatants were able to inhibit adherence of *H. pylori* to gastric epithelial cells via interference with inflammation. The *Lactobacilli* exerted an anti-inflammatory effect by inhibiting TLR-4 mediated pro-inflammatory signaling cascades which lead to decreased expression of IL-8. This inflammatory process is hypothesized to maintain the disease state in *H. pylori* infections, instead of contributing to bacterial eradication.[133] It needs to be noted, however, that conflicting results exist, and other studies did not confirm the positive effects of probiotics in the treatment of *H. pylori* infections. Possible reasons might be the choice of the exact strain used for the respective studies. The following paragraphs highlight different bacterial species that have shown promising therapeutic effects in past studies: [125126127]Similar to *Lactobacilli*, *Bifidobacteria* were shown to exert beneficial effects in the treatment of inflammatory diseases of the intestine.[25] These rod-shaped, saccharolytic, gram-positive bacteria are part of the phylum of *Actinobacteria* and more than 50 species are known.[134,135] They are part of the natural human gut microbiome, as well as the microbiome of most mammals.[136] Their biological effect varies between the different subspecies and immunemodulatory, as well as antiviral effects have been observed.[137]

In clinical studies, a positive effect in the treatment of inflammatory bowel diseases was detected.[25] One example is the use of *Bifidobacterium longum,* which significantly improved clinical outcome in Crohn's disease patients.[138] Here, patients of mild to moderate Crohn's disease were treated with a defined amount of live bacteria and a mixture of fibres. The treatment lead to improvement of disease scores and decreased levels of TNF in the tissue, while the levels of other pro-inflammatory cytokines were not affected.
Other strains, such as *B. lactis* showed *in vivo* amelioration of colitis in a mouse model. Here, the authors observed that treatment of mice with the probiotic bacteria lead to a decrease of dextrane-sulfate induced apoptosis, thereby ameliorating the colitis symptoms.[139]

A gram-negative, strain known for its probiotic effects is *E. coli* Nissle 1917, a facultative anaerobic strain, which is already on the market in a life-bacteria formulation as an adjuvant over-the-counter therapy for irritable bowel syndrome.[108] It has been proven to elicit a variety of biological effects, such as suppression of pain via an analgesic lipopeptide, as well as suppression of neuroinflammation in an mouse model for multiple sclerosis.[140,141] However this strain is also known to possess a silent gene associated with genotoxic effects. This makes application of *E. coli* Nissle 1917 life-bacteria of this strain a risk, as activation of this gene could theoretically occur.[142]

1.2.4 Mechanisms of action of probiotic bacteria in skin diseases and wound healing

Positive effects on wound healing were demonstrated for *L. reuteri,* in a study where the live bacteria were incorporated into a hydrogel, which was then used to promote wound healing. Here, the authors propose, the bacteria-loaded hydrogel to be effective via different mechanisms. Via the inclusion of *L. reuteri,* the pH of the wound site was lowered caused by the lactic acid produced by the bacteria. This lower wound pH then inhibited the growth of pathogens, such as *Staphylococcus aureus* in the wound.[143]

For example, the strains *L. plantarum* and *L. paracasei* were recently shown to promote wound healing in a mouse model, where the cell wall component lipoteichoic acid was identified as a main contributor to this effect.[91]

The use of probiotics has shown promising results in other inflammatory skin disorders. Here, like wound healing, an overshooting type 1 immune reaction can be the cause of various autoimmune diseases, with psoriasis being an example of a type 1 immunity-induced autoimmune disease with symptoms mainly on the skin. Similarly, autoimmune reactions can also be triggered by an overshooting type 2 immune reaction. In this case, the symptoms are triggered by the recruitment of mast cells, which then leads to allergy-like symptoms. The most well-known example of an inflammatory skin disease triggered by a type-2 immune reaction is atopic dermatitis.

Due to the similarity regarding the targeted physiological processes, such as the involved immune responses this also highlights the potential of probiotic bacteria in the context of wound healing. There have been reports of successful application of probiotic bacteria in this context before. Ong et al showed one example. They also highlight another mechanism of action, which does not interfere with the immune system: Their results indicate *L. plantarum* treatment

can disrupt pathogenic biofilms, namely *Staphylococcus aureus* in a model of infected porcine skin. This led to a faster wound healing compared to untreated controls in a rat wound model.[144] In a similar study, Tsai et al described the antifibrotic effect of *L. plantarum* and *L. paracasei.* In this study, the authors used heat-killed bacteria and showed, the preparations induced matrix-metalloproteases involved in re-epithelization. They further verified this effect in an *in vivo* mouse model and demonstrated a higher re-epithelization for the mice, treated with the heat-killed bacteria.[91]

A second known mechanism of action for probiotics in wound healing is their influence on cell migration. Cell migration is a highly organized process of cells moving via the remodeling of their cytoskeleton. In cell migration, cells move collectively, while being connected via tight junctions.[145] This process occurs mainly at the edges of the wound, while in 1.5 cm of distance from the wound edge, mainly proliferation occurs.[146] Various probiotic bacterial strains have shown a stimulating effect on cell migration, which then accelerates wound healing as well. This has been demonstrated for the *Lactobacillus* strain *L. rhamnosus* and to a lesser extent *L. reuteri.*[147] In a similar study, comparable effects were also shown for the strains *L. salivarus* and *L. plantarum,* while *L. casei* and *L. paracasei* slightly inhibited cell migration.

1.2.5 Potential safety concerns regarding the application of live probiotic bacteria

Treatment with probiotic bacteria shows a generally acceptable biocompatibility in healthy patients. However, the use of life-bacteria bears the risk of adverse effects.[148] In immunocompromised patients and immunosuppressed, their nature as proliferating organisms has been found to be problematic, as bacterial clearance is not optimal. In these vulnerable patients, strong adverse effects up to septic conditions and bacteremia may occur.[149] This is concerning because some probiotic strains either still express virulence factors or could acquire them via horizontal gene transfer.[142,150]One notable example can be found in *E. coli* Nissle, which still has the gene cluster for genotoxic metabolites, such as colibactin. Even though, efforts have been made to remove this gene cluster by genetic engineering, it was found that removal of the genotoxicity gene cluster also led to a loss of the probiotic activity.[142]

Bacteremia is defined as a condition, where bacteria reach the bloodstream and thus, can spread throughout the whole body. The bacteria can then infect all tissues where they find suitable growth conditions. This leads to a generalized inflammatory response in the affected patients. The combination of wide-spread infection and the resulting inflammation leads to sepsis, a major cause of death in hospitalized patients.[151] One example of this adverse effect

was shown in a case report by Haziri et al. Here, a patient diagnosed with both IBD and HIV, who was under anti-adhesion molecule antibody therapy using Ustekinumab, developed a systemic infection with *Lactobacilli.*[152]

Thus, alternatives to the application of live bacteria are needed to fully harness their therapeutic potential and make them available for a wider patient collective.

1.3. Extracellular vesicles and bacterial membrane vesicles

The studies described in this thesis aim to circumvent this problem by using the membrane vesicles (MVs) secreted by the probiotic bacteria. These phospholipid-based particles with a size range of 50-150 nm transport protein and nucleic acid cargo that are also found within the bacteria and have also been found to correlate to their biological effect.[119,153,154] An overview of extracellular vesicles and bacterial membrane vesicles is shown in Figure 5

Various subtypes of EVs were described, both for the extracellular vesicles from eukaryotic origin and prokaryotic origin. EVs and MVs serve multiple purposes, mainly in cell- cell communication. This communication can occur within one organism, within tissues across the extracellular space and across the bloodstream with different organs. Both, EVs and MVs occur in communication between different organisms of the same species as well as communication between different species.[155,156] In this case, bacterial membrane vesicles are the most prominent class.

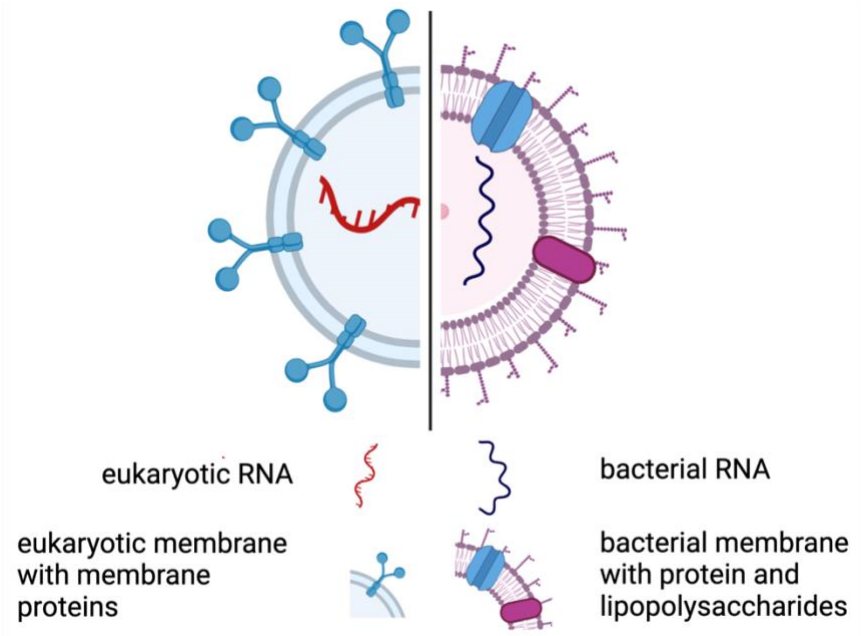

Figure 5: General composition of eukaryotic and prokaryotic MVs. Both are composed of a phospholipid membrane and carry specific protein and RNA cargo.

Over recent decades, several methods of MV isolation were tested, each possessing a different set of advantages and disadvantages and a different possible area of application. To isolate MVs, various properties of the MVs can be exploited. These include their size and density, interaction with solid-phase materials via their surface markers, as well as precipitation and microfluidic methods.[157,158] While affinity-based and precipitation methods, are mainly used for diagnostic applications, other methods, such as ultracentrifugation at centrifugal forces of more than 100 000 x g and size-exclusion chromatography (SEC) can be used for higher batch sizes.[159] The choice of the isolation method was also shown to have a substantial influence on the size and purity of the resulting MVs. One example for this observation was shown by Buschmann et al. who compared a variety of isolation methods including SEC, ultracentrifugation, as well as membrane-affinity and precipitation methods. They observed, MVs isolated by SEC showed the highest particle/protein ratio, indicating a high purity and a low abundance of co-isolated soluble proteins.[160] It was further detected, using transmission electron microscopy, that MVs isolated with a precipitation method, showed a higher concentration of small-sized MVs with a diameter smaller than 100 nm. SEC-based isolation of MVs also bears the advantage of a possible compatibility with current good manufacturing practice (cGMP) regulations. The major disadvantage of using SEC is that resulting MVs tend to be diluted and a further concentration step is often required.[161,162] Another frequently used method is tangential flow-filtration (TFF). This technique has been used for industry-scale production of biomacromolecules and viruses before, which shows it's potential to also isolate similarly sized MVs.[163] The disadvantage of this technique, however, is the lower purity of the resulting MVs, when compared to other isolation methods.[164] A typical MV isolation protocol as used in this work is shown in Figure 6

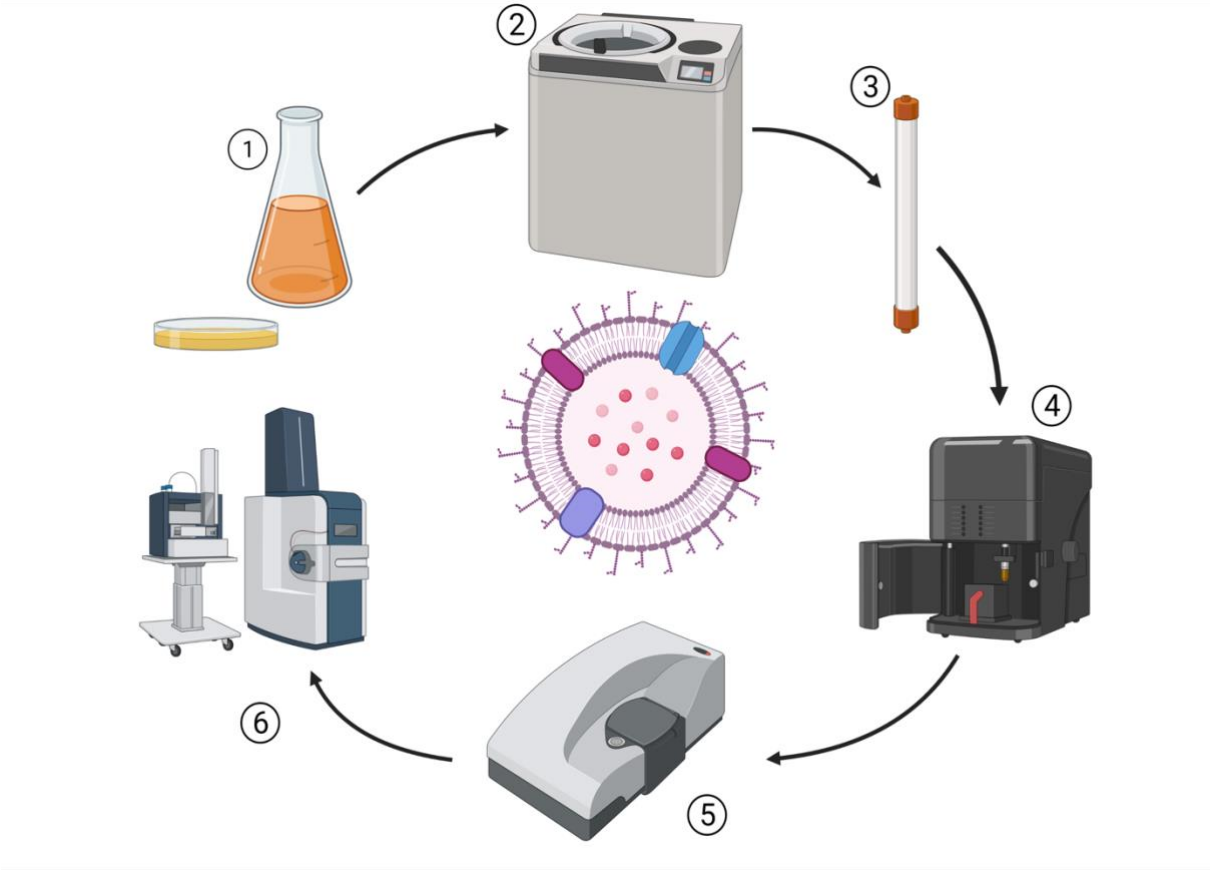

Figure 6: Typical isolation and characterization protocol as used in this work. Bacteria are grown under different culture conditions. (1) Then, MVs are harvested using ultracentrifugation (2) and purified using size-exclusion chromatography (3) Purified MVs can be characterized regarding size and concentration using nanoparticle tracking analysis (4) as well as zetapotential using dynamic light-scattering and laser doppler anemometry. (5) Proteins within the MVs can be characterized using liquid chromatography / mass spectrometry (6).

For eukaryotic EVs three main classes exist, namely exosomes, microvesicles and apoptotic bodies. Exosomes are the most studied of the three groups in the context of their therapeutic and diagnostic application.[165] They are the smallest out of the three groups with a typical size of <150 nm.[166] Their biogenesis was described as an intracellular pathway, where they are shed from the Golgi apparatus. This allows for selective sorting of cargo into the MVs. Microvesicles are characterized by a larger size, typically 100-1000 nm.[167] They are shed from the cell membrane and predominantly carry membrane-bound proteins as their cargo. They were first described as coagulation-mediating carriers for blood platelets.[167] Apoptotic bodies are the largest of the eukaryotic extracellular vesicles. They are formed during apoptosis, the process of cell death. They occur in size ranges of more than one µm.[168] Much like the other two types of eukaryotic EVs, their function was described to be intercellular communication, in this case delivering protein, phospholipid and nucleic acid cargo from a dying cell to its surrounding cells.[169]

Currently, while in the field of exosomes, some preparations, mainly using mesenchymal stem cells (MSCs), have already reached clinical evaluations, eukaryotic EVs retain a range of important shortcomings.[166] Namely, since most eukaryotic cells need to grow in adherent cultures, yields of purified EVs are low compared to the surface needed to grow the cells. Some progress has been made, using e.g. hollow-fiber bioreactors, where cells grow on semipermeable fibers in a cartridge.[170] This technique greatly reduces the laboratory space needed to harvest enough EVs. This, combined with the circumstance that every single cell usually produces limited amounts of MVs makes upscaling for the industrial-scale production of therapeutic EVs a challenging endeavor. [156,]

The use of bacterial membrane vesicles (MVs) may present a viable alternative to using EVs derived from eukryotic cells. Bacterial culture in large scales has been used for several years, e.g. in the production of therapeutic proteins, and similar production methods may be used to produce MVs.[171] This is facilitated by the fact that bacteria can grow in suspension cultures with no need for surface area, which would be needed for the culture of adherent eukaryotic cells. Like their eukaryotic counterparts, bacterial MVs are produced by almost all bacteria and serve multiple purposes, which will be described in the following.[155]

Bacterial MVs were described as means of communication for bacteria, when confronted with adverse environmental conditions, such as nutrient scarcity, environmental toxins, or immune reactions, helping the bacteria to form biofilms. MVs are important in the communication between microorganisms and their host.[172] In prokaryotes, multiple types of bacterial MVs were described. They are present in size ranges between 20 and 400 nm.[155] Depending on the type of bacteria, various mechanisms influencing MV production were described (Figure 7):

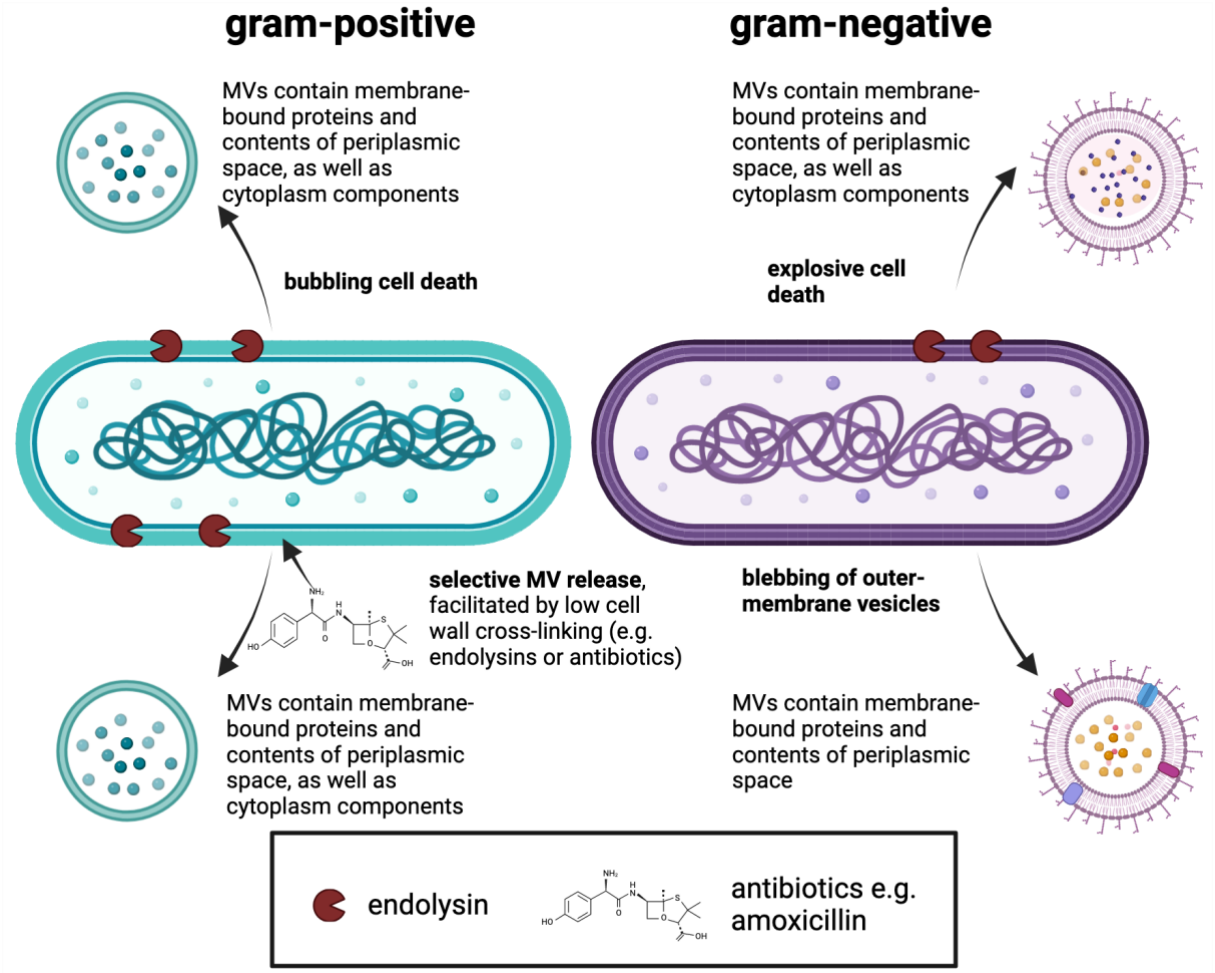

Figure 7: Most important factors influencing MV-production in bacteria: for gram-positive bacteria, MVs originate from the cytoplasm membrane and need to cross the cell wall (facilitated by cell-wall remodeling enzymes). Gram-negative bacteria produce vesicles originating from the outer membrane.

For gram-negative bacteria, the dominant mechanisms of MV production are blebbing from the outer membrane, followed by intercalation of hydrophobic molecules into the membrane, which leads to outer-membrane vesicles (OMVs) as well as endolysin-triggered cell lysis leading to explosive outer-membrane vesicles (see Figure 5) (EOMVs).[173,174] These hypotheses can be supported by the fact, that in many cases, only low amounts of intracellular cargo can be found in the resulting MVs. A wide range of biological effects of the MVs derived from gram-negative bacteria has been described. For instance, OMVs derived from the opportunistic pathogen *Pseudomonas aeruginosa* were shown to deliver virulence factors, such as β-lactamase and alkaline phosphatase into host cells.[175] However, other bacteria were also shown to release OMVs with more beneficial effects to the host, which were found to have therapeutic potential. OMVs from non-pathogenic *Myxobacteria* were found to possess inherent antibiotic properties.[176] Another example for health benefits elicited by vesicles derived from gramnegative bacteria can be found for probiotic *E. coli* Nissle 1917 OMVs. They were described to possess anti-inflammatory as well as neuroprotective properties.[140] In the context

of inflammatory bowel diseases, they were also shown to lower the levels of several proinflammatory cytokines including and Interleukin-2 (IL-2) or Tumor necrosis factor (TNF).

To form MVs in gram-positive bacteria, the MVs first need to cross the peptidoglycan cell wall. This may be achieved using different mechanisms. MVs may either be pushed through the cell wall by turgor pressure, or release can be facilitated using cell-wall remodeling-enzymes, such as muropeptidase, as known for *Lactobacillus* species.[177,178] Another way that allows grampositive bacteria to release MVs is bubbling cell death (see Figure 7).[179] Similar to gramnegative OMVs, MVs from gram-positive were proven to elicit a wide range of biological effects, ranging from virulence factors to antibiotic and anti-inflammatory effects. A typical isolation and characterization protocol of MVs, as used in this work, is shown in Figure 6.

An example for MVs from gram-positive bacteria acting as virulence factors can be seen for *Staphylococcus aureus* MVs that were described to contain cell-lytic proteins. These MVs were shown to induce apoptosis in a dose-dependent manner.[180] However, like MVs derived from gram-negative bacteria, many potential therapeutic effects were described for MVs derived from gram-positive bacteria. These effects often result from interaction of the MVs with the host immune system, by either by inducing or by suppressing immune responses. For example, OMVs derived from Streptococci were found to elicit mild immune-stimulatory effects which makes them suitable vaccine candidates.[181,182] MVs from probiotic bacteria are another example notably *Lactobacilli* and *Bifidobacteria,* MVs which were shown to possess antiinflammatory properties.[121,183]

1.4. Probiotic microvesicles as potential therapeutic agent of autoimmune diseases

1.4.1. Probiotic MVs in the context of autoimmune diseases of the intestine

The use of probiotic bacteria MVs, as well as extracellular vesicles from eukaryotic origin have the potential to be a therapeutic alternative for inflammatory diseases of the intestine. In the past, the use of probiotic bacteria showed therapeutic potential in various clinical studies, even though higher-level clinical studies are needed.[184] Extracellular vesicles have been considered as experimental therapeutic options for autoimmune diseases of the intestine. One example was shown by Eichenberger et al. who used the vesicles obtained from parasitic hookworm *Nippostrongylus brasiliensis*. They demonstrated, EVs are uptaken in an *in vitro* organoid model and demonstrated a protective effect against chemically induced colitis. The authors concluded that the effect was caused by the proteins and miRNAs contained in the EVs.[185,186] Another interesting approach was followed by Labruna et al. who investigated the use of

Lactobacillus MVs in the context of Celiac disease. They investigated the uptake of gluten and gliadin, the disease-provoking peptides into intestinal cells. They found that treatment with the cell-free supernatants of *Lactobacilli*, which contain secreted proteins as well as the MVs, showed inhibiting effects on gluten uptake. This highlights the question, whether *Lactobacillus* MVs might represent a promising therapeutic option. These findings also lie in accordance with the discovery, that in Celiac disease patients, lower levels of *Lactobacilli* are found in the intestinal flora.[187]

Strains like *L. kefir* and *L. kefirgranum* as well as the membrane vesicles released by these strains were shown to suppress the production of proinflammatory cytokines Interleukin-8 and Tumor-necrosis-factor.[188,189]

In this thesis, I assess the use of the probiotics' MVs to circumvent the shortcomings of lifebacteria therapy. Their therapeutic potential was shown before regarding the IBDs. Here, Jacouton et al showed the potential of *L. casei* MVs in the prevention of colitis associated cancer. They found, treatment with the probiotic strain *L. casei* BL23 showed anti-inflammatory as well as anti-proliferative effects in a mouse-model of colitis. This effect was caused by an increase in caspase-activity, which leads to increased apoptosis.[121] Similar results were also shown by Park et al, who demonstrated the ability of three different *Lactobacillus* strains in an *in vivo* mouse model, demonstrating the ability to ameliorate trinitrobenzene-sulfonic acid induced colitis. They used a formulation composed of the MVs from *L. kefir, L. kefirgranum* and *L. kefiranofaciens.* This study also demonstrated the *in vitro* anti-inflammatory effects, in human intestinal cell-line Caco-2 inflamed with TNF. They observed, this effect was caused by inhibition of the p65 subunit of the inflammatory transcription factor NF-κB.[188]

These examples highlight the therapeutic potential of probiotic bacteria, and, as a safer therapeutic option, their MVs in the context of inflammatory diseases of the GIT.

1.4.2. Probiotic MVs in the context of skin autoimmune diseases and wound healing disorders

Probiotic bacteria and their MVs were shown to be effective in various examples of overshooting immune reactions. One example for the immune regulating effect of probiotic MVs can be seen for neurogenic skin inflammation. This disease is characterized by a stressinduced release of pro-inflammatory cytokines, which then inhibits hair growth and keratinocyte proliferation.[190] In this context, it was shown that a lysate from *Bifidobacterium longum* could ameliorate the inflammation in an *ex vivo* model.[191] Another example was demonstrated by Chen et al. They demonstrated the anti-inflammatory effects of *Lactobacillus*

pentosus in a mouse psoriasis model.[192] Psoriasis as an autoimmune disease of the skin, is characterized by an overshooting T_H1 and T_H17 T-cell reaction, which leads to keratinocyte hyperproliferation.[193] Here, treatment with *Lactobacillus pentosus* led to a decrease in skin lesions as well as a decrease in proinflammatory cytokine production. This effect can further be observed not only for the live bacteria but also for membrane vesicles derived from *L. plantarum.* Kim et al described, that MVs from the strain *L. plantarum* APsulloc 331261 MVs elicited an increase in M2 cell markers and a decrease in M1 cell markers *in vitro* on macrophage-like cell-line THP-1. They also found an increased IL-10 production caused by the MVs in an organ culture skin model. This indicates that *L. plantarum* MVs can show suitable therapeutic properties for the treatment of type-1 immune response driven inflammatory skin diseases, such as psoriasis or chronic non-healing wounds.[194]

Similar results were also observed in the context of atopic dermatitis. Atopic dermatitis is a chronic inflammatory skin disease, in which an imbalance between T-cell subpopulations leads to an IgE antibody driven, chronic inflammatory skin response, with similar symptoms to an allergic reaction. This leads to constant itching and a severe decrease in quality of life for affected patients.[195] Here, Kim et al found a mixture of four probiotic strains, *L. casei, L. rhamnosus, L. plantarum* and *Bifidobacterium lactis* was effective in reducing the allergy-like symptoms and reducing T2-mediated hypersensitivity.[190,196] Similarly, MVs released by *L. plantarum* were found to be effective against atopic dermatitis, induced by *S. aureus* MVs.[197] Thereby, as excessive inflammation is known to lead to poorly healing, chronic wounds, this highlights their potential to treat inflammation-induced wound healing disorders.

These examples highlight the potential of probiotic bacteria in the treatment of skin diseases. Promotion of wound healing has been reported for bacterial MVs, such as the MVs secreted by *Synechococcus elongatus,* a photoautotrophic cyanobacterium. Here, Yin et al found, the MVs secreted by this strain had an angiogenic effect which promoted the healing of burn wounds. Interestingly, they also found the MVs to promote production of the pro-inflammatory cytokine IL-6, which is otherwise known to counteract the wound healing process.[198]

Another promising therapeutic route, which has gained interest in the scientific community is the use of human extracellular vesicles, such as the ones derived from mesenchymal stem cells (MSCs). These have demonstrated multiple effects beneficial to wound healing before. They showed antimicrobial properties, e.g. against *P. aeruginosa, S. aureus* as well as *Candida albicans.*[199] Their role in various regenerative therapy and anti-inflammatory applications was demonstrated in the past. In one example, Shigemoto-Kuroda et al demonstrated the MSC's inhibitory effects on pro-inflammatory T_H1 and T_H17 T-helper cells in two autoimmune disease models.

Efforts were made in the development of suitable formulations for the skin-application of probiotics in wound healing. In a recent study, *Lactobacillus reuteri* live-bacteria were trapped in a hydrogel matrix. Here, antibacterial effects *in vitro* were shown. This formulation also elicited a faster wound healing in a mouse model, and wounds were closed after 10 days, which was not observed in the hydrogel only, without the bacteria incorporated.[143] Similarly various examples can be found for hydrogel formulations of EVs and MVs. One example was described by Yang et al. They used eukaroytic MVs derived from mesenchymal stem cells and incorporated them into a F127-polycitrate-polyethylenimine hydrogel matrix, which lead to a long-lasting release of MVs.[200]

2. Project synopsis and aims of the project.

Inflammatory diseases pose a great burden on health systems worldwide, with inflammatory GIT diseases alone affecting around 1 million people in the US and approximately 3 million patients in Europe, according to recent estimates.[25] Similarly, the incidence of wound healing disorders, to which overshooting inflammation is a major contributor, has increased severely, with chronic wounds affecting 6.5 million patients and treatment costs of 20 million dollars annually in the US alone.[72,73] These numbers show that novel therapeutic options for these diseases are needed. Probiotic bacteria have shown potential in therapy of anti-inflammatory diseases in both the GIT and the skin before, however, therapeutic application of live-bacteria is linked to various disadvantages, such as possible bacteremia and sepsis in susceptible patient groups.[152,201]

A possible solution might be the use of MVs derived from the probiotic bacteria, however, the therapeutic use of MVs is limited by their poor local bioavailability after systemic or oral application. Their small size (~100nm) makes them prone to fast clearance after systemic application via rapid clearance through the mononuclear phagocyte system.[202,203] Intestinal application of MVs limited by their low capability to penetrate intestinal mucus. This decreases their bioavailability at the inflamed sited of the intestinal mucosa upon oral application.[204] This work aimed to circumvent these disadvantages of applying native MVs. To do so, bigger particles, coated with MVs were utilized to ensure a high local MV concentration at the site of inflammation. Thus, it was aimed to circumvent the risks associated with the use of live bacteria through the use of their MVs while at the same time avoiding the disadvantages of the native MVs cause by their small particle size. This resulted in a bacteriomimetic therapeutic system composed of synthetic microparticles with MVs coupled on their surface. This system was characterized *in vitro* and *in vivo*.

Thus, the following research aims were addressed in this project (Figure 8):

1. To **assess the effect of different culture conditions** on the most promising vesicles producers, namely *Lactobacilli.*
2. To extensively **characterize** the *Lactobacillus* MVs **physicochemical properties**.
3. To circumvent disadvantages of applying the native MVs by **fabricating a novel MVbased therapeutic system**.
4. To characterize the MVs as well as the MV-loaded microparticles *in vitro* regarding their **anti-inflammatory effect** on immune and epithelial cells.
5. To characterize the MVs and the MV-loaded microparticles in an *in vivo* **wound healing model** in mice.

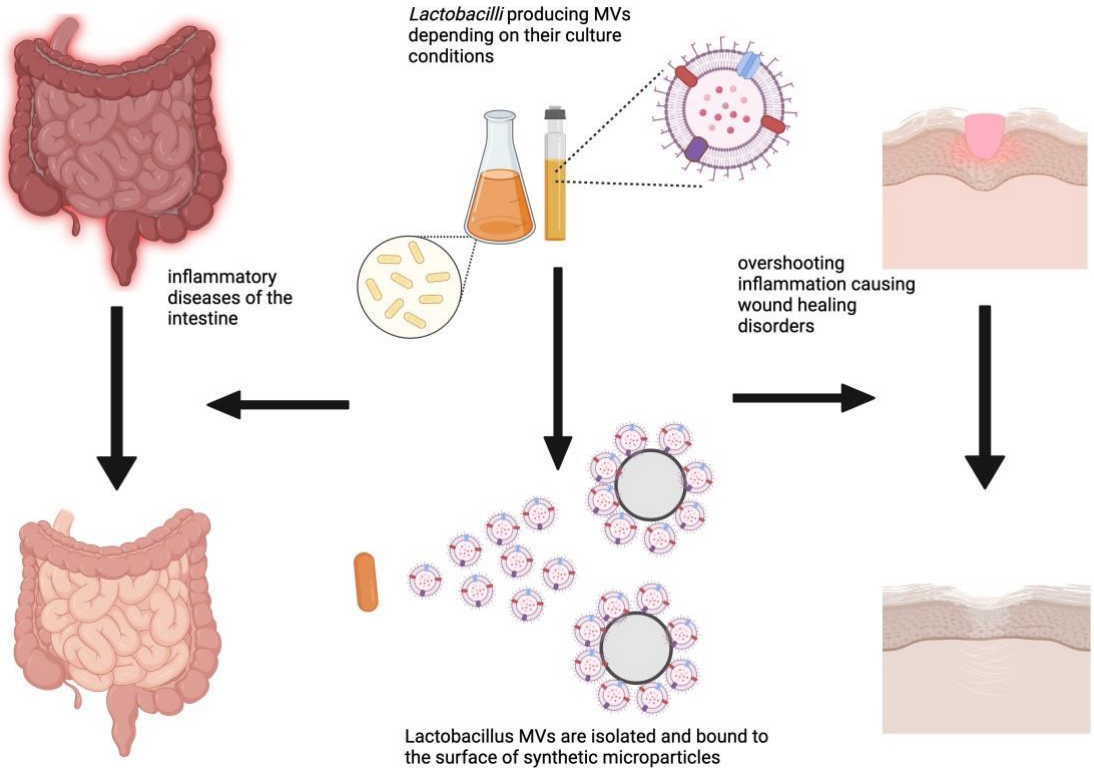

Figure 8: Concept of the thesis: Various culture conditions of Lactobacilli *and the effect on the MVs produced by the bacteria were assessed. The MVs were subsequently fabricated onto the surface of of syntetic microparticles to create a bacteriomimetic system and tested in models for inflammatory diseases of the intestine as well as wound healing* in vivo *models.*

3. Main Findings

In this work, the results that were published in three peer-reviewed papers are shown and summarized. Their impact is presented, and results shown in the different papers are combined to compare the data. The original publications can be found in chaper (7. Scientific output)

3.1. Bacterial culture conditions influence the properties of *Lactobacillus* MVs

Bacterial culture conditions have a strong influence on the MVs they produce. Thus, *L. casei* and *L. plantarum* were cultured under conditions that deviated in pH, oxygen supply and mechanical mixing of the culture. Then the MVs produced under every culture condition were isolated, using the same isolation procedure (differential ultracentrifugation followed by a sizeexclusion chromatography purification) for all culture conditions. The particle concentration of the obtained purified MVs was measured using nanoparticle-tracking analysis (NTA), which revealed significant differences depending on the culture condition (Figure 9).

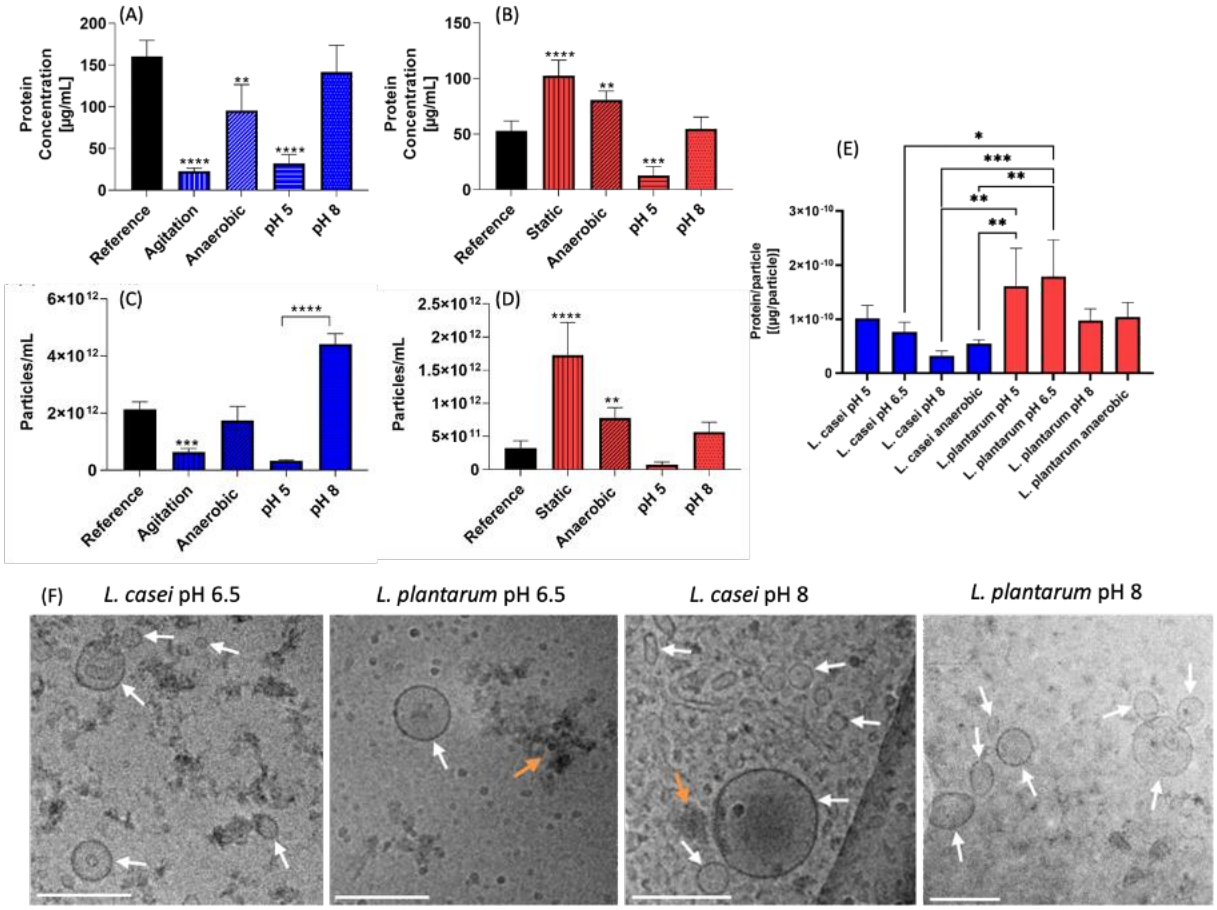

Figure 9: Properties of the MVs harvested from L. casei and L. plantarum depending on the culture conditions: (A) Protein concentration of purified L. casei *MVs, (B) Protein concentration of purified* L. plantarum *MVs (C) Particle concentration of purified* L. casei *MVs, as measured by NTA (D) Particle concentration of purified* L. plantarum *MVs, as measured by NTA (E) resulting ratio of protein/MV. Reference culture conditions are the conditions described by the supplier of the strain. Results are displayed as mean out of 3 biological replicates ± SD (F) Cryo-TEM images of MVs from various culture conditions. Scale bars indicate 200 nm in all images.*

For *L. casei* the highest particle number of 5 x 10^{12} particles/ml, was measured for the pH 8 culture, the lowest paticle concentration was measured for the pH 5 culture (Figure 9 C). For *L. plantarum,* the static, non-shaken culture yielded the highest particle concentration (1.5 x 10^{12}) while the pH 5 culture showed the lowest concentration (Figure 9 D). This trend could also be observed regarding the protein concentration of the purified MVs, where for both strains, the pH 5 condition showed low protein content in the MVs (Figure 9 A and B). However, on a single-MV level, the pH 5 conditons showed the highest concentration of protein / MV for *L. casei* and the second-highest for *L. plantarum* (Figure 9 E). This indicates, that in pH 5 cultures, both strains produce a low number of MVs with a high amount of protein cargo for every single MV. Contrarily, for the pH 8 cultures, *L. casei* produced a high number of MVs with

low amounts of protein cargo. In summary, we also found, that for every condition, *L. plantarum* produced more proteins per single MV than *L. casei* (Figure 9).

In addition to these differences in the total amount of protein in the MVs, the identity of the proteins varied between the MVs from the same specied harvested under the different culture conditions. Proteomic analyses showed presence of common proteins (125 for *L. casei* and 101 for *L. plantarum)*, such as the p40 and p75 muramidases, in all culture conditions. A number of unique proteins for the various bacterial culture conditions were identified. Here, pH showed a stronger influence on the protein composition of the MVs than the availability of oxygen. We found that differences between the standard culture and the anaerobic culture were minor, compared to the difference between the cultures with different pH.

The pH 8 condition for both strains showed a high abundance of intracellular proteins, such as metabolism-related and ribosomal proteins. In contrast, the MVs from pH 5 and pH 6,5 cultures showed mainly cell surface and cell-membrane related proteins. [204]This data set shows that protein contents of MVs greatly vary depending on the pH value of the culture. The difference in protein cargo raised the question, if different culture conditions can be used to tailor the MVs to a specific effect. In the following, *in vitro* effects of *Lactobacillus* MVs harvested from the different culture conditions were explored.

3.2. *Lactobacillus* MVs show no cytotoxic effects on epithelial and immune cell lines

Cytotoxicity is a possible issue that needs to be assessed in the development of novel therapeutics. For MVs, it can be dependent on the strain used to isolate the MVs as well as the culture conditions. In one study, Kwon et al found that *Staphylococcus aureus* MVs elicited cytotoxic effects in keratinocytes. [205] Similar effects were described by Briaud et al, who showed cytotoxicity of *Staphylococcus aureus* vesicles to be dependent on temperature during cultivation in an erythrocyte hemolysis model. [206] We thus tested whether the *Lactobacilli* MVs from any of the culture conditions used in this work exerted toxic effects on relevant cell lines for the therapeutic settings to be researched in this project. [207-208]

We chose the following cell lines for further analysis: First, we used macrophage-like differentiated THP-1 cells, as macrophages are involved in the pathogenesis of most autoimmune conditions. We also investigated the effect of the MVs on enterocyte-like CaCo-2 HTB-37 cells, as a model cell line for intestinal application. Last, we tested the effect of the MVs on keratinocyte-like cell line HaCat, as a model for dermal application. We used two different assays, namely PrestoBlue and Lactate dehydrogenase (LDH) assay. PrestoBlue is

a formazane dye, which undergoes reduction in living cells, leading to the formation of a fluorescent product. Thus, living, metabolically active cells lead to a high read-out in this assay.[207] The LDH-assay on the other hand, reacts to the presence of the intracellular enzyme LDH in the cell supernatant. This enzyme is only found outside the cells in case of membrane damage, thus indicating cell death. Thus, high read-outs in this assay would indicate cell death and thus cytotoxic effects of the substance, the cells are treated with.

We found, that for all culture conditions and tested cell lines, neither of the assays indicated relevant cytotoxic effects. In our case, we used the definition of ISO-10993-5 guideline, which defines cytotoxicity as a reduction of cell viability by more than 30%. Tested on macrophagelike dTHP-1 cells, we observed the highest reduction in viability in the PrestoBlue assay for *L. plantarum* MVs obtained from pH 6.5 cultures (76%, Figure 10 A). All other conditions revealed no reduction in cell viability. In LDH-Assay this could not be confirmed as all samples except *L. casei* MVs from pH 6.5 cultures showed negative cytotoxicity values (which means fewer cell lysis than in the PBS-treated control). On HaCaT cells, the lowest observed cell viability was for *L. plantarum* pH 6.5 MVs (Figure 10 C) as well. Here this corresponded with the cytotoxicity value observed in the LDH-assay (17 %) which was also the highest observed cytotoxicity value. It can be concluded that MVs from every culture condition showed no cytotoxic effect.

These results match those of Bhuyan et al. who similarly found no cytotoxic effects for Lacticaseibacillus casei MCJ protein-based metabolites as well as Azami et aks who examined possible cytotoxic effects of L. casei metabolites in an NIH/3T3 cell line.[208,209]

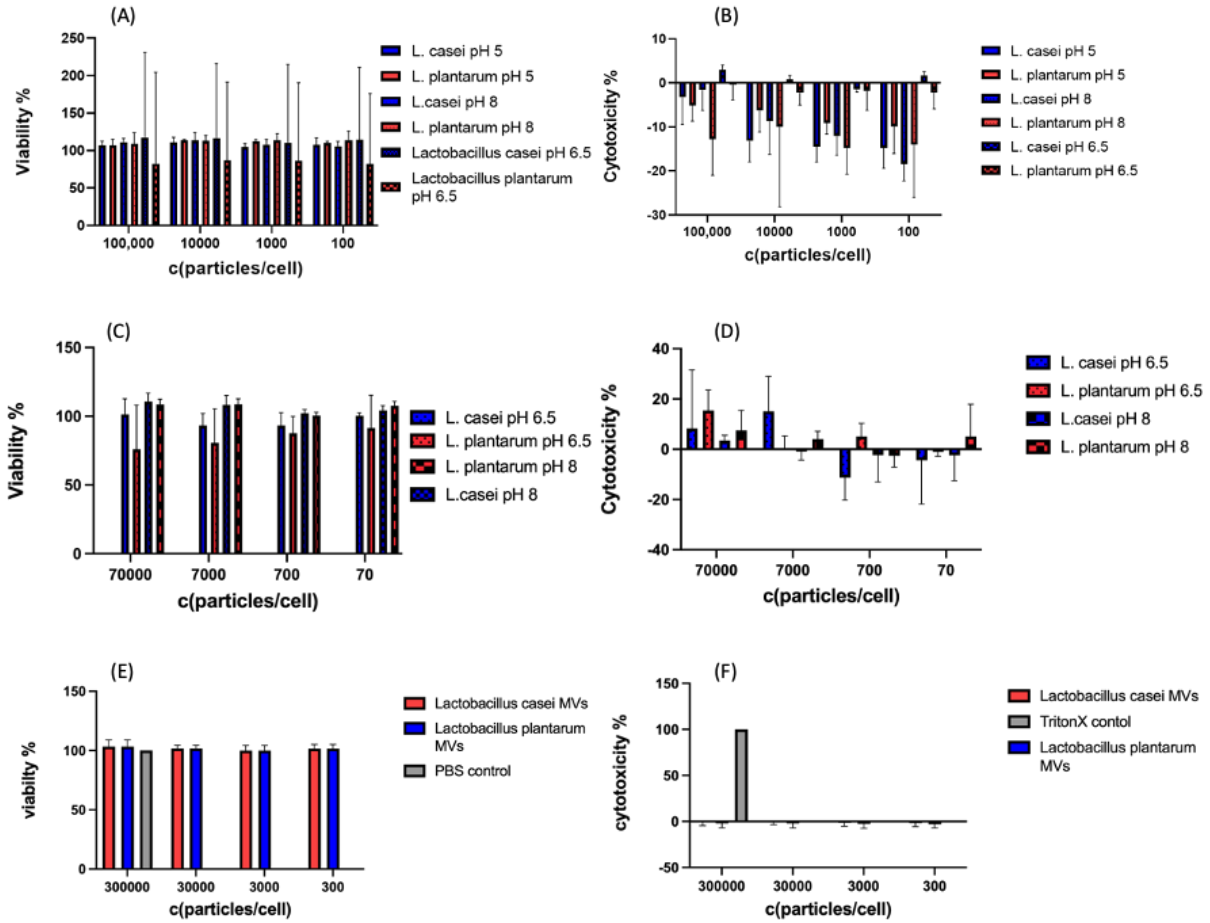

Figure 10: Viability testing on different cell lines shows no cytotoxic effect of Lactobacillus MVs. Shown are the results obtained with PrestoBlue ® assay (A),(C) and (E) and Lactatedehydrogenase assay (B), (D) and (F) for three different cell lines: (A) and (B): macrophagelike cell line dTHP-1; (C) and (D) keratinocyte-like cell line HaCaT; (E) and (F): enterocyte-like cell line Caco-2 HTB37. Values are shown are Mean +SD of 3-9 biological replicates.

3.3. MVs can be formulated to the surface of microparticles (Probiomimetics) which show anti-inflammatory properties in vivo

We then formulated the MVs in a novel bacteriomimetic approach, to improve their therapeutic potential. This is necessary as natural MVs are typically found in a size range of 50150nm.[176,210] [213]We coated the MVs on the surface of synthetic microparticles in the size range of 3 – 4 µm, a size range, in which live-bacteria are typically found.[211] Size and shape also influence the recognition of particles by immune cells and thus have the potential to improve anti-inflammatory properties.[212]

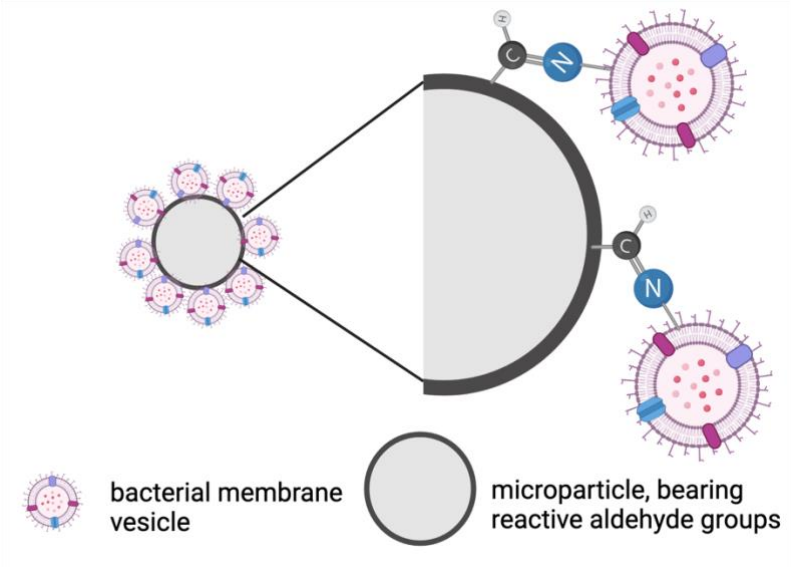

Figure 11 MV-covered microparticles: We used synthetic microparticles bearing aldehyde groups on their surface. These can react with amine groups of lysine residues found in the proteins on the MV surface forming imine bonds.

To test this, we used synthetic microparticles, covered with free reactive aldehyde groups, which can then react with lysine residues found on the surface proteins of the MVs. Using this mechanism, MVs can be covalently linked to the particle surface. We then determined the amount of MVs bound to the microparticle surface by measuring the protein content of the supernatant after the coupling reaction and imaged the resulting MV covered microparticles using scanning electron microscopy (Figure 12).

Here, we found distinct differences of microparticle coverage between the MVs harvested from different bacterial culture conditions. In general, pH 5 cultures showed the highest particle coverage (35-40% for both, *L. casei* and *L. plantarum* MVs), leading to the assumption that in this culture condition, MVs with more lysine-bearing surface proteins are produced. Contrarily *L. casei* MVs derived from the ph 6.5 condition showed the lowest particle coverage, both from the aerobic and anaerobic cultures (both < 5%). We also found, that *L. plantarum* MVs adhered more to the microparticles than *L. casei* MVs in every culture condition. This is particularly interesting, as particle numbers after the purification are consistently higher for *L. casei* MVs. A possible explanation can be found in the protein concentration per single MV (Figure 9), which was shown to be higher for *L. plantarum* than for *L. casei*. It can, thus, be concluded that the protein content per MV determines the MV's ability to be loaded onto the microparticles. This also highlights the potential of tailoring the MVs by altering the bacterial growth conditions.

Alteration of culture conditions can be used to modify the membrane vesicles produced by eukaryotic and prokaryotic cells alike. [213,214] In eukaryotic cells, the most prominent example has been depleting the medium from fetal bovine serum, which is commonly used in most cell culture media.[214] For bacterial vesicles, several techniques were applied to modify the MV

production, ranging from genetic modifications to the producing bacteria to altering culture conditions.[215] In a study by Lynch et al, it was found that gram-negative bacterium *Vibrio fischeri* shows pH-dependent alterations in the proteins contained in their outer membrane vesicles.[216] The present work showed, that this was also the case for gram-positive bacteria, in this case *L. casei* and *L. plantarum.* We further showed that altering culture conditions has effects not only on protein composition of the MVs but also on the amount of MVs produced in each culture as well as the biological effect. This needs to be considered when performing similar experiments, as slight alterations in media pH can have significant effects on the MVs properties. This can also be used to tailor specific MV properties or to increase MV yield.

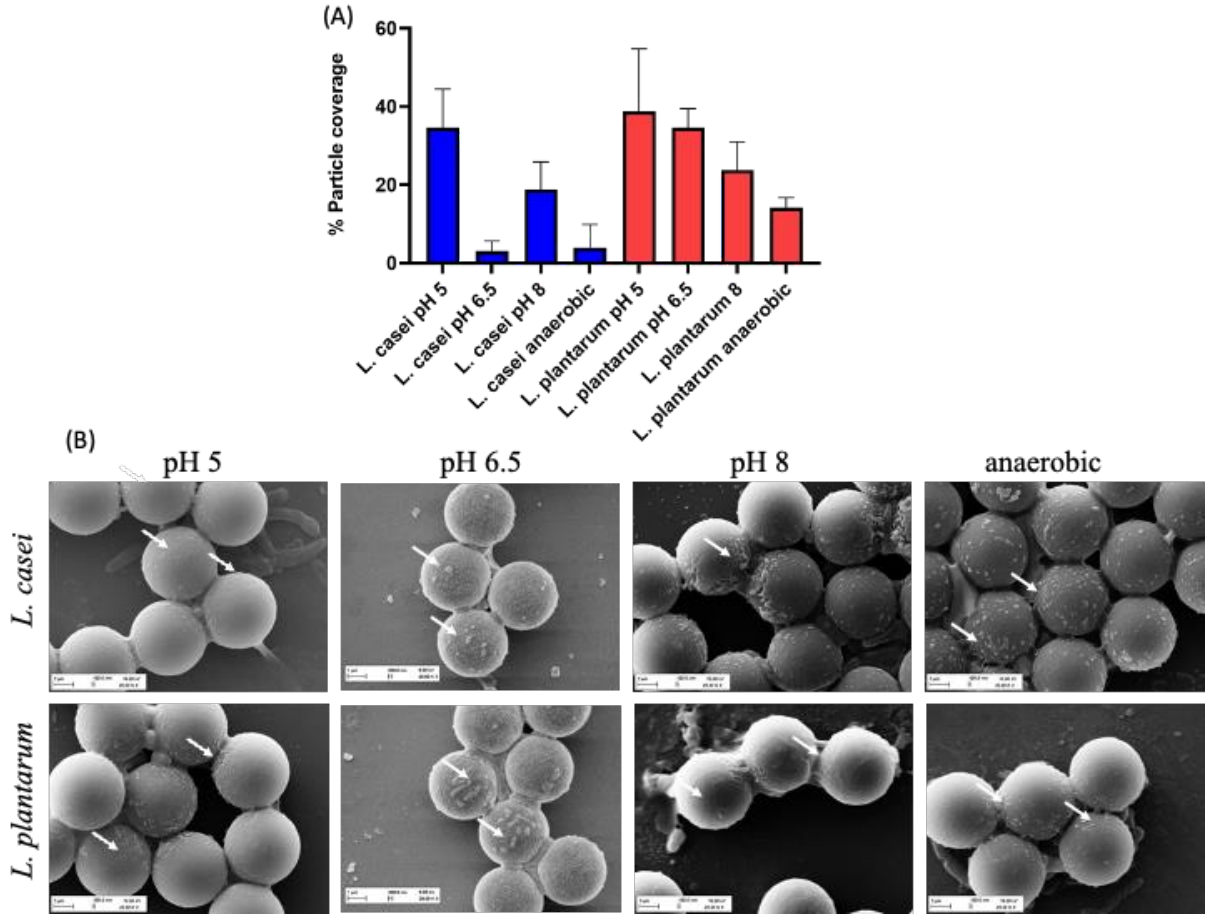

Figure 12 Characterization of MV coated microparticles: A Calculated partice coverage and B SEM images of the MV-covered microparticles. MVs from L. plantarum *showed a higher particle coverage than* L. casei *across all culture conditions.*

3.4. *In vitro* effects of MVs and Probiomimetics

Then the effect of the MVs and the MV-coated microparticles on cytokine production was examined in several inflamed *in vitro* models. It was tested on both, macrophage-like cell line dTHP-1 as well as primary blood mononuclear-cells, if the MVs and MV-coated microparticle

have the potential to ameliorate lipopolysaccharide-induced inflammation. Therefore, cells were co-treated with lipopolysaccharide as a pro-inflammatory stimulus and the MVs and MVcoated microparticles. It was demonstrated (Figure 13) that, normalized to the treatment dose, distinct differences occur regarding the cytokine production. For example, *L. casei* had the strongest anti-inflammatory effect, in the agitation condition, where the bacteria were shaken at a velocity of 180 rpm, contrary to the reference condition, where bacteria where cultured statically, which leads to lower oxygen supply.

The lowest anti-inflammatory effect for *L. casei* was shown in the reference condition (static, microaerophilic culture at a pH of 6.5). Here, the highest TNF and the lowest IL-10 release was measured. For *L. plantarum*, the strongest IL-10 increase on THP-1 cells was observed for the pH 5 culture. This culture condition also lead to the second-highest decrease in TNF production.

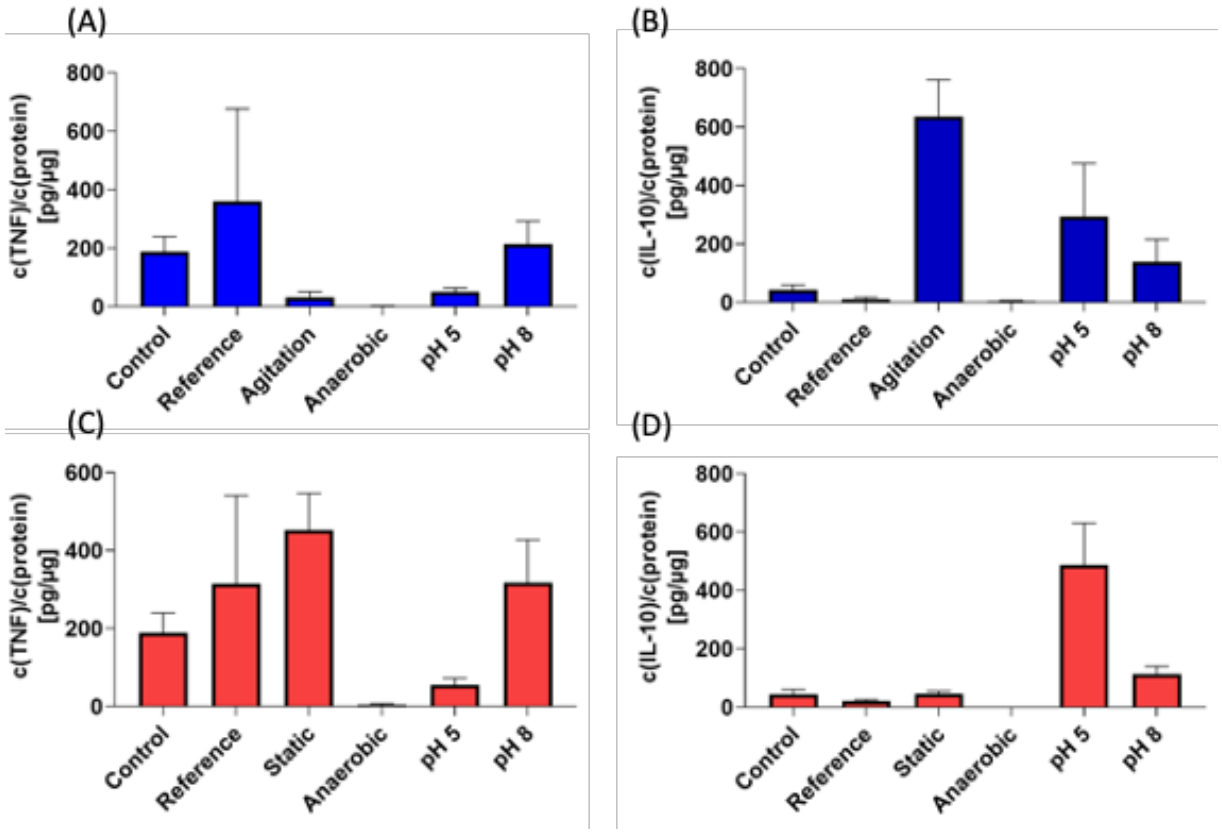

Figure 13: Anti-inflammatory effect of MVs macrophage-like dTHP-1 cells. TNF and IL-10 were determined in the supernatants of the cells using ELISA (A) and (B): dTHP-1 cell line treated with LPS and L. casei *MVs (C) and (D) dTHP-1 cell line treated with LPS and* L. plantarum *MVs*

Subsequently, the effect of the MVs from the different culture conditions and respective MVcoated was tested on primary cells. The cells examined were peripheral blood mononuclear cells (PBMCs). LPS was used to stimulate inflammation. Over all samples, a trend to lower TNF-release could be observed, (Figure 14 A) while IL-10 release remained at a similar level

as the LPS control (Figure 14 B). IL-10/TNF ratio was calculated for each culture condition. It was shown to be predictive for the healing of burn wounds before.[217] Here, IL-10/TNF ratios for the anaerobic MVs from both strains, microparticles from the pH 6.5 culture, and the anaerobic *L. plantarum* conditions were increased. In those two conditions, the MVs did not show an increase in IL-10/TNF ratio in the same way as the MV-coated microparticles (Figure 14 E).

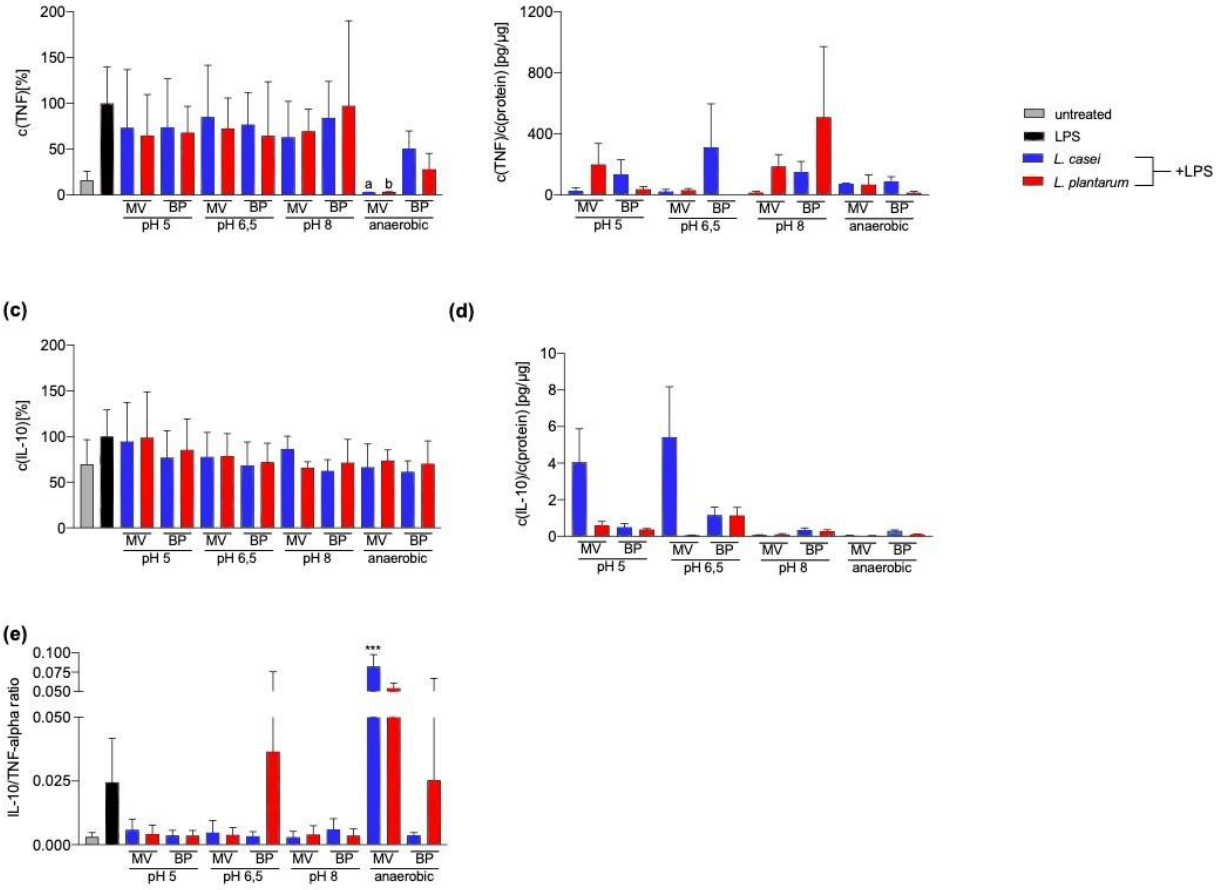

*Figure 14: PBMCs after incubation with 1 µg/ml of lipopolysaccharide (LPS) and the MVs or MV-coated microparticles (probiotic particles, BPs) respectively, for 4 h. TNF and interleukin10 in cell supernatant were determined using ELISA. (a) TNF, normalized to the inflamed control (LPS) to allow better comparability of effects induced by BPs. (b) IL-10 normalized to the inflamed control (LPS), and (c) IL-10/TNF ratio. Quantitative data are shown as means + SD calculated from three independent experiments. Statistical differences were analyzed by ANOVA followed by Dunnett's post-hoc test. ***p<0.001, a: p=0.0516, b: p=0.0534.*

We also observed the effect of the MVs and MV-coated microparticles on healing of tissuerepair *in vitro* models. We used two model cell lines representative of the diseases we targeted with this project. The cell lines used were enterocyte-like Caco-2 cells, representative for autoimmune intestinal diseases (TEER-value assay), as well as keratinocyte-like HaCaT (scratch assay) cells, for the wound healing disorders.

We found, that the MV-coated microparticles could ameliorate the inflammation-induced barrier damage of the Caco-2 cells, an effect which was not observed for the MVs alone. In this assay,

we inflamed the cells with lipopolysaccharides derived from *E. coli,* which lead to an approximately 20% decrease in the trans-epithelial electrical resistance (TEER) after 6h (Figure 15 B and C). Co-treatment of the cells with LPS and the MVs lead to a similar result. Treatment with the MV-coated microparticles however lead to a different result. Here. TEER values declined for the first three hours in a similar extent as for the LPS-only and the MVtreated. After six hours however, the TEER values recovered to above 90% of the original value. This highlights the potential of the MV-coated microparticles to ameliorate inflammationinduced barrier damage.

We also tested the MVs and MV-coated microparticles in a keratinocyte cell scratch assay (Figure 15 A), which determines the effects of a treatment on cell migration. Here, the observed, effects were less pronounced. Comparison between the MVs and the coated MPs shows, that, for some conditions, such as pH 5 culture MVs, both the MVs as well as the coated MPs showed a 5% increase in cell migration. Interestingly, L. plantarum pH 5 culture MVs had no effect on the closure of the scratch, while the coated MPs from this culture condition showed a 10% decrease in wound closure. Contrarily, the pH 6.5 culture from this strain showed the opposite trend. Here, the MVs alone lead to an 8% decrease in scratch closure, while the coated MPs lead to a slight increase.

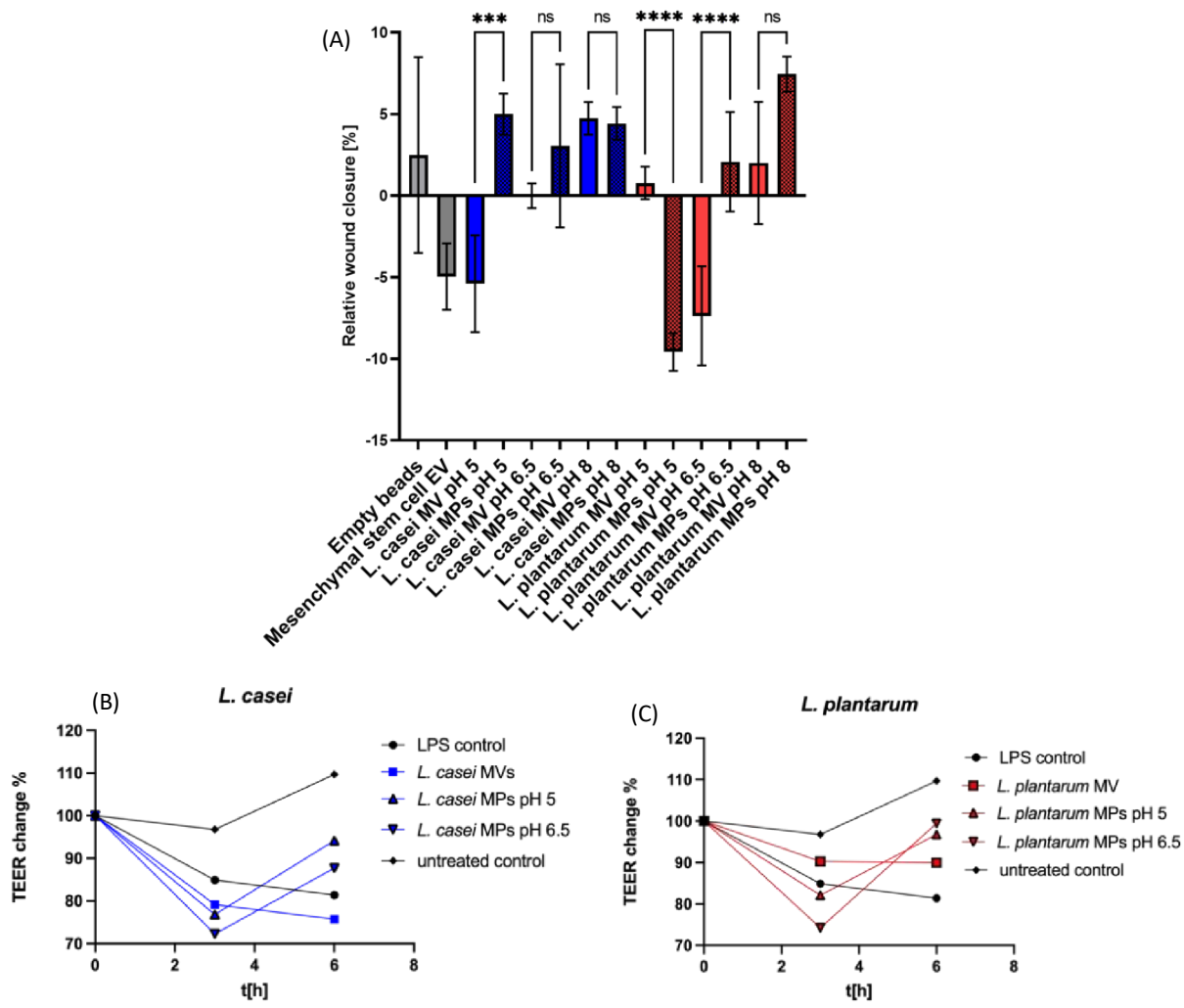

Figure 15: In-vitro effects of MVs and the MV-coated microparticles on (A) wound healing in a keratinocyte-cell line scratch-assay and (B) and (C) repair of damaged intestinal barrier in enterocyte cell line Caco-2 htb37

3.5. *In vivo* effects of MVs and MV-coated microparticles

Finally, we determined the *in vivo* effects of the MVs and MV-coated microparticles in a wound healing mouse model (Figure 16). Here, wounds of 1 cm in size were cut into the mice tail and were subsequently treated with MVs or MV-coated microparticles embedded in a hydroxyethylcellulose hydrogelmatrix (bacteriomimetic hydrogel) as well as the hydrogel matrix alone. Over the course of 30 days, the wounds were observed regarding their morphology. The thickness of the tail was measured as a surrogate for inflammation occurring in the wound site. After the wounds were closed entirely, histological cuts were prepared and microscopically characterized.

While no difference was observed regarding the time until complete wound closure, we found significant differences regarding the thickness of the mice tail. Thickness of the tail can be regarded as a surrogate marker for inflammatory processes, which occur during wound healing, as inflammation leads to swelling of the tissue.[70,127]Here, after wounding, tail thickness

increased significantly for the group treated with the empty hydrogel until day 6 post-wounding. Afterwards, tail thickness slowly decreased until day 22, when the original thickness was restored. A similar trend, even though less pronounced, could also be observed for the group treated with the MV hydrogel. It is worth noting, for this group, the tail thickness only increased by 0.2 cm, however the difference compared to the empty hydrogel control was not statistically significant (Figure 16 B). Interestingly, for the MV-loaded microparticle treated mice, the increase in tail thickness could only be measured until day 2 post-wounding. This difference was statistically significant, highlighting the anti-inflammatory effect of the MV-coated microparticles. Similar trends also became obvious when observing the thickness of the dermis after the wounds healed, whereas a high increase in dermis thickness is associated with high scar formation.[218] Here, the dermis thickness strongly increased for the MV-hydrogel as well as the empty hydrogel and reached values more than three times as high as for unwounded control animals (Figure 16 C). In contrast, for the mice treated with bacteriomimetic hydrogel containing the MV-loaded microparticles, dermis thickness only doubled in comparison to the unwounded control animals.

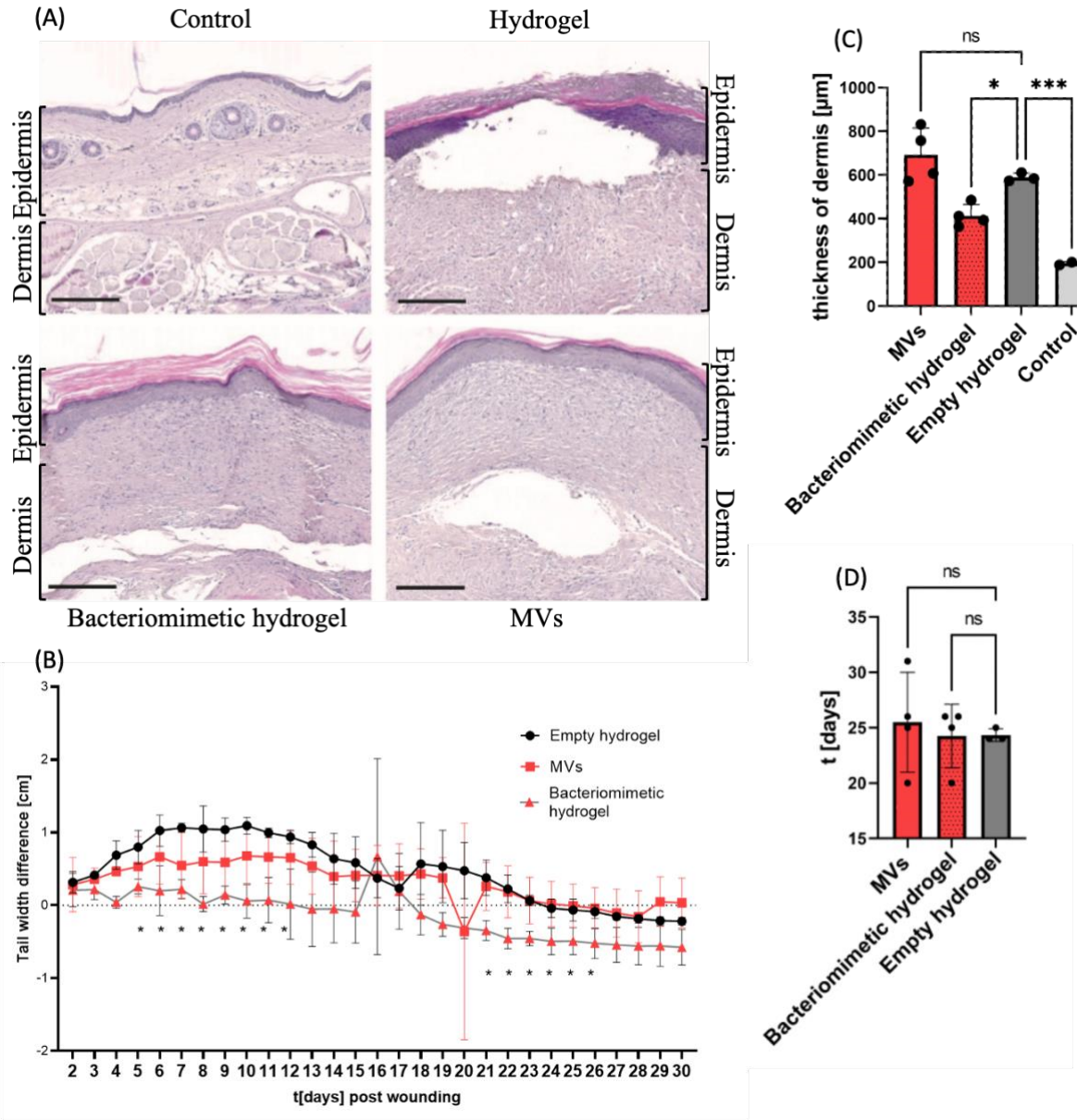

Figure 16: In vivo effects of L. plantarum MVs and MV-coated microparticles in a tail-wound mouse model. (A) histological images of the wound after 30 days of healing (B) difference in tail-thickness of the mice. Tail thickness indicates inflammatory activity in the wound site. (C) thickness of the dermis after 30 days of healing, whereas increased thickness indicates scarring. (D) time until wound closure

These results show the potential of microparticles with *Lactobacillus* membrane vesicles on their surface as a novel therapeutic system. They show the potential of this therapeutic system for immune modulatory therapy in the context of intestinal inflammatory diseases and wound healing disorders.

4. Conclusion and Outlook

In this thesis, I characterized anti-inflammatory membrane vesicles secreted by probiotic bacteria, namely *Lactobacillus casei* and *Lactobacillus plantarum*. The bacteria were grown under different culture conditions to assess, which culture conditions lead the the bacteria producing suitable MVs. I further developed a novel bacteriomimetic formulation to improve their pharmacokinetic properties as well as harnessing advantageous effects of larger-sized microparticles on the immune regulatory properties. The strains *L. casei* DSMZ1011 and *L. plantarum* NCIBM were cultivated under various culture conditions, including different media pH values as well as varied oxygen supply and mechanical stress via agitation of the cultures. Under the different culture conditions, the resulting MVs substantially differed regarding their physicochemical properties, the yields that could be obtained from a given volume of bacterial culture as well as the amount of protein detected within the MVs. Proteomic characterization revealed a high number of unique proteins for the MVs obtained from the respective culture conditions. As a result, the MVs also differed regarding their anti-inflammatory effect in various *in vitro* models as well as their effect on the healing cultivated cell layers injured mechanically or via pro-inflammatory stimuli. The different culture conditions drastically changed the ability of the MVs to be loaded onto synthetic microparticles.

I also could show the advantages of coupling the MVs to the surface of synthetic microparticles. Thereby, we developed a bacteriomimetic system decorated with MVs on the surface. The MV coated microparticles showed wound healing promiting effects in a mouse *in vivo* model

Based on the obtained results, future research should aim to optimize the formulation of the bacteriomimetics. Primarily, the synthetic microparticles should be formulated using biodegradable polymers, to ensure safe applicability, especially for oral or a possible parenteral application route.[219,220] Using a different polymer for the particle will also influence reaction conditions and thus loading degree of the particles, which can be used to increase loading degree of the microparticles with MVs. Loading degree of the MVs on the microparticles can be further optimized by altering reaction conditions to achieve higher coverage of the microparticle surface. Here, the use of different coupling reactions can be assessed. Click-chemistry (copper(I)-catalyzed azide alkyne cycloaddition after azidemodification of the MVs' surface proteins can be a promising tool that has been tested on MVs before.

For gastrointestinal application, it also need to be assessed whether the bacteriomimetics are stable to the harsh conditions in the stomach (acidity as well as protein degrading enzymes). If stability is diminished by the GIT conditions, finding a GIT stable formulation is of great importance.[221] This can be achieved by encapsulating the bacteriomimetic microparticles into bigger dosage forms with GIT stable properties (e.g. granules via spray-drying, tablets or

capsules).[222–224] Here, especially the low pH in the stomach is of concern. Thus, a formulation should be developed, which is insoluble in gastric acid, but dissolves in the higher pH in the intestine. In this case, the MVs and MV-coated microparticles can be protected from the harsh conditions in gastric acid and be released in the intestine, to reach the inflamed tissues in e.g. IBD or celiac disease.

In this case, release of the bacteriomimetics out of the dosage forms needs to be examined and optimized.

5. References

Figures 1-8 and 11 were created with BioRender.com

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6. List of publications and oral presentations

Manuscripts published in international, peer-reviewed journals

Kuhn, T., Koch, M., & Fuhrmann, G. (2020). Probiomimetics—Novel Lactobacillus-Mimicking Microparticles Show Anti-Inflammatory and Barrier-Protecting Effects in Gastrointestinal Models. *Small*, 16(40), 2003158.

Kuhn, T., Aljohmani, A., Frank, N., Zielke, L., Mehanny, M., Laschke, M. W., Koch, M., Hoppstädter, J., Kiemer, A. K., Yildiz, D., & Fuhrmann, G. (2024). A cell-free, biomimetic hydrogel based on probiotic membrane vesicles ameliorates wound healing. *Journal of Controlled Release*, 365, 969–980.

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Poster Presentations:

Poster Presentation at HIPS Symposium, 2018, Saarbrücken

Poster at International Symposium on Biological Barriers, 2018, Saarbrücken

Poster Presentation at HIPS Symposium, 2019, Saarbrücken

Poster presentation at Doktorandentag der Universität des Saarlandes, 2021, Saarbrücken

Poster presentation and short-talk at Controlled Release Society Local Chapter Meeting, 2020, München

7. Scientific output

7.1. Probiomimetics—Novel Lactobacillus-Mimicking Microparticles Show AntiInflammatory and Barrier-Protecting Effects in Gastrointestinal Models.

Thomas Kuhn, Marcus Koch and Gregor Fuhrmann

Small, 2020, 2003158

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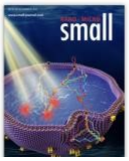

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Probiomimetics – Novel *Lactobacillus*-Mimicking Microparticles Show AntiInflammatory and Barrier-Protecting Effects in Gastrointestinal Models

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Keywords: inflammatory bowel diseases, biomimetics, bacteriomimetics, anti-inflammatory therapy

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. We developed a novel cell-free and anti-inflammatory therapeutic system – probiomimetics – based on MVs of probiotic bacteria (*Lactobacillus casei* and *L. plantarum*) coupled to the surface of microparticles.

The MVs were isolated and characterized for size and protein content. MV morphology was determined using cryoelectron microscopy and was reported for the first time in this study. MVs were non-toxic against macrophage-like dTHP-1 and enterocyte-like Caco-2 cell lines. Subsequently, the MVs were 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%) was observed with probiomimetics but not with native MVs. Moreover, we demonstrate that probiomimetics have the ability to ameliorate inflammationinduced 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 *E. 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 administering therapeutics based on bacterial membrane vesicles (MVs), 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]-[27]

The use of eukaryotic vesicles (EVs) to treat autoinflammatory diseases has been reported earlier.[28]-[30] For example, the application of EVs, produced by human mesenchymal stem cells in inflammatory diseases, was shown to ameliorate autoimmune reactions.[31],[32] However, it is difficult to produce large quantities of these mammalian vesicles for multi-patient clinical applications.[28] In addition, the activity of eukaryotic EVs varies depending on the cell culture conditions and the passage of the cells.[33]

An alternative group of vesicles with inherent anti-inflammatory properties are MVs of probiotic bacteria.[34] These vesicles have shown promising anti-inflammatory effects in earlier studies.[35] They can be obtained in substantially higher amounts and can be easily standardized compared with mammalian EVs.[36] 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.[37]

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.[38] 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.

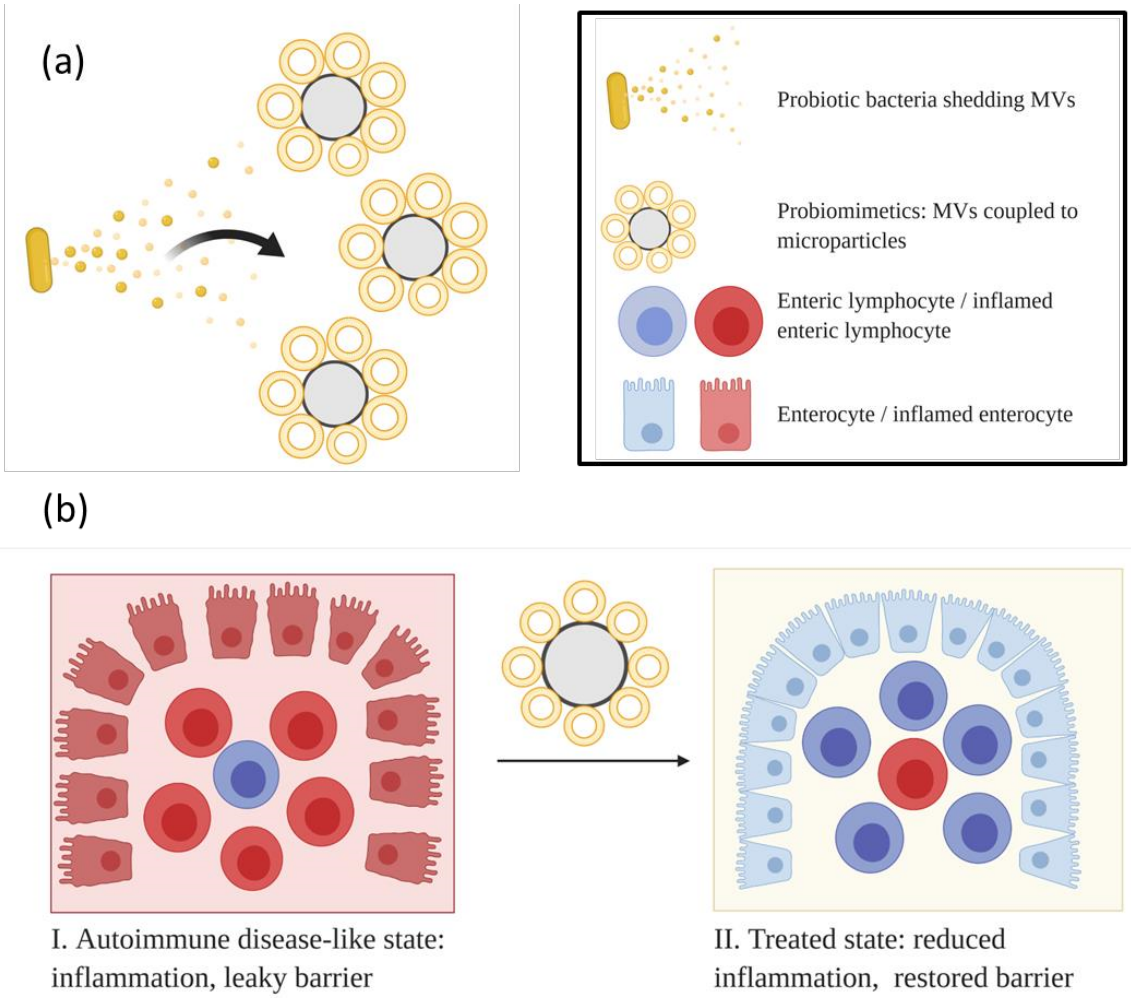

Figure 1. Concept of the study: (a) Probiotic *Lactobacilli* shed membrane vesicles that are chemically coupled to the surface of microparticles. (b) The obtained probiomimetics are characterized and assessed in suitable *in vitro* models. Using the immune-modulatory effects of the probiotic bacteria,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

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 analysis (NTA). The mode sizes of *L. casei* and *L. plantarum* MVs were 113 ± 12 nm and 117 ± 24 nm, respectively. These results are consistent with previous studies on MVs from other *Lactobacillus* species.[39] The particle concentrations ranged from approximately 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 with the peak containing free proteins (**Figure S3**). Similar to the particle concentration, the protein content in *L. casei* (120.8 µg/mL ± 7.6 µg/mL) was higher than that in *L. plantarum* (20.3 µg/mL ± 5.9 µg/ml).

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 measured the hydrodynamic diameter while electron microscopy showed the MV morphology. The MVs appeared to be round-shaped particles, with an electrondense 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.

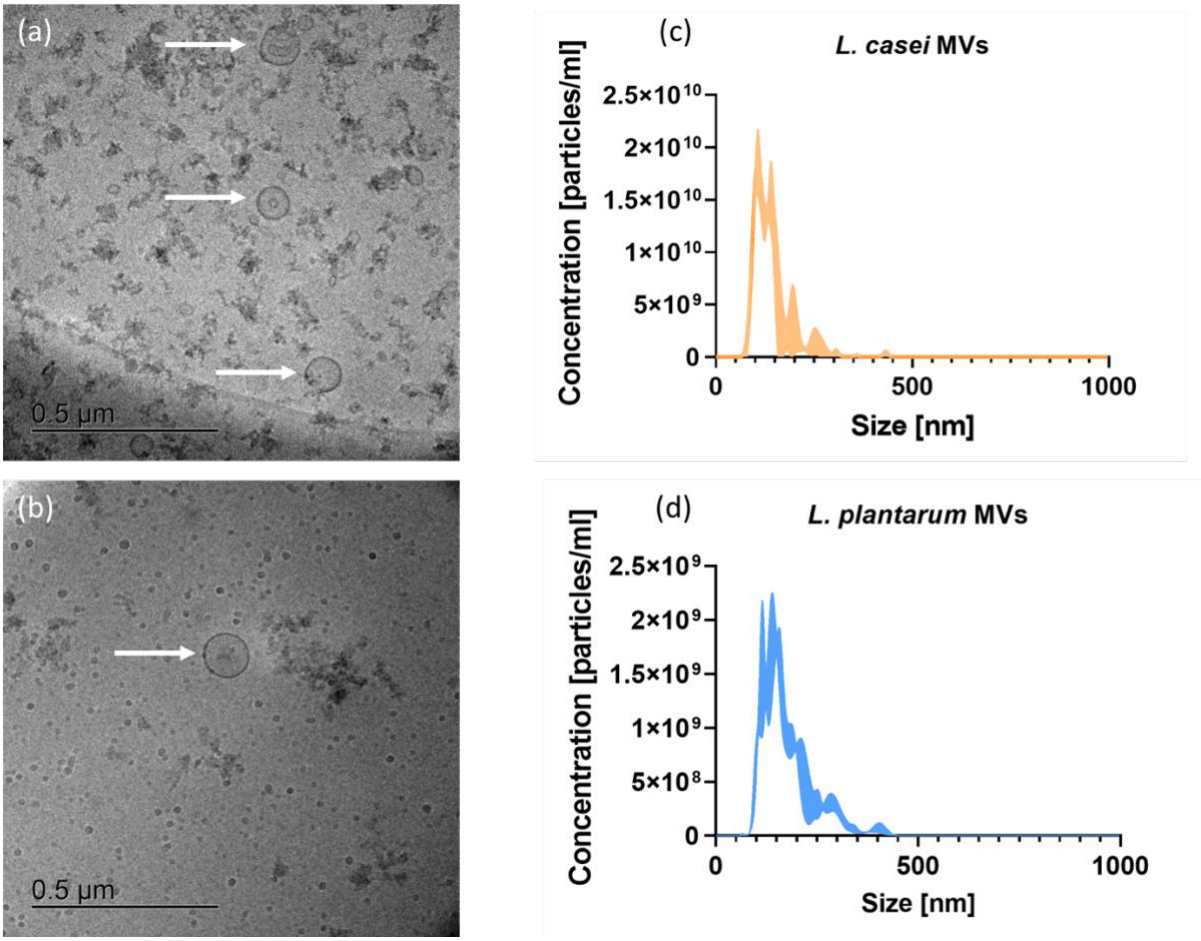

Figure 2. Characterization of the *Lactobacillus casei and L. plantarum* MVs used in this study. Representative Cryo-transmission electron microscopy image of (a) *L. casei* MVs and (b) *L. plantarum* MVs. Images were acquired of MVs from the ultracentrifugation pellet before further purification by size-exclusion chromatography to ensure sufficient MV concentrations for imaging. MVs are marked with arrows. Co-pelleted proteins are visible. Typical size

distribution of (c) *L. casei* MVs and D) *L. plantarum* MVs measured via nanoparticle tracking analysis.

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,[41] 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 phosphatebuffered saline (PBS), despite treatment with a very high concentration of MVs. Concentrations used were as high as 300,000 MVs/cell for Caco-2 and 100,000 MVs/cell for dTHP-1, with no toxic effects observed.

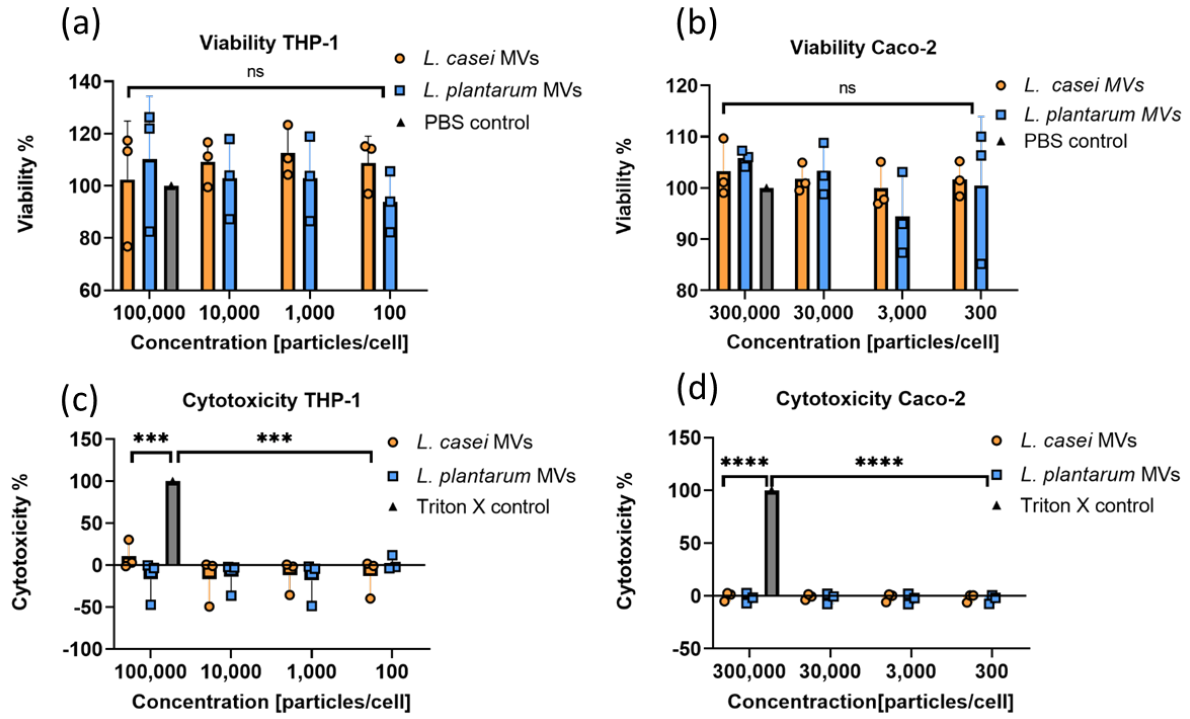

Figure 3. Cytotoxicity testing of the *Lactobacillus* MVs. A) and B) PrestoBlue assay, measuring the metabolic activity of the two tested cell lines Caco-2 and THP-1 treated with MVs. C) and D) Lactate dehydrogenase assay for assessing the intracellular enzyme lactate dehydrogenase released in case of 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

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 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.[42] 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.

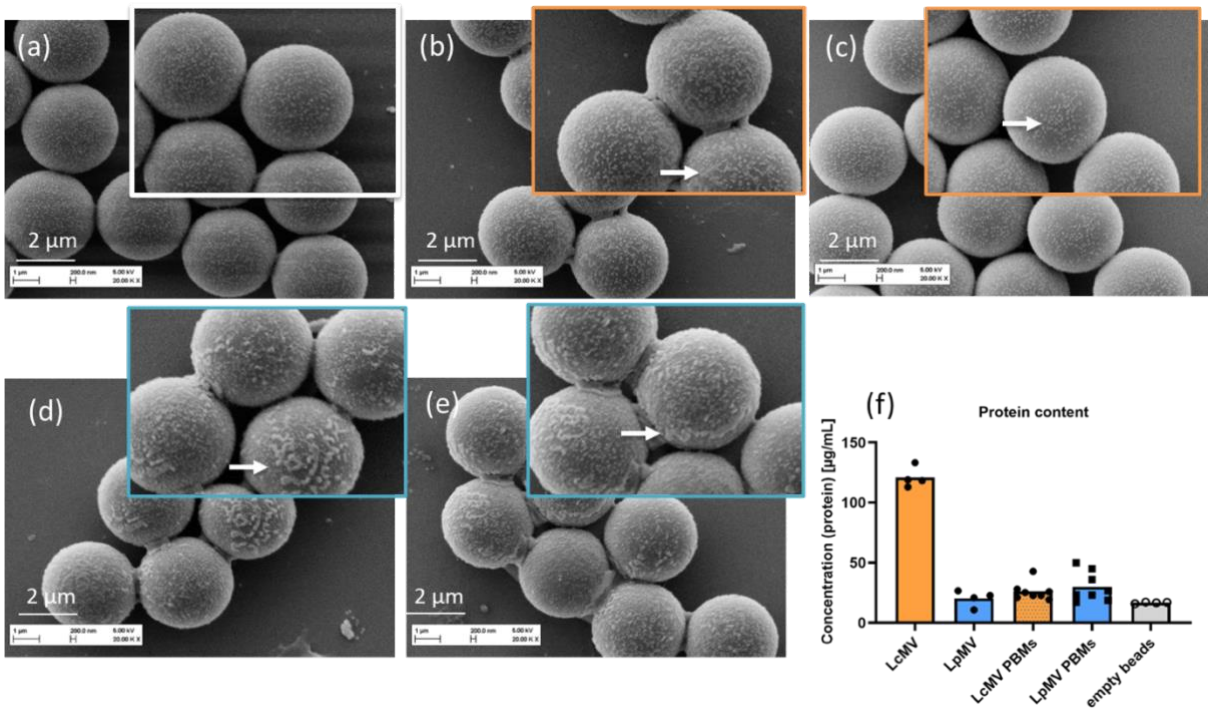

Figure 4. Morphological characterization of the probiomimetics via scanning electron microscopy. (a) Untreated beads as control; (b) probiomimetics coated with *L. casei* MVs at pH 5 and (c) at pH 7. (d) Probiomimetics coated with *L. plantarum* MVs at pH 5 and (e) at pH 7. *L. plantarum* MVs are arranged 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 mean values of all experiments.

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.[43] 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)**.[44]

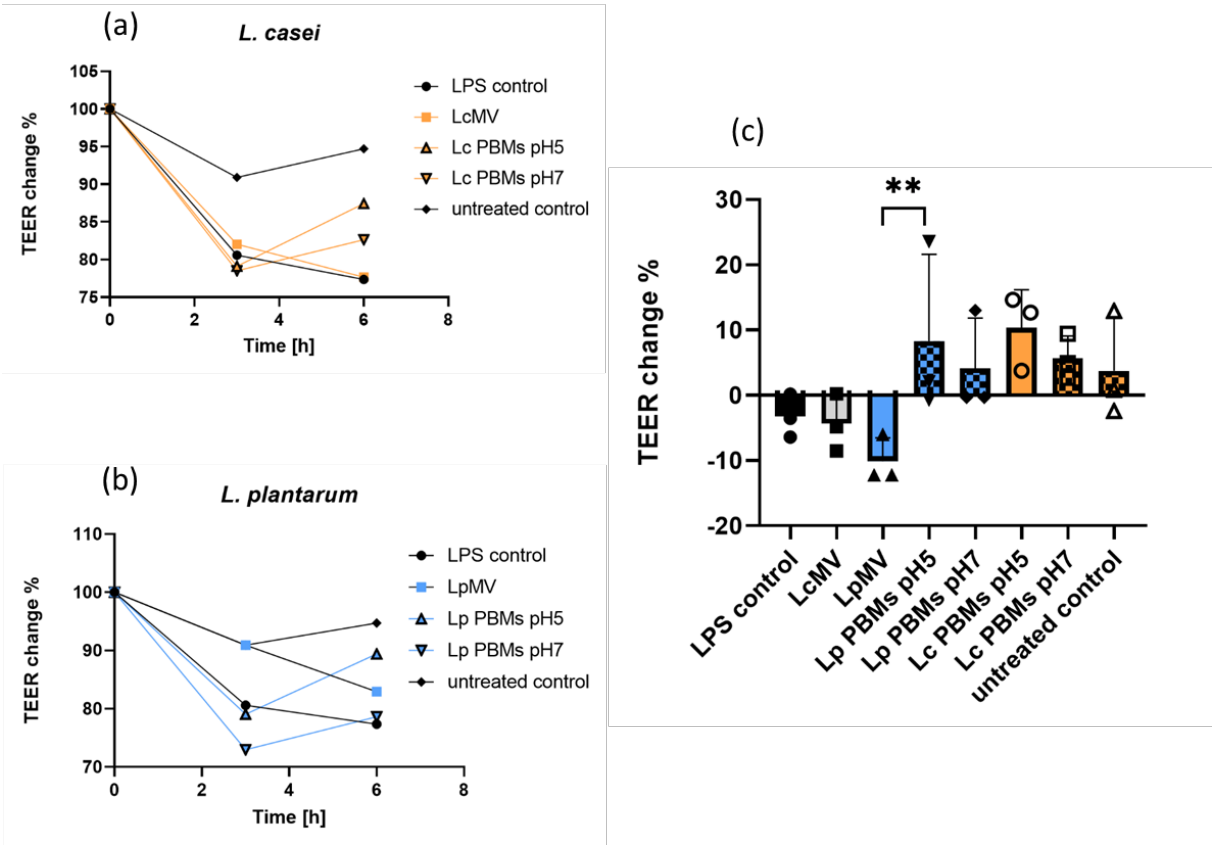

Figure 5. Trans-epithelial electric resistance values of enterocyte-like Caco-2 cells as a surrogate for the intestinal barrier function. (a), (b) Change in the TEER values over the course of the experiment, as an average of three individual experiments. When treated with probiomimetics for 3 h, the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

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 MV and probiomimetics. Macrophage-like dTHP-1 cells and enterocyte-like Caco-2

cells were stimulated with LPS (10 µg/mL) to mimic inflammatory processes present in the GIT environment. Cells were co-treated with the native MVs or coated microparticles for 6 h and 24 h, respectively and the concentration of released cytokines was measured using enzymelinked 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.[45] 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 the supporting information **Figures S2 and S3**, as this normalization could not be applied there.

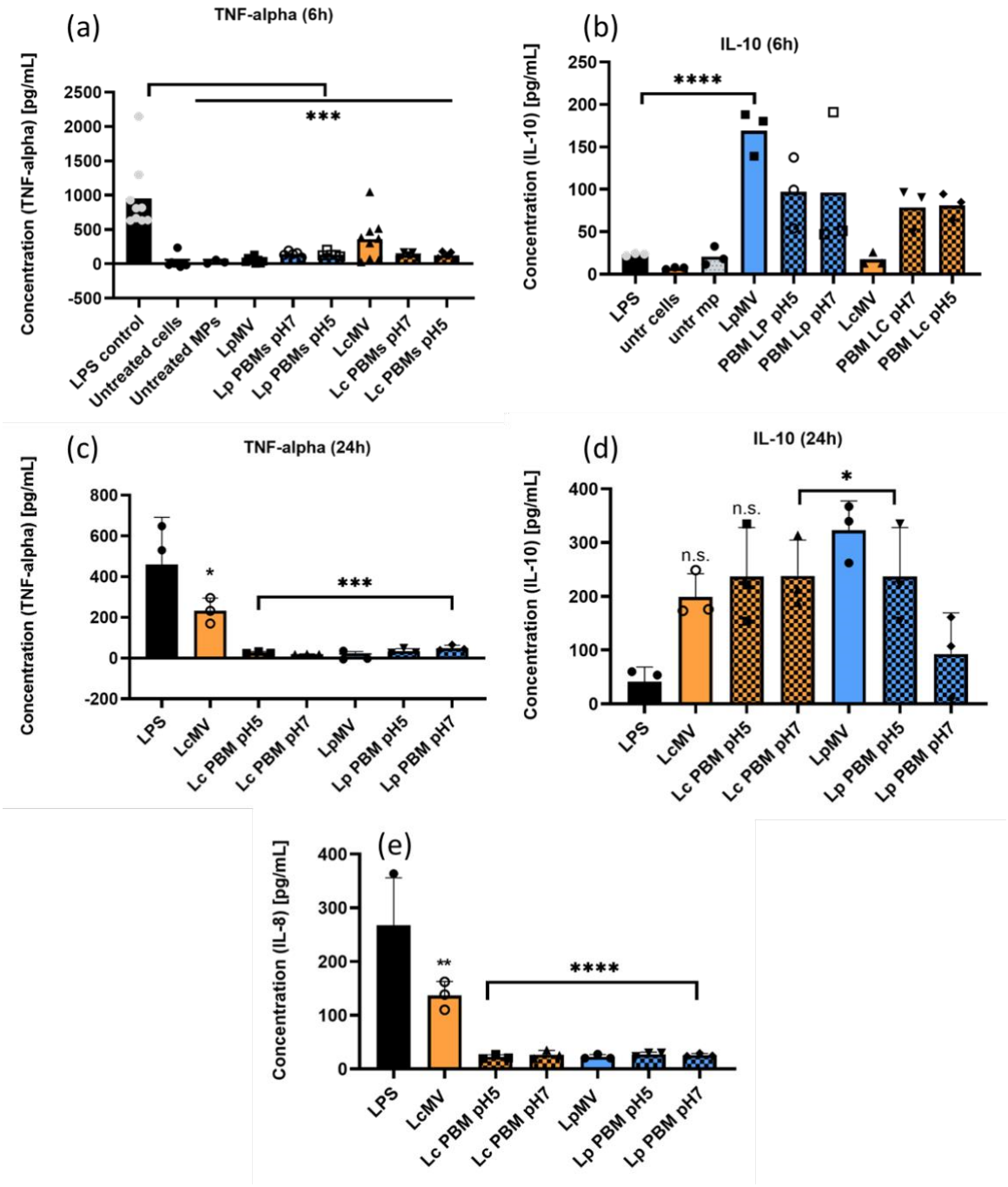

Figure 6. Cytokine production in inflamed macrophage-like dTHP-1 cells as well as in enterocyte-like Caco-2 cells. Cells were stimulated with lipopolysaccharides (10 µg/ml) and co-treated with probiomimetics or MVs. Supernatants were harvested after 6 h or 24 h, and the protein content was analyzed using 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 pro-inflammatory 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 pro-inflammatory IL-8 in enterocyte-like Caco-2 cells after 24 h. Values represent the mean of 3-9 biological replicates.

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 pro-inflammatory effects, indicating that the reduction in TNFα 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.[46] 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* MVcoated microparticles, even though their concentrations were consistently 5-fold lower. This effect seemed to be of 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 antiinflammatory 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, approximately 12-fold, inhibition of IL-8 release, while the effect observed from Lactobacillus casei MVs was 2-fold.

Similar effects were have been seen with MVs from different *Lactobacillus strains,* such as *Lactobacillus rhamnosus* and *L. reuteri*.[46] This effect has been proven to be linked to the innate immune system, which is also consistent with the effects seen in the dTHP-1 cells. Other Lactobacillus strains, such as *L. kefir,* have anti-inflammatory effects on Caco-2 cells and they ameliorated colitis in a mouse model.[47] According to a recent study by Choi et al. these effects seem to be related to multiple mechanisms, such as the reduction of NOproduction and the amelioration of endoplasmic reticulum stress.[48]

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.[49] 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.[50] 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 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

4.1. 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% non-essential amino acid mix (Gibco, Waltham, MA). Cells were supplemented with fresh medium every 2-3 days. 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 days, 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) and seeded into 96-well plates, at a density of 1×10^5 cells/well. Cells were then allowed to differentiate for 24 h for viability testing and 48 h for cytokine assays.

4.2. 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 days at 37°C and 5% CO_2. The medium was changed every 2-3 days (500 µL in the apical compartment and 1500 µL in the basolateral compartment). After 11-12 days, 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) 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 h and 6 h. Then, the supernatant was collected for the quantification of cytokines.

4.3. Bacterial culture

L. casei (DSMZ, Braunschweig, Germany) was cultivated on deMan-Rogosa-Sharpe agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 5 days 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 deManRogosa-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 days 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 supplementary **Figure S1**.

4.4. 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.

4.5. Vesicle characterization

4.5.1. 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.

4.5.2. 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.

4.5.3. 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 1ml of sterile filtered PBS.

4.6. 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 (approx. 5 × 10^{11} - 5 × 10^{12} EVs/mL) 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 λ = 492 nm.

4.7. Cytokine assay and enzyme-linked immunosorbent assay

THP-1 cells were differentiated with 7.5 ng/mL 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 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 h 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.

4.8. 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 approximately 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)

4.9. 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.

4.10. 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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Supporting Information

Probiomimetics – Novel *Lactobacillus*-Mimicking Microparticles Show AntiInflammatory and Barrier-Protecting Effects in Gastrointestinal Models

*Thomas Kuhn, Marcus Koch, Gregor Fuhrmann**

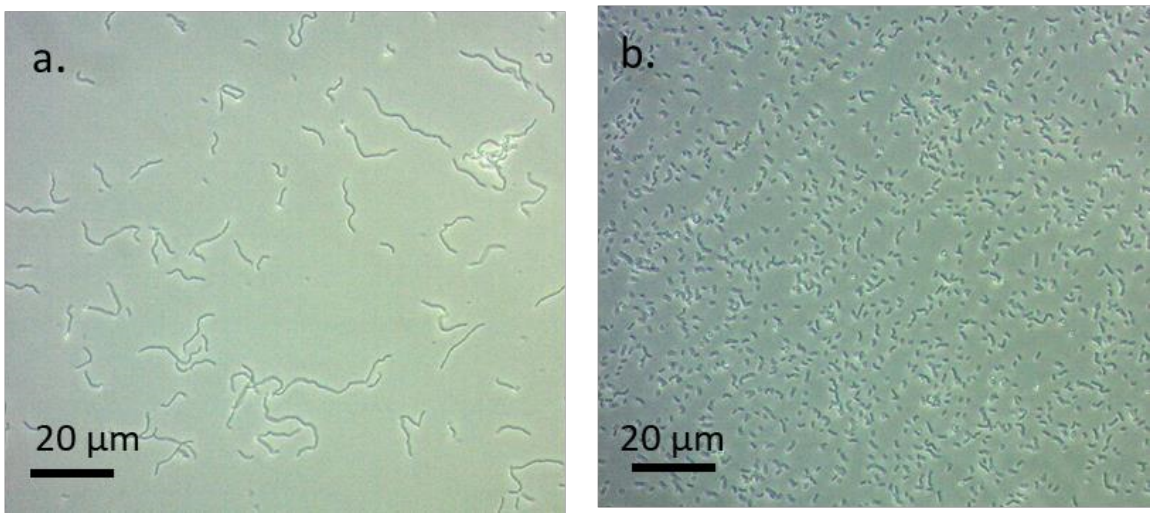

Figure S1 light microscopy images of a. *Lactobacillus casei* and b. *Lactobacillus plantarum.* While *Lactobacillus casei* appear as shapes of several individual bacteria*, Lactobacillus plantarum* appear to be planktonic. Bacteria supernatants with MVs were harvested after 48h of liquid culture which equals the late stationary phase.

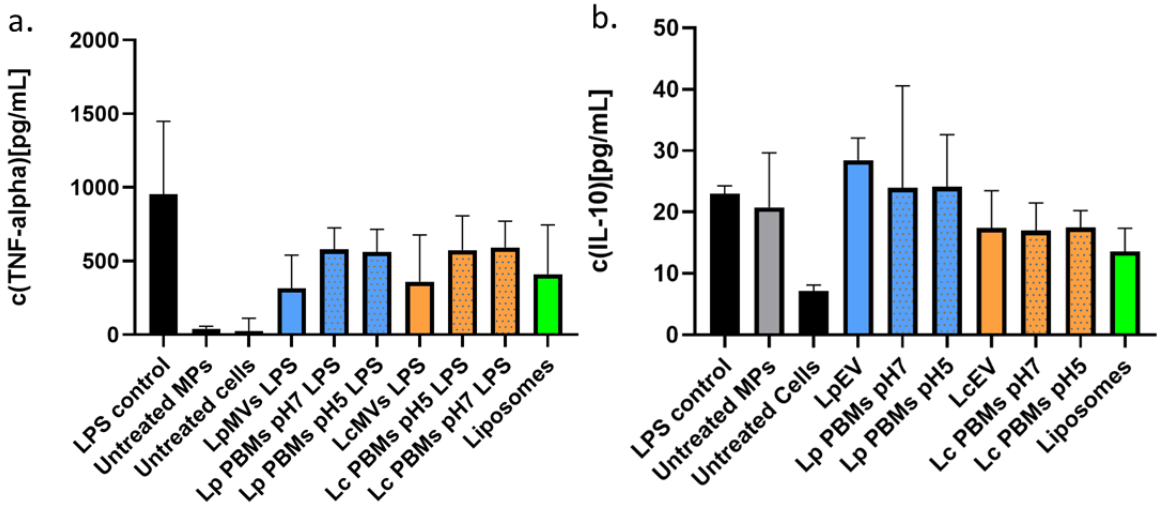

Figure S2 Raw data of the 6h ELISA-measurements before normalization. a. for TNF-alpha; b. for IL-10. Also shown are phosphatidylserine-containing liposomes as synthetic particles with an anti-inflammatory effect.

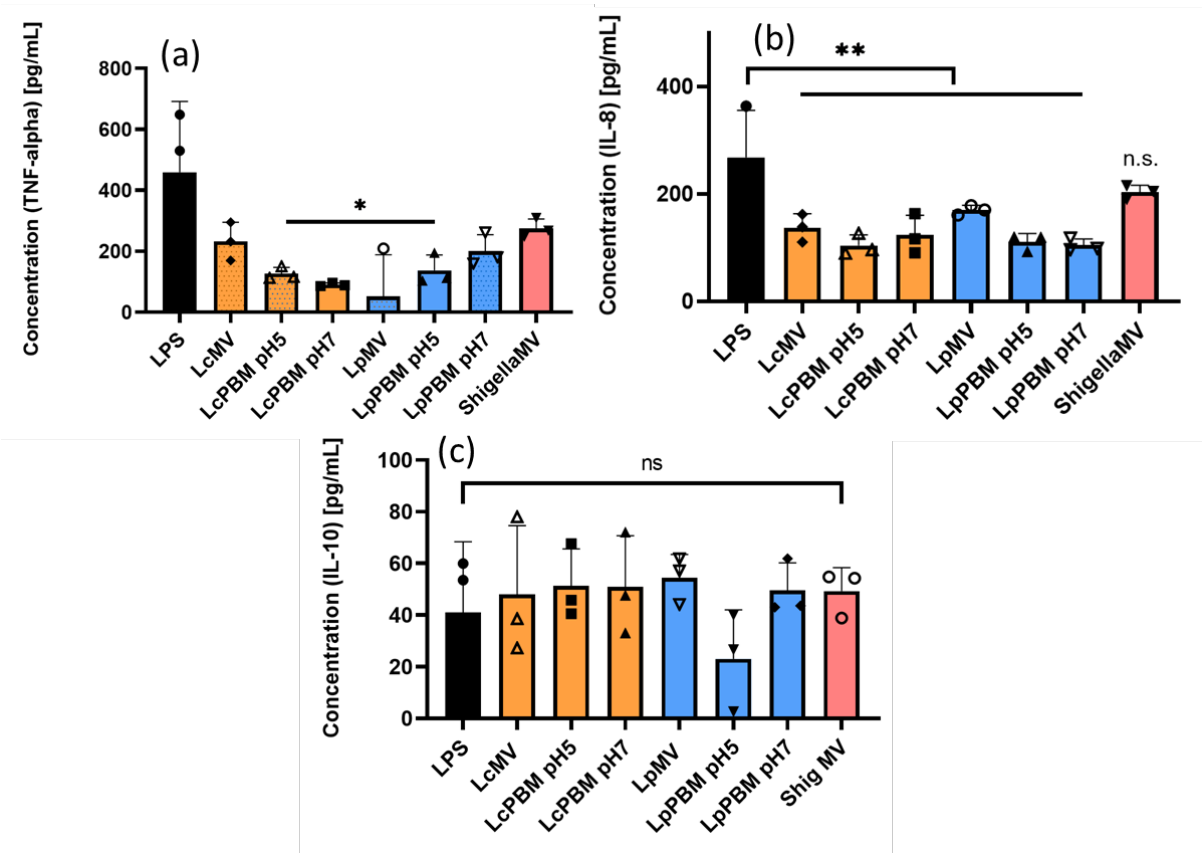

Figure S3: Raw data of the 24h ELISA-measurements before normalization. a. for TNF-alpha; b. for IL-8; c. for IL-10. Also shown are MVs from the intestinal pathogen *Shigella flexneri.* The experiment was performed in three biological replicates.

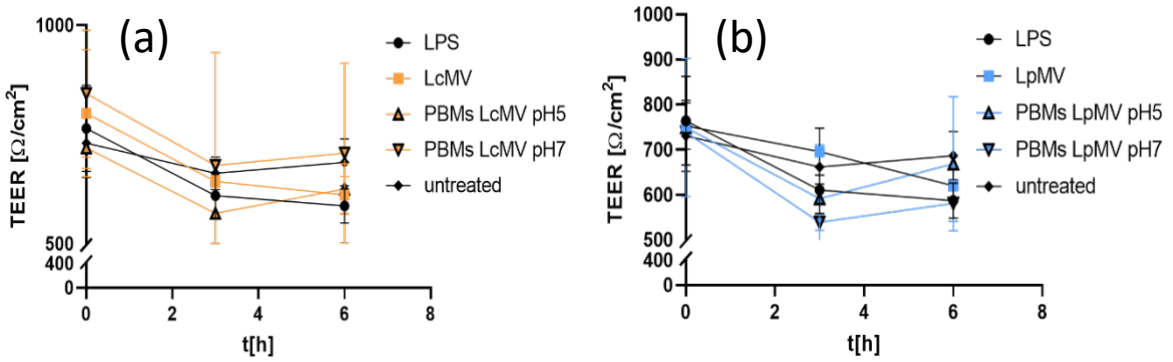

Figure S4: Raw data of the TEER-value measurements: For the Caco-2 cells treated with PBMs, it could be observed that the TEER values were stabilized in the time between 3 and 6 hours compared to the samples treated with LPS and the MVs only.

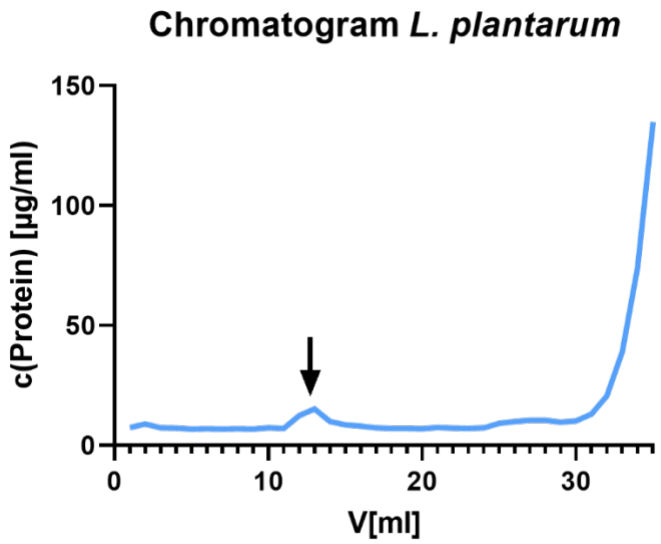

Figure S5: Representative chromatogram of the size-exclusion chromatography used to purify the membrane vesicles (MVs). Fractions with the volume of 1ml were collected. Fraction 13 marks the peak where the MVs can be found. The big peak starting at fraction 32 marks the co-pelleted proteins. This demonstrated successful baseline separation of the MVs and the contaminating soluble proteins.

Table S1. Properties of the MVs of both *Lactobacillus* strains

	***Lactobacillus casei* MVs**	***Lactobacillus plantarum* MVs**
Size	100-120 nm	100-120 nm
Zeta-potential	6.3 ± 0.68 mV	3.3 ± 0.22 mV
Protein concentration (in highest concentrated SEC fraction)	~120 µg/ml	~20µg/ml
Obtainable concentration	$2*10^{12}$/ml	$5*10^{11}$/ml
Anti-inflammatory effect	Moderate	High

7.2. A cell-free, biomimetic hydrogel based on probiotic membrane vesicles ameliorates wound healing

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A cell-free, biomimetic hydrogel based on probiotic membrane vesicles ameliorates wound healing

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Keywords: bacteriomimetics, probiotic bacteria, Lactobacilli, bacterial membrane vesicles, extracellular vesicles, inflammation, wound healing

Abstract

Probiotic bacteria, such as *Lactobacilli,* have been shown to elicit beneficial effects in various tissue regeneration applications. However, their formulation as living bacteria is challenging, and their therapeutic use as proliferating microorganisms is especially limited in immunocompromised patients.

Here, we propose a new therapeutic avenue to circumvent these shortcomings by developing a bacteriomimetic hydrogel based on membrane vesicles (MVs) produced by *Lactobacilli*. We coupled MVs from *Lactobacillus plantarum* and *Lactobacillus casei*, respectively, to the surface of synthetic microparticles, and embedded those bacteriomimetics into a pharmaceutically applicable hydrogel matrix. The wound microenvironment changes during the wound healing process, including adaptions of the pH and changes of the oxygen supply. We thus performed proteomic characterization of the MVs harvested under different culture conditions and identified characteristic proteins related to the biological effect of the probiotics in every culture state. In addition, we highlight a number of unique proteins expressed and sorted into the MVs for every culture condition. Using different *in vitro* models, we demonstrated that increased cell migration and anti-inflammatory effects of the bacteriomimetic microparticles were dependent on the culture condition of the secreting bacteria. Finally, we demonstrated the bacteriomimetic hydrogel's ability to improve healing in an *in vivo* mouse full-thickness wound model. Our results create a solid basis for the future application of probiotic-derived vesicles in the treatment of inflammatory dispositions and stimulates the initiation of further preclinical trials.

Introduction

Skin is the largest organ of the human body and the one that is most exposed to environmental influences and exogenous noxae, such as pathogens, toxins and physical trauma.[1,2]Healing of wounds caused by such traumata is a complex interplay of various cells and mediators. Typically, wound healing occurs in three phases consisting of an inflammatory phase, a proliferative phase and a remodeling phase resulting in a mature scar and recovery of the injured tissue.[3,4] However, an overshooting inflammatory reaction may impair wound healing, leading to delayed wound closure, chronic wounds, and the formation of hypertrophic scars.[5,6] Wound healing disorders pose a substantial burden on healthcare systems worldwide, with more than eight million patients and treatment costs of more than 25 billion $ in the US alone.[7,8] However, current therapy guidelines do not address overshooting inflammation as a main contributor to wound healing disorders, but focus mostly on wound cleaning and management of the wound milieu.[9] Moreover, there has been numerous evidence of scar less, inflammation-free wound healing that was observed in the healing of fetal wounds. These observations support the hypothesis that modulating overshooting immune reactions may also lead to an improved healing process. This highlights the potential of targeting the immune reaction with suitable therapeutics to augment wound healing.[10,11]

A novel therapeutic route is the use of probiotic bacteria.[12,13] These have been shown to elicit multiple biological effects in the context of wound healing, including antimicrobial effects against pathogenic bacteria in the wound, interactions with the host inflammatory response, as well as pH alteration of the wound milieu.[13] However, safety and applicability concerns have been raised regarding the use of live, proliferative bacteria as therapeutics, especially in the context of immunocompromised patients.[14]

A promising alternative might be the use of probiotic bacterial membrane vesicles (MVs) as explored in the recent studies. Bacterial MVs are a class of biogenic nanoparticles shed by almost every microorganism.15,[16] They are characterized by a diameter of 50-150 nm and are composed of a phospholipid membrane, as well as multiple types of cargoes.[17] Possible cargoes include a variety of nucleic acids and proteins. They have been shown to fulfill many biological roles in cell-cell communication, in particular in the communication of bacteria and their host immune system, exerting similar biological effects to those of the live bacteria.[18] One example are the Gram-positive *Lactobacillus (L.)* strains *L. casei* and *L. plantarum*, which possess immune-regulating properties.[19–21] Their potential to induce the regulatory M2 phenotype in macrophages makes them interesting candidates for the treatment of poorly healing wounds.[22]

Therefore, in our recent study, we assessed a hydrogel containing bacteriomimetic microparticles as alternative to augment wound healing. These bacteriomimetics are a

biomimetic therapeutic system composed of MVs derived from probiotic *Lactobacilli* coupled onto synthetic microparticles. We formulated the bacteriomimetics in a pharmaceutical hydrogel for better applicability and as a cutaneous therapeutic option for wound healing disorders.[23,24] Probiotic and commensal bacteria are physiologically relevant in wound healing but different pH and oxygen-supply conditions that occur during wound healing, may overall influence their biological activity.[25] To optimize the biological effect of the MVs used, we mimicked such conditions during bacterial culture and investigated their effect on the MVs' biological effects (Fig. 1). We evaluated the anti-inflammatory effect of the bacteriomimetics on primary human peripheral blood mononuclear cells and further demonstrated their ability to reduce scar formation in a mouse *in vivo* model. In our study, we combined the inherent positive effects on wound healing of both hydrogels and our bacteriomimetic microparticles. With our easy-to-prepare hydrogel loaded with bacteriomimetic microparticles, we underline the potential of using bacterial MVs as a therapeutic avenue for wound healing disorders.

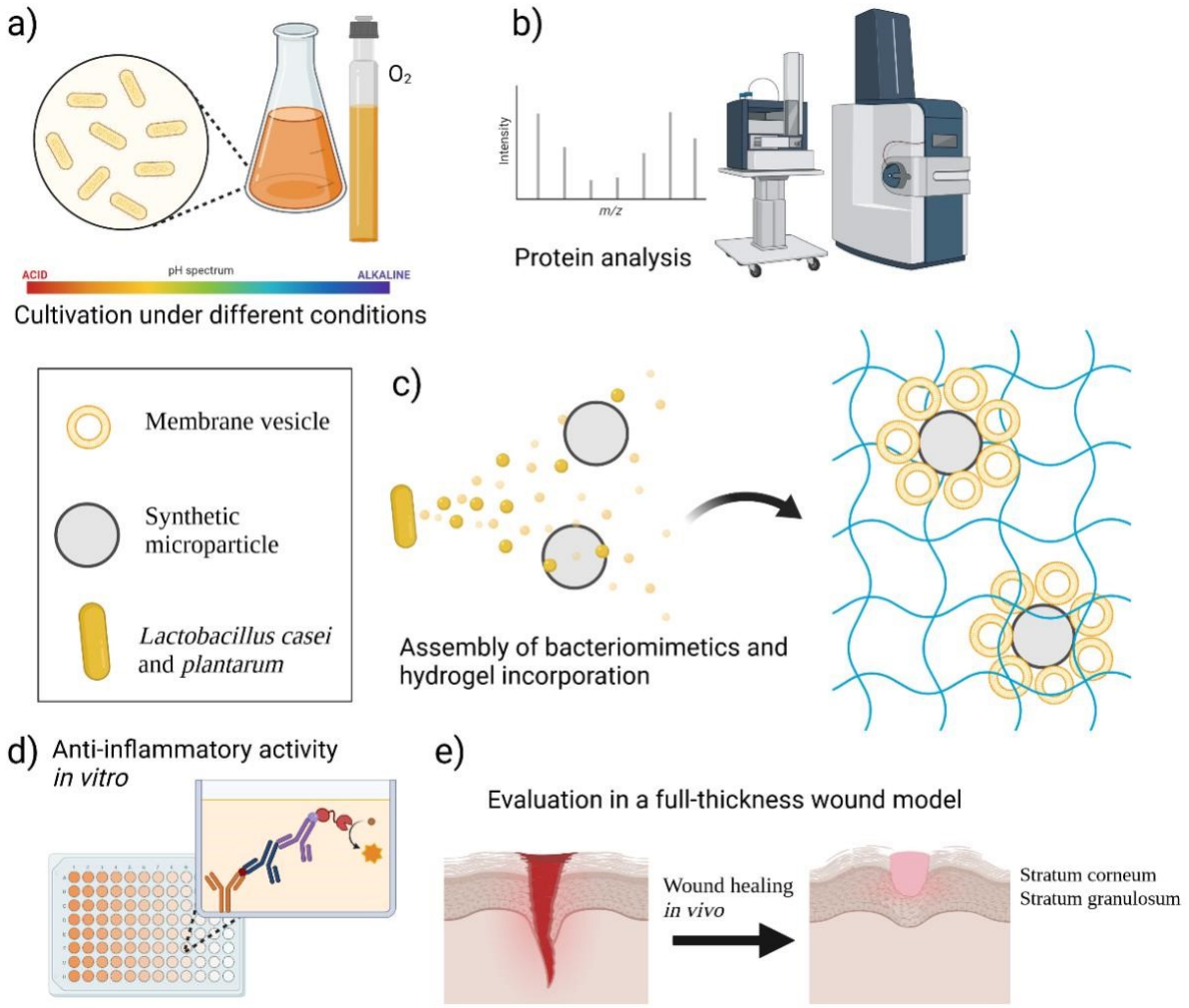


Figure 1. Schematic overview of the present study. (a) Lactobacillus strains *L. plantarum* and *L. casei* were cultivated under various culture conditions, including different media pHvalues and oxygen supply. (b) The cargo proteins within and on the surface of the MVs were characterized using LC-MS. (c) The harvested membrane vesicles were coupled to synthetic microparticles, creating a bacteriomimetic therapeutic system, which could then be formulated

in a skin-applicable hydrogel. (d) The anti-inflammatory activity and their potential to improve wound healing and reduce scarring were studied in different *in vitro* models (determination of their influence on cytokine production via ELISA, as well as cell migration via keratinocyte scratch assays) and (e) in a mouse model. The figure was made using BioRender software. LC-MS, liquid chromatography–mass spectrometry; MV, membrane vesicles.

Results and Discussion

Proteomic characterization of the purified MVs

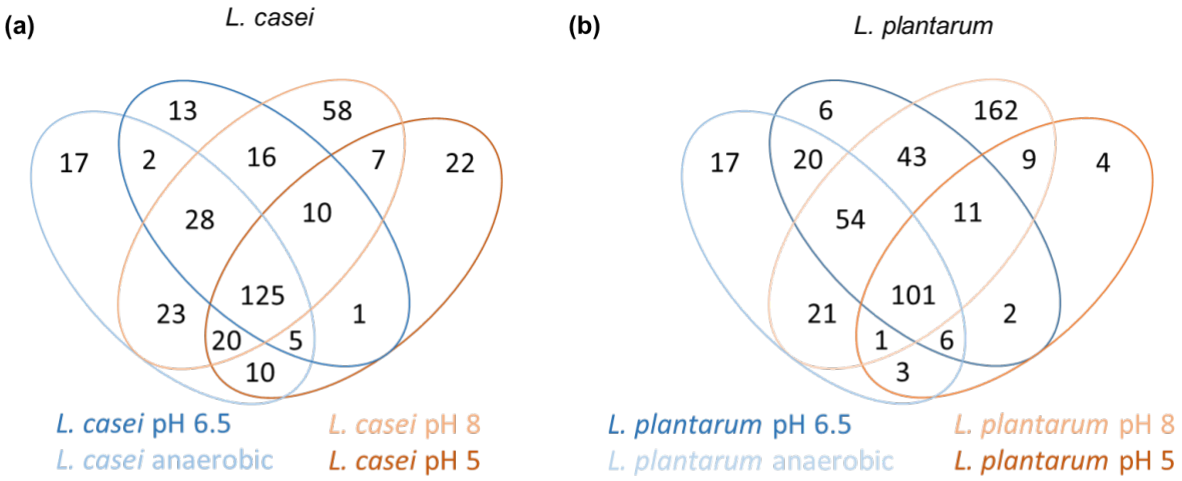
We cultured the *Lactobacilli* in various culture conditions resembling the conditions in the human intestine where the living bacteria's probiotic effects were first proven[26] to further characterize their MVs. Supplementary Fig. S1 shows size profiles and MV yield in comparison to media controls, and purification profiles of MVs by size exclusion chromatography. The morphology of the vesicles was verified by cryoelectron microscopy (Supplementary Fig. S2). To specify the composition and the cargos of the MVs, mass spectrometry analysis was performed. The unique proteins found with the highest coverage for every culture condition are listed in the Supplementary Table S1, and a full list of proteins is found in the Supplementary List (all proteins).

For both strains, a high number of proteins, namely 125 for *L. casei* and 101 for *L. plantarum* were present in all conditions (Fig. 2). For *L. casei,* all samples showed an abundance of p40 and p75 muramidases, which have been described as contributors to the probiotic, effect of the live bacteria before.[27,28] The *L. casei* pH 5 cultures showed an abundance of universal stress proteins, indicating a preference for higher pH-values, which was also highlighted for MVs harvested from this culture condition. [29] *L. casei* pH 6.5 cultures presented the identification of thioredoxin, a protein associated with response to oxidative stress, which was neither abundant in the anaerobic culture nor the pH 5 and pH 8 cultures.[30] In contrast, in the pH 8 cultures of *L. casei* we identified various enzymes involved in nucleotide metabolism, such as S-adenosylmethionine:tRNA ribosyltransferase-isomerase and Proline-tRNA ligase. Interestingly, these proteins are typically present in the cytoplasm. These findings were in contrast to the proteins identified at pH 5, pH 6.5, and anaerobic cultures, where more proteins linked to the cell wall and the bacterial membrane were detected.

In *L. plantarum,* common cell surface proteins were found in all culture conditions included hydrolases such as autolysin Acm2, and metabolic enzymes such as Glyceraldehyde-3phosphate dehydrogenase.[31] Overall, the MV proteins from *L. plantarum* differed strongly in relation to the culture condition applied. In the pH 5, pH 6.5, and anaerobic cultures, mostly extracellular and cell-wall related proteins occurred, such as hydrolases and ion transporters. Interestingly, pH 8 cultures showed a considerably higher abundance of intracellular, metabolism-related proteins, as well as ribosome components. This higher abundance of

intracellular proteins in both strains could indicate a different and more random process of MV biogenesis at higher pH medium, like bubbling cell death, whereas the high abundance of cellwall degrading enzymes could indicate active MV secretion in the other culture conditions.[15,32] We could also demonstrate in both strains the overlap of the proteins found to be highest for pH 6.5 culture and the anaerobic culture, whereas the number of unique proteins was highest in pH 8 cultures. We thus concluded that medium pH has a stronger influence on the protein content of the resulting MVs than the deprivation of oxygen.

These results are in line with previous findings that culture conditions have a strong impact on the physicochemical properties of the MVs produced by the bacteria.[29] Overall, our proteomic analyses showed presence of common proteins, such as the p40 and p75 muramidases, across all MV samples but also high abundance of unique proteins depending on the bacterial culture conditions.



(c)

Protein	Function
p40 cell wall hydrolase	peptidoglycan hydrolase, mucosa-binding[36]
p75 cell wall hydrolase	peptidoglycan hydrolase, cell separation[37]

(d)

Protein	Function
p60 muropeptidase	control of cell length and cell division[38]
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	extracellular matrix binding[39]

Figure 2. Proteomic characterization of the Lactobacillus MVs harvested under different culture conditions. (a) *L. casei* and (b) *L. plantarum*. Venn plots show the overlap of the detected proteins and the number of unique proteins for every culture condition. For both strains, a high number of unique proteins could be found for the pH 8 cultures. (c) Common proteins include p40 and p75 cell wall hydrolases and peptidases for *L. casei* and p60 muropeptidase for *L. plantarum. L. plantarum* samples showed mainly extracellular proteins and proteins involved in cell wall remodelling. Each condition was measured in biological triplicates and technical replicates.

Assembly and characterization of the bacteriomimetics

As mentioned above, the therapeutic use of living bacteria is limited, especially in immunocompromised patients. Therefore, we followed a bacteriomimetic approach to

investigate the functional impact of the different protein compositions of MVs. To obtain bacteriomimetics, MVs were purified by ultracentrifugation and size-exclusion chromatography, and coupled to the surface of the commercially available beads with reactive aldehyde groups on their surface creating bacteriomimetic microparticles, BP.[23] We used beads with a size of 3.7 µm, which is within the typical size range of bacteria mimicking direct bacterial interaction. MVs were functionalized to the microparticle surfaces in a simple one-pot reaction, utilizing the reaction between aldehyde groups on the microparticles and lysine residues on proteins abundant on the MV surface.[23]

Subsequently, aimed to assess the amount of MVs bound to the microparticle surface. We measured the concentration of proteins in the purified MVs and in the supernatant after the coupling reaction, and quantified thereby the microparticle coverage using equation (1) from the method section (Fig. 3a). Interestingly, the lowest surface coverage of 3 and 4% was seen for the MVs harvested from *L. casei* under the standard culture conditions in medium with pH 6.5 and anaerobic conditions, respectively. However, MVs harvested from pH 5 and pH 8 cultures showed a higher particle coverage of 34% and 18%, respectively. While no differences were observed at pH 5 (L. plantarum 38%), a tendency towards higher coverage was observed for *L. plantarum* than for *L. casei.* The lowest coverage for *L. plantarum* MVs was observed for anaerobic cultures (14%).

These variations can be partially explained by the differences in protein concentration per single MV (Fig. 3b). Here, we observed that *L. plantarum* pH 5 and pH 6.5 cultures had the highest protein content per particle. These were also the conditions that yielded the highest particle coverage. A similar trend was visible for the *L. casei* pH 5 culture MVs, which yielded both the highest protein amount per particle as well as the highest particle coverage out of all *L. casei* samples. Interestingly, *L. casei* pH 8 did not follow the same trend. MVs harvested from this culture condition showed a high degree of coupling to the microparticles while also containing the lowest amount of protein per MV. In this case, the fraction of membrane-bound proteins as well as the number of free lysine residues may contribute to overall coupling efficiency because similar studies showed the extent of surface modification of model liposomes was proportional to the amount of reactive groups on the surface.[33]

The obtained BP were additionally characterized regarding the MVs morphology by scanning electron microscopy and depending on the culture conditions used for the production of the MVs. MVs harvested from anaerobic cultures and the pH 6.5 cultures of both strains were visible as single MVs on the microparticle surface. MVs obtained under pH 5 and pH 8 culture conditions resulted in a uniform coverage of the surface where individual MVs cannot be distinguished (Fig. 3c-d). Having established the microparticle coverage as a reliable inprocess control in the manufacturing of the BPs, we subsequently were interested in the biological activity as function of the different protein concentration of the bacteriomimetics.

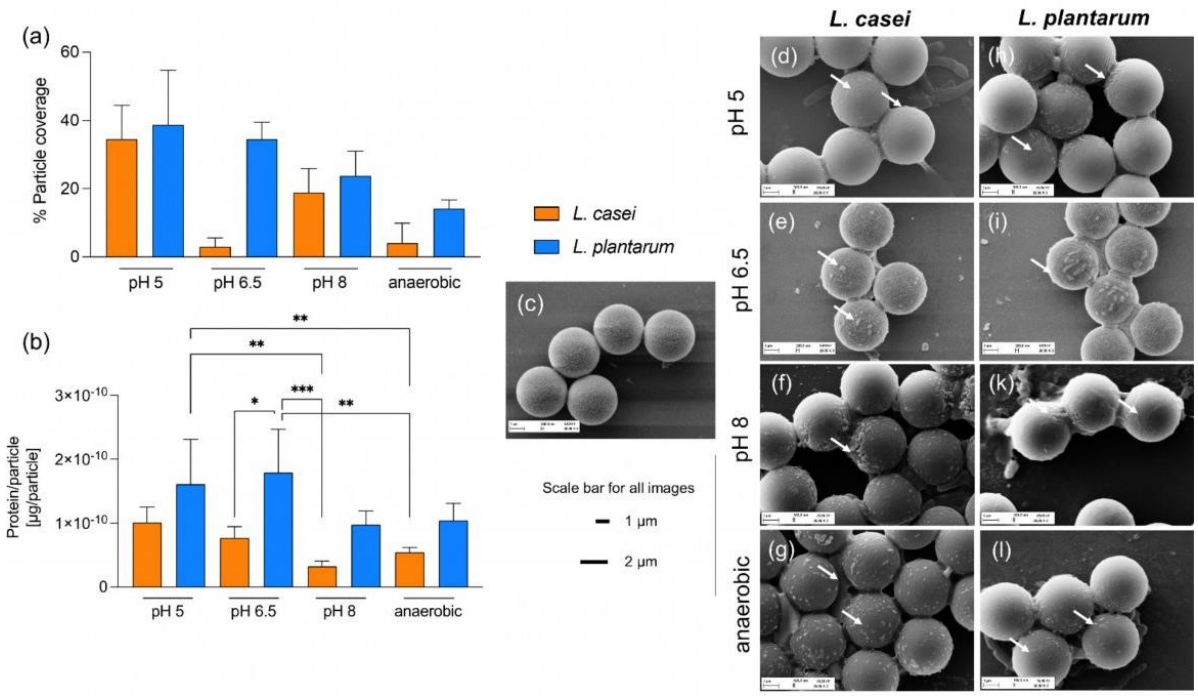


Figure 3. Characterization of the bacteriomimetic hydrogels. (a) Particle coverage calculated through protein quantification of biomimetics and (b) average protein content per MV for the different culture conditions were determined by bicinchoninic acid assay. In b, the protein content of bulk MVs was normalized to the particle concentration determined by nanoparticle tracking analysis. (c) Untreated beads without MVs and beads coated with MVs (BP) harvested under the different culture conditions and from (d-g) *L. casei* and (h-l) *L. plantarum* as indicated were subjected to scanning electron microscopy. Quantitative data are shown as means + SD calculated from three independent experiments. Statistical differences were analyzed by one-way ANOVA and Tukey's post-hoc test. *p<0.05, **p<0.01, *p<0.001. In addition for data in b), a two-way ANOVA analysis was performed (Supplementary Fig. S3).

In vitro biocompatibility of MVs and bacteriomimetic microparticles (BP)

First, we tested whether *Lactobacillus* MVs are tolerated by different cell lines to exclude possible cytotoxic effects during wound healing. We used keratinocyte-like HaCaT cells and monocyte-like THP-1 cells as model for skin and immune cells. The THP-1 cells were differentiated into a macrophage-like phenotype using phorbol-12-myristate-13-acetate. As shown in Supplementary Figure S4, the MVs influenced cell viability only at very high concentrations and for few conditions with high standard deviation. Cytotoxicity in HaCat cells was always below 15% and absent in THP-1 cells, making MVs suitable for further experiments on their biological effects.

During wound healing, cell migration - the process of cells actively moving into the wound site - is physiologically relevant. This process is characterized by a sequence of cellular extension, attachment, contraction and detachment, and is prone to inhibition by the secretome of various bacteria.[34,35] In some models, bacterial treatment was shown to improve the capability of cells to migrate into wounded sites.[36,37] However, for biocompatibility at least no negative effect on

migration is essential to avoid inhibition of wound closure. Therefore, we examined the effects of our *Lactobacillus* MVs and the BPs on keratinocyte cell migration using a scratch assay as *in vitro* wound healing model as straightforward technique to study cell migration in real-time. The scratch closure might not only occur upon migration, but also through proliferation of nonmigrating cells. However, this was ruled out by mitomycin pretreatment. Extracellular vesicles from mesenchymal stem cells served as a positive control [38,39]. We observed only minor changes compared to an untreated control; all changes in wound closure were found to differ by less than 10%. Overall, no significant effect on *in vitro* wound closure was observed. (Fig. 3a,b), which in our hands rules out any overshooting cell migration or strong inhibition of cell migration that could lead to insufficient wound closure in an *in vivo* setting. Interestingly, mesenchymal stem cell vesicles did not induce a better wound healing in our hands, although they are general considered to be anti-inflammatory. However, the anti-inflammatory action may not directly occur on the level of cell migration, but involve cytokine release and the interplay between different cell types.

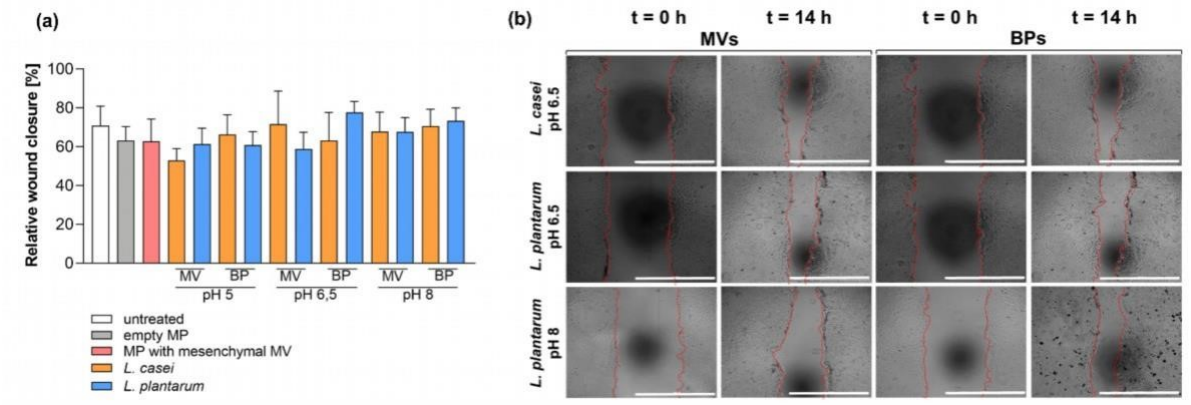


Figure 4. Scratch assay results obtained from keratinocyte cell line HaCaT. (a) HaCaT cells were grown to confluence, incubated with mitomycin (2 µg/ml) for 2 h to exclude cell proliferation, and subjected to automated scratch for high reproducibility. Subsequently, cells were incubated with MVs, BPs, the empty microparticles (negative control), or extracellular vesicles from mesenchymal stem cells (positive control). Images were automatically taken every 2 h, and wound percentage of wound closure was determined after 14 h in comparison to an untreated control. (b) Representative images of the scratch after t=0 h and t=14 h for selected conditions are shown. The red lines indicate the borderline between cell edges and the cell-free area; scale bars indicate 1000 µm. Quantitative data are shown as means + SD calculated from six independent experiments.

Immunomodulatory effects of Lactobacillus MVs and BPs are dependent on the bacterial culture conditions

Wound healing is orchestrated by the release of inflammatory mediators. Next, we examined the effect of the MVs and the BP on primary human immune cells by measuring their effect on the cytokine production of peripheral blood mononuclear cells (PBMCs). Reduced cytokine release could be also linked to reduced cell survival. However, microscopic control of cell

morphology, attachment and density revealed no influence of MV or BP treatment on these parameters excluding artifacts of reduced viability in the cytokine quantification (Supplementary Fig. S5). Furthermore, we previously showed that unmodified microparticles did not influence cytokine release.23 Tumor necrosis factor (TNF) and interleukin-10 (IL-10) were chosen as readout parameters based on their pro-inflammatory and regulatory action, respectively, in wound healing, and LPS served as pro-inflammatory stimulus and comparator to assess relative changes.

The LPS-induced release of TNF was inhibited by treatment with MVs derived from anaerobic cultures of both *L. casei* and *L. plantarum*, with a slight reduction by the equivalent BPs (Fig. 4a). The release of IL-10 showed a tendency of reduction. In general, we observed a higher standard deviation than observed in other experiments. However, this was expected as the primary PBMCs show a high donor variability with respect to both stimulation by LPS, and the release of cytokines. We subsequently calculated the ratio of IL-10 and TNF because it has been reported to be an important predictor for wound healing. A higher IL-10 to TNF ratio was shown to correlate with healing outcomes in various conditions such as coronary artery disease, infections, and wound healing before. Tsurimi *et al.* demonstrated that an increase in this marker leads to a better healing outcome in burn wounds of adult patients.[40–43] Here, we detected elevated IL-10/TNF ratios for the anaerobic MVs from both strains, microparticles from the pH 6.5 culture, and the anaerobic *L. plantarum* conditions (Fig. 5e).The immune modulatory effects of *Lactobacillus* MVs have been demonstrated in the past.[44,45] To take the donor variability into account we calculated the IL-10/TNF ratio as measure of the antiinflammatory action. We observed the strongest anti-inflammatory effect for the MVs from anaerobic cultures of both *Lactobacillus* strains. Interestingly, a similar trend was observed for *L. plantarum* pH 6.5 BPs and anaerobic BPs. Combining these observations with the findings made in the proteomics characterization, we conclude that the changes observed in the proteins included in the MVs have a considerable impact on the biological effect of the MVs. We hypothesize that the 22 proteins found for *L. plantarum* both in the pH 6.5 and the anaerobic culture MVs are contributors to the biomimetics' anti-inflammatory effect. For *L. casei*, a similar pattern was observed, and the main contributors to the anti-inflammatory effect appear to be most expressed in pH 6.5 and in the anaerobic culture. Interestingly, for the anaerobic culture from both strains, we found that the BPs were less active in suppressing inflammation than the MVs. However, the BPs resembling a larger particle similar in size to bacteria. Thus, this physical stimulation *per se* could account for this weaker suppression, and requires further investigations.

In conclusion, culture of *L. plantarum* at pH 6.5 resulted in the highest concentration of MVs, which were already proven to improve healing in an intestinal cell line model after inflammation induced barrier damage.[23] Further, these MVs showed the highest loading degree in BPs and

the highest anti-inflammatory potential. Thus, MVs and BPs derived from *L. plantarum* pH 6.5 seemed to be the most promising candidates for initial *in vivo* experiments.

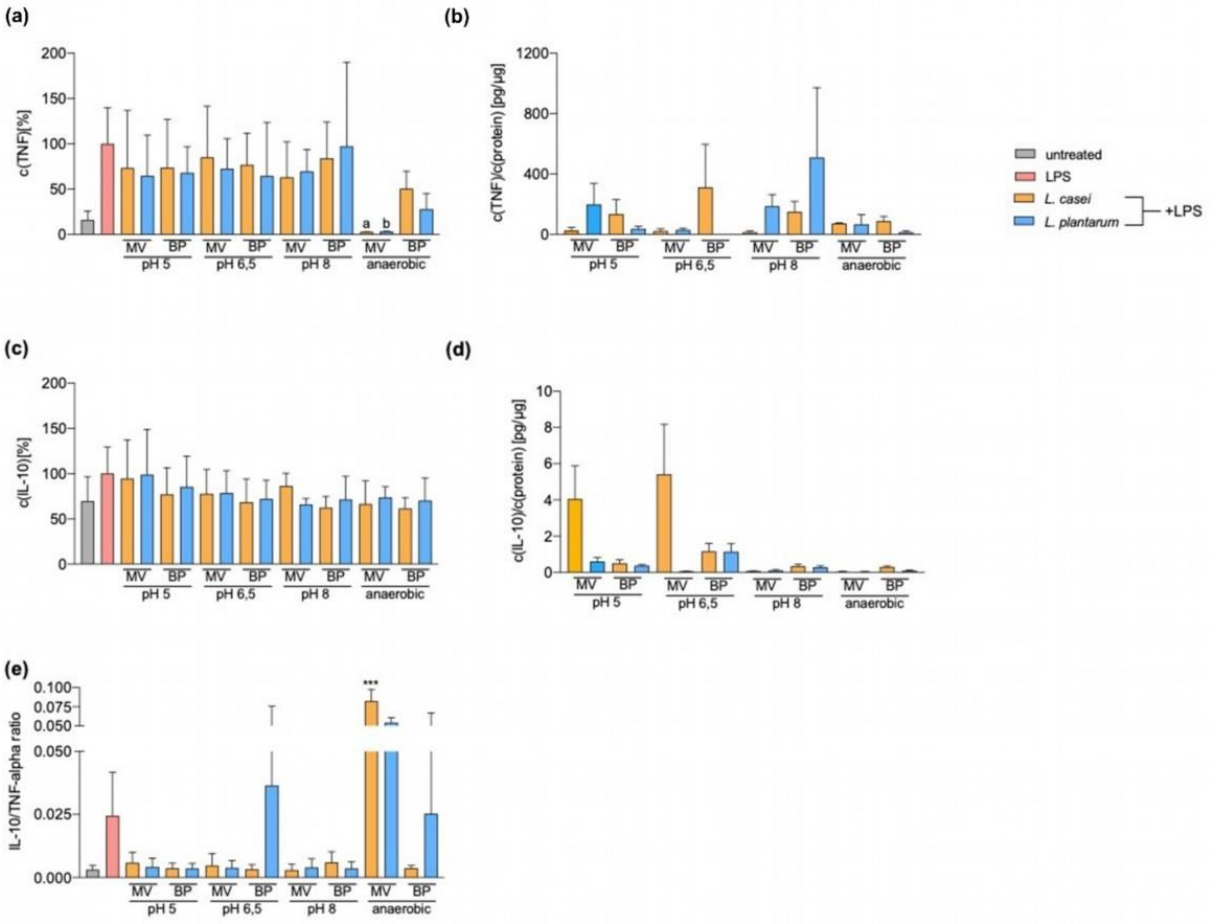


Figure 5. Influence of the MVs on peripheral blood mononuclear cells. PBMCs were incubated with 1 µg/ml of lipopolysaccharide (LPS) and the MVs or BPs, respectively, for 4 h. The cytokine release of TNF and interleukin-10 to the supernatant were determined using ELISA. (a) TNF, data shown as a percentage, normalized to the inflamed control (average of LPS) to allow better comparability of effects induced by BPs. (b) IL-10, data shown as a percentage, normalized to the inflamed control (average LPS), and (c) IL-10/TNF ratio. Quantitative data are shown as means + SD calculated from three independent experiments. Statistical differences were analyzed by ANOVA followed by Dunnett's post-hoc test. ***p<0.001, a: p=0.0516, b: p=0.0534.

In vivo testing of MVs and BPs in hydrogel formulations

Essential prerequisites for the application of MVs and BPs on wounds are efficient delivery and deposition. Therefore, MVs and BPs were formulated into a hydrogel using a readily available pharmacopoeia hydroxyethyl cellulose (HEC) gel of 1% and 2.5% concentration. The same hydrogel mixed with phosphate-buffered saline was used as a negative control (hydrogel). Prior to animal experimentation, the release of fluorescently stained MVs from the gel was verified using fluorescence-assisted nanoparticle tracking analysis. A fast release of the MVs could be detected within 12 hours and from both formulations (Supplementary Fig.

S6), which confirmed that the hydrogel did not hamper the particle release and that no additional fixation in the wound bed would be required to allow efficient delivery

We finally tested our formulations in a full-thickness mouse-tail *in vivo* wound model to get first indications regarding the applicability and safety of the MVs and BPs. Over the course of 30 days, changes of the wound and the tail were monitored, followed by detailed histological assessment. This model allows for sequential measurements and better clinical monitoring of the wound because only short hair but not fur is growing on the animal's tail.[46] Most importantly, the wound closure occurs without shrinkage from the wound edges and within a period comparable to human settings.[47]

In our model, tail width and wound width served as surrogates to assess the inflammation, which occurs during the wound healing process, modulated by edema and exudate formation. The tail width increased by 1 mm during the first week for the empty hydrogel treated animals while the increase was lower with an earlier recovery in MV and BP hydrogel treated animals, respectively (Supplementary Fig S7a). The wound width increased by more than 1 mm after 1 week for the empty hydrogel treated animals as well as the group treated with MV loaded hydrogel. The BP hydrogel led to a quick reduction in the wound width, which was significantly different already at day 5 post wounding compared to the other groups (Fig. 6a,b). While for the BP loaded hydrogel the initial width was restored already 12 days after wounding, it took between day 23 and 24 for empty hydrogel and the MV-loaded hydrogel. The length and width of the inner wound area served as macroscopic measurements of re-epithelialization. Hydrogel treated wounds showed the fastest reduction in wound length (Supplementary Figure S7b). However, the allover wound closure time is not affected (Supplementary Fig S7c). Especially, MV hydrogel treated animals showed a significantly reduced reduction in wound length within the first two weeks after wounding (Supplementary Figure S7b). Interestingly, the inner wound width was faster reduced in BP hydrogel treated animals compared to hydrogel or MV treated animals (Fig. 6c, significance between MV and BP hydrogel treatment). Thus, BP loaded hydrogels seem to dampen the inflammatory response during the wound healing process, supporting faster re-epithelialization.

To gain further evidence, we performed detailed (immune)histological analyses. Restoration of the damaged tissue is one criterion for advances in wound care. None of the treatments reduced the increase of the epidermis thickness (Fig. 6d, Supplementary Fig. S7d). The structured epidermis is characterized by a basal cell layer (*Stratum basale*), which are the source for the keratinocytes forming the *Stratum spinosum* and Stratum *granulosum*. BPloaded hydrogel reduced the percentage of non-structured epidermis within the wound site significantly in comparison to hydrogel treated wounds, while MV-loaded hydrogel showed a high variability (Fig. 6e).[48] An increased dermal thickness correlates to a higher infiltration with fibrocytes and was shown to indicate a higher risk of scarring.[49] For the mice treated with BP

loaded hydrogel, a significant decrease of dermis thickness compared to treatment with hydrogel and the MV loaded hydrogel, respectively, was observed (Fig. 6f). Additional measures of scare formation are the cell number (indication of hypertrophy) and the collagen deposition. Therefore, cells within the wounded sites (hematoxylin-eosin staining) and the collagen deposition (Ladewig trichrome staining) were automatically counted. All three treatments showed an increase of cellularity, but without significant differences amongst the treatment groups (Fig. 6g). Further, we determined the total wound area and calculated the overall cell number, again without obvious differences (Supplementary Fig. S7e). Similarly, no differences in collagen deposition to the dermal wound area were observed (Supplementary Fig. 7f,g). Nevertheless, dermal thickness has been described as a risk factor for hypertrophic scarring.[50] The healthy tail dermis is characterized by dense and organized collagen fibers. MV and BP hydrogel, respectively, treated wounds showed a tendency towards an increase of recovered collagen organization at the wound edges in comparison to hydrogel treated wounds, which was not reaching significance (Fig. 6h). However, with respect to the 3R only low animal numbers were used in this initial testing, potentially not allowing for the observation of slight differences. Furthermore, formation of the granulation tissue and re-epithelialization are part of the proliferation phase in wound healing. The maturation phase, including the decision between resolution of the granulation tissue and the scar formation, occurs in a time frame of several month to one year. This phase is not covered by the used basic model and would require further investigation in, for example, chronic or diabetic wound healing models. One additional parameter of wound healing is the induction of re-vascularization. Within hydrogel treated wounds, no revascularization sites were detected in the histological staining. However, both MV and BP loaded hydrogels induced revascularization, with BPs showing a higher efficiency (Fig. 6h).

Our *in vitro* experiments showed a change of the IL10 to TNF ratio upon treatment with MVs and the bacteriomimetic microparticles, which could change the presence of inflammatory cells within the wound area. Based on the late time point, TNF release into the wound are could not be detected (data not shown). Further, we did not observe changes in $CD68^+$ macrophage numbers or $CD3^+$ T lymphocyte infiltration after full wound closure (Supplementary Fig. S7h-j). In general, macrophage density always decreases after wound closure.[51] Thus, it might be that the change in the inflammatory behavior accounts in more early phases of the wound healing process, which may also explain the lack of neutrophil detection (early phase markers). Indeed, treatment with BP loaded hydrogel resulted in an early resolution or less pronounced inflammatory response as indicated by wound width and less exudate during experiments. Neutrophils are beneficial for wound healing preventing wounds from infection, but the damage by the proteolytic activity of protesases can lead to delayed healing and scar formation.[55] Wound treated with MV loaded hydrogel showed a slight increase in myeloperoxidase (MPO)

as neutrophil marker in comparison to hydrogel treated wounds, whereas the number of neutrophils was reduced by BP hydrogel treatment (Fig. 6j, p = 0.0361). These findings are in line with the delayed re-epithelialization in MV hydrogel versus BP hydrogel treated wounds (Fig. 6c).

Taking all evidences from this initial *in vi*vo-testing together, the application of bacteriomimetic hydrogel seems to be save and offers a therapeutic potential to care delayed wound healing. This initial study stimulates for future investigations in more complex wound healing models like diabetic ulcera or infected wounds.

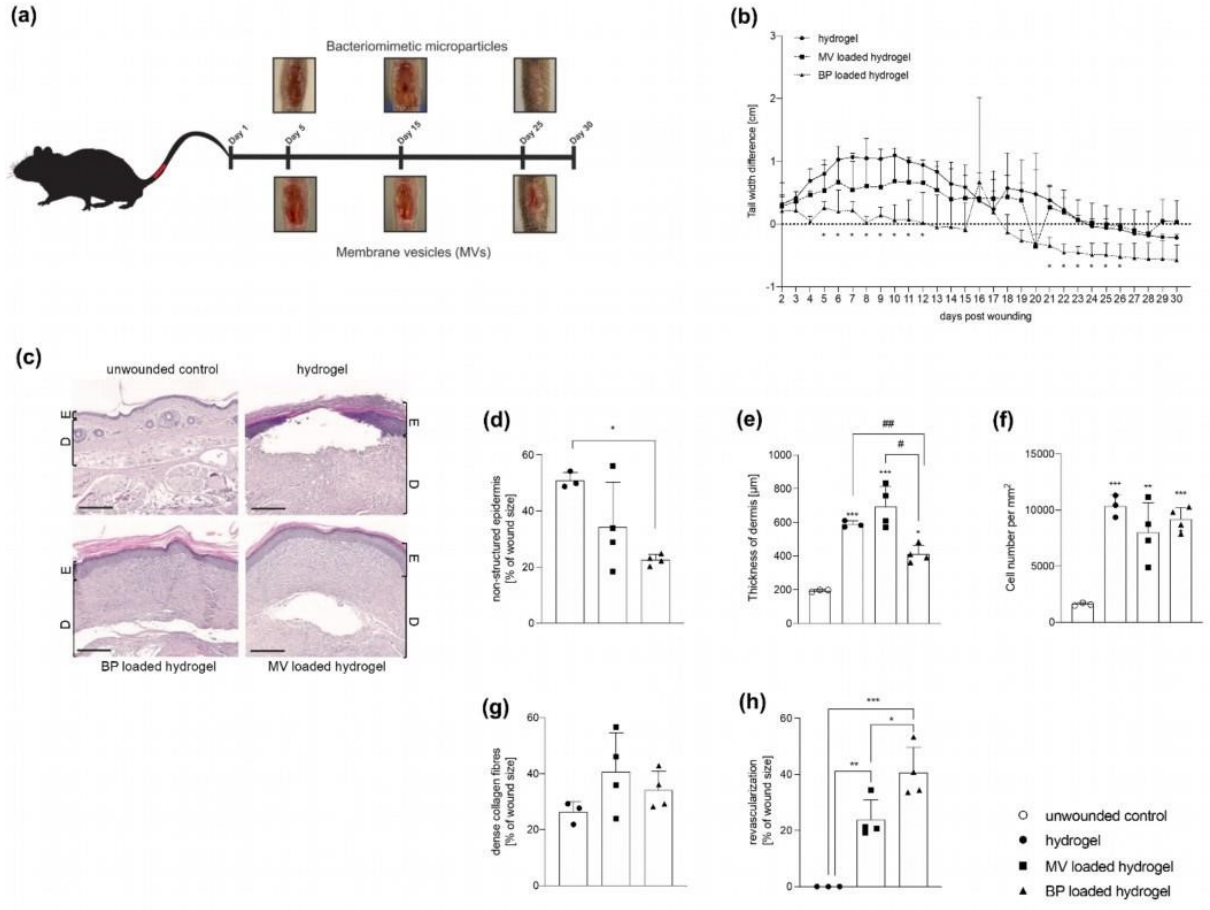


Figure 6. Full-thickness wound healing mouse model. Full-thickness wounds of 10 x 3 mm size were set in the tails and covered with either hydrogel, MV loaded hydrogel or BP loaded hydrogel (MVs obtained from *L. plantarum* cultured at pH 6.5). Wound size and tail width were measured each day. (a) Representative pictures of the wounds at different time points after wounding. (b) Differences in total wound width. (c) Re-epithelialized wound area. (d-j) Hematoxylin-eosin (exemplary images shown in d, scale bare indicate 200 µm), Ladewig trichrome and immunohistochemical stainings were analyzed for epidermal structure (e), thickness of dermis (f), cell density (g), collagen structure (h), neovascularization (i) and neutrophil number (j). Quantitative data are shown as means + SD calculated (n=3-4 in a-j, n=2-3 in j). Statistical differences were analyzed by One-way ANOVA followed by Tukey's post test. Asterisks indicate differences to the unwounded control, hashes between treatment groups. $^{*,\#}p<0.05$, $^{**,\#\#}p<0.01$, $^{***}p<0.001$.

Conclusion

In this study, we explored a bacteriomimetic hydrogel as therapeutic system in the context of wound healing. We harvested membrane vesicles from two *Lactobacillus* strains – *L. casei* and *L. plantarum* – and showed their cell tolerability and their anti-inflammatory effects. We subsequently designed a novel non-proliferating bacteriomimetic therapeutic system and revealed its promising anti-inflammatory effects *in vitro*, and its capability to improve wound healing and decrease scar formation in an *in vivo* full thickness mouse model.

In recent years, various reports have emerged, highlighting the therapeutic effects of probiotic bacteria in wound healing, *e.g.*, hydrogels with live-bacteria.[24] *Lactobacillus reuteri* bacteria were further used as drug delivery vectors in a similar model.[52] Comparable approaches were explored in recent years, using heat-killed or lysed bacteria.[19,53] Within our present study, we showed that wound healing can also be increased using the bacteriomimetic microparticles, reducing the risk of live bacteria treatment or reaction to other bacterial components and offering novel treatment options for non-healing wounds and immunocompromised patients. Nevertheless, the effect could be improved by packing the vesicles more densely on the microparticle surface. In addition, dose-response studies may reveal enhanced anti-inflammatory activity. Further studies should also include bacteriomimetic hydrogel testing in the context of other cutaneous diseases involving inflammation such as autoimmune diseases of the skin, as well as further developing the system using biodegradable polymers as carrier materials.

Experimental Section

Cell Culture

HaCaT cells were cultured in Dulbecco's modified eagle medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, USA). Medium was exchanged every 2-3 days. Cells were passaged to a new flask every 7 days, when 80-90% confluence was reached. THP-1 cells were cultured in a suspension culture in RPMI (Gibco, USA) medium supplemented with 10% FCS (Gibco, USA). Every 3-4 days, 10^6 cells were passaged to a new T75 flask and supplemented with 10ml of fresh medium.

Bacterial Culture

Lactobacillus casei DSM20011 (DSMZ, Germany) were cultured in deMan-Rogosa-Sharpe (MRS) medium (Carl Roth). Initially, bacteria were cultured on an MRS agar plate for 4 days at a temperature of 37°C under a 5% CO_2 atmosphere. This plate was then stored at 4°C for up

to 4 weeks. For liquid cultures, 2 single colonies were used to inoculate 100 ml of liquid MRS medium. Liquid cultures were then placed in an incubator without shaking at a temperature of 37°C and allowed to grow for 48 h.

Lactobacillus plantarum NCIBM 8826 (NCIBM, UK) were cultured in deMan-Rogosa-Sharpe (MRS) medium (Carl Roth, Germany). Initially, bacteria were cultured on an MRS agar plate for 2 days at a temperature of 30°C. This plate was then stored at 4°C for up to 4 weeks. For liquid cultures, 2 single colonies were used to inoculate 100ml of liquid MRS medium. Liquid cultures were then placed in an incubator shaking at a temperature of 30°C and allowed to grow for 48 h.

For the different pH-conditions medium pH was adjusted to pH 5 or pH 8 using HCl and NaOH. Regular medium had a pH value of 6.5. For anaerobic culture, the medium was additionally supplemented with 0.05% sodium thioglycolate, and the incubations were performed in airtight bottles completely filled with medium.

EV isolation

After 48h of growth, bacteria were separated from the supernatant using centrifugation at 9500 x *g* for 5min. The bacterial pellet was discarded, and supernatants were further purified using 0.45µm pore size vacuum filtration using Stericup-HV 150 mL Durapore PVDF 0.45 filter bottles, (Merck, Germany). Then, the filtered supernatants were centrifuged for 2h at 4°C at a centrifugal force of 100000 x *g* using an ultracentrifuge with a Type45 Ti fixed angle rotor. (Beckmann, USA). After ultracentrifugation, supernatants were discarded and the pellets, containing EVs and co-pelleted proteins were resuspended in 400 µl of filtered phosphatebuffered saline.

Resuspended pellets were further purified from co-pelleted proteins using size-exclusion chromatography. Pellets were transferred to a chromatography column containing 40 mL of Sepharose CL-2B (Gibco, USA) and eluted with filtered phosphate-buffered saline. Fractions of each 1ml were collected.

Cryoelectron microscopy

3 µL of MV sample was transferred to a copper grid and blotted for 2 s. The grid was then plunged into undercooled liquid ethane (–165°C; Gatan Cryoplunge3) and transferred under liquid nitrogen to a cryo-TEM sample holder (Gatan model 914). Low-dose bright-field images were acquired at –170 °C on a JEOL JEM-2100 LaB6 Transmission Electron Microscope equipped with a Gatan Orius SC1000 CCD camera.

Proteomics

Lysis of the MVs

To determine the proteins contained inside the MVs, MVs were diluted to $1x10^{11}$ particles/ml for *L. plantarum* and $1x10^{12}$ particles/ml for *L. casei*, and lysed using a buffer containing 1% Triton-X 100 (SigmaAldrich, USA) with 50 mM Tris-HCl (Serva, Germany) and 150 mM NaCl for 4h cooled to 0°C with intermediate vortexing every hour.

Filter assisted protein digestion

Lysed samples were transferred onto a filter tube with a MWCO of 10 kDa. Excess liquid was removed centrifuging at 8000 x g at 4°C. Proteins were then reduced using 450µl of a 1M solution of Dithiothreitol in 0.1 M ammonium hydrogen carbonate for 45 min at 56°C. After the liquid was removed via centrifugation, 450 µl of alkylation buffer (0.5 M iodoacetamide in water) was added and the mixture was incubated for 30 min at RT. The liquid was removed again, and, after two washing steps with 50 mM ammonium hydrogen carbonate. Next, 450 µl of digestion buffer (49 ng/ml of Trypsin in 50 mM of ammonium hydrogen carbonate) were added and the mixture was incubated overnight at 37°C. On the next day, the digested peptides were centrifuged into an Eppendorf tube, and the filter was washed twice with 300 µl of 5% formic acid. Next, the liquid was removed using a vacuum centrifuge at 60°C for 2 h. The dried peptides were dissolved in 10% formic acid and stored at -20°C for LC-MS analysis.

LC-MS/MS analysis

The tryptic digests are analyzed on a Dionex UltiMate 3000 rapid separation liquid chromatography (RSLC) system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Bruker timsTOF, a high-resolution hybrid trapped ion-mobility spectrometry-quadrupole timeof-flight mass spectrometer equipped with a high-resolution electrospray ionization (HRESI) source (Bruker Daltonics, Billerica, MA, USA). Separation of 10 µL sample was achieved with a gradient of acetonitrile with 0.1% formic acid (B) in ddH2O with 0.1% formic acid (A) on an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 µm dp) (Waters, Eschborn, Germany) equipped with a Waters VanGuard BEH C18 1.7 µm guard column at a flow rate of 0.6 ml/min and 45 °C. The initial gradient was held at 5% B for 1 min and then elevated to 45% B within 20 min. After that, the B level was elevated to 95% within 3 min and held there for 5 minutes. Finally, the gradient was ramped back to 5% B in 1 min and re-equilibrated for the next injection for 1 min. Detection is performed by a diode array detector at 200–600 nm. The LC flow is split into 75 µl/min before entering the mass spectrometer, which was externally calibrated to a mass accuracy < 1 ppm and a collisional cross section (CCS) accuracy < 0.5%. Mass spectrograms were acquired in parallel accumulation and serial fragmentation (PASEF) mode ranging from 100–1700 m/z and 0.6–1.6 V*s/cm2 1/k0 in positive MS mode. Source parameters are set to 500 V end-plate offset, 4000 V capillary voltage, 1.5 bar nebulizer gas pressure, 6 L/min dry gas flow and 200 °C dry gas temperature. Ion transfer and quadrupole parameters were set to 350 VPP funnel 2 RF, 400 VPP multipole RF, 5 eV ion energy and 100 m/z low-mass cut-off. TIMS settings are 350 VPP funnel 1 RF and 250 V collision cell in.

Collision cell was set to 80 µs transfer time, 1200 VPP collision RF and pre-pulse storage was set to 12 µs. PASEF parameters were set to 10 MS/MS scans (total cycle time: 1.89 s), charge range 0–5, active exclusion for 0.4 min, scheduling target intensity was set to 10,000, intensity threshold was set to 1000 and CID collision energy is 20-59 eV, depending on precursor mass and charge. The HPLC-MS system was operated by HyStar 6.0.30.0 (Bruker Daltonics, Billerica, MA, USA), and LC chromatograms, as well as UV spectra and mass spectrograms were analyzed with DataAnalysis 5.3 (Bruker Daltonics, Billerica, MA, USA).

Data Analysis

The acquired raw data were submitted to PEAKS X Pro, PEAKS Studio 10.6 (Bioinformatics Solutions, Waterloo, ON, Canada) to search against FASTA protein databases constructed for *Lactobacillus casei* DSM 20011 (UniProt Proteome ID UP000015560) and *Lactobacillus plantarum* NCIMB 8826 (UP000000432). For de-novo search, the mass error tolerance was set to 10 ppm and the fragment mass error tolerance to 0.1 Da. Trypsin was defined as digestion enzyme with three missed cleavages allowed. Variable modifications are carboamidomethylation of cysteine and oxidation of methionine, histidine and tryptophane allowing for 10 variable modifications per peptide. PTM search was conducted with the default 312 built-in modifications allowing for 3 variable modifications per peptide. Maximum false discovery rates (FDRs) were set to 5%. Protein identifications were considered true when at least one unique peptide was identified per protein with a confidence score of –10lgP ≥ 20. For protein identification, cultivation of Lactobacilli, EV isolation and sample preparation was performed in biological triplicates. Each replicate was analyzed by LC–MS/MS in technical duplicates, resulting in a total of six measurements per condition.

Coupling of MVs to microparticles

300 µl of MVs as harvested were mixed with 700 µl of phosphate-buffered saline and 10 µl of pre-washed Aldehyde/sulfate latex beads (ThermoFisher, USA). The mixture was incubated for 16 h while shaking at 300 rpm. Afterward, the coated microparticles were pelleted by centrifugation of 5 min at 5000 x *g* and washed using 1 mL of phosphate-buffered saline. This procedure was then repeated twice. Then, the coated particles were suspended in 1 mL of phosphate-buffered saline. For electron microscopy, the coated microparticles were additionally washed three times with deionized water.

Determination of Protein Content

To determine the protein content, the QuantiPro BCA assay kit (Merck, Germany) was used according to the manufacturer's recommendations. In brief, 150 µL of each SEC fraction was pipetted into a 96-well plate and mixed well with 150µL of QuantiPro BCA assay working reagent (Sigma Aldrich, USA). Bovine serum albumin standard was used for calibration. After

incubation for 1h at 60°C, the absorbance at 562 nm was measured using a plate reader (Tecan, USA). The amount of MVs on the microparticles was determined using the following equation:

Equation (1)

$$c(protein\ on\ MPs) = c(protein\ in\ SEC\ fraction) - c(protein\ in\ supernatant)$$

*MVs on microparticles % = {{[[c(particle/mL of MV in formulation) * c(protein on MP surface)]/ c(protein in MV)]/ c(particle/ mL of MPs in formulation)}/ (surface of single MP m^2/ circular area of a 100 nm MV in m^2)} x 100*

With c(MPs in formulation) = $1.43*10^7$/ml (determined using the formulae on the ThermoFisher website); c(MV average) was averaged to $3.33*10^{10}$/ml based on nanoparticle tracking analysis[23] The instrument used was a NanoSight (Malvern Panalytical, Malvern, UK) under constant settings (camera level: 15, detection threshold: 5) for every measurement. The NTA 3.3 software was used for analysis; surface of single MP m^2/ circular area of a 100 nm MV in m^2 was calculated with aldehyde/sulfate latex beads of 4 µm and 100 nm MVs: calculated factor = 22.89 m^2/ 0.00785 m^2 = 2916

Scanning electron microscopy

A 3 µl volume of each sample was transferred to a silica wafer and allowed to dry at RT for 23 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 - 10 kV and a beam current of 1.978 pA (Zeiss EVO MA15 LaB6).

Viability Assay

Cells were seeded into 96-well plates, two types of cells were used, namely HaCaT (keratinocyte-like) and dTHP-1 (differentiated THP-1, macrophage-like). For the assays using HaCaT cells, approximately 2×10^4 HaCaT cells suspended in 200 µL of DMEM, supplemented with 10% FCS and 1% nonessential amino acid (Invitrogen, Waltham, MA). For the assays using dTHP-1 cells, THP-1 cells were seeded at a density of 100,000 cells per well in RPMI medium supplemented with 10 % FCS and 7.5 ng/ml phorbol 12-myristate 13-acetate (PMA).

After allowing the cells to grow for 48 h, the medium was aspirated, and 100 µL of fresh medium (without FCS, in order to prevent interference of FCS with the LDH assay) was added, followed by the addition of 100 µL of the sample. The controls used were death-control (medium supplemented with 2% TritonX-100) and live-control (PBS).

Cells were incubated with EVs of the highest-concentrated SEC fraction (≈5 × 10^{11} for *L. plantarum* and ≈5 × 10^{12} for *L. casei* 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, the 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, the absorbance of the solution was measured at λ = 492 nm.

Scratch Assay

For live-cell analysis of scratch-induced wound closure, 2 × 10^4 cells per well were seeded on collagen G (40 µg/ml) (Biochrom, Germany) coated 96-well plates near confluence and allowed to grow overnight in standard medium. At confluence, cells were pre-treated for 2 h with mitomycin (5 µg/ml) to inhibit cell proliferation followed by 3 times washing with PBS.

Subsequently, a defined scratch was performed in each well using the certified BioTec autoscratch (BioTec, Highland Park, USA) for 96-well plates ensuring an equal scratch of around 1.2 cm2 in each well, as described previously[54]. The medium was removed and 100 µl standard medium were added to the wells. The closure of the wounded area was monitored using the Lionheart (FX) Automated Microscope system (BioTec, Highland Park, USA) by taking images of each well every 2 h over a period of 24 h. The reduction of wound width was determined over time using the Gen5 software version 3.05.11. For accurate measurement of control cells, wound closure was determined after 14 h. An automated primary mask that quantifies the area in the image containing cells was used to quantify the progression of cell migration.

Isolation of peripheral blood mononuclear cells (PBMCs) from Buffy Coats

We isolated PBMCs from adult healthy blood donors (Blood Donation Center, Saarbrücken, Germany). Human material use and handling was approved by the local Ethics Committees (permission no. 173/18; State Medical Board of Registration, Saarland, Germany). PBMCs were isolated using density gradient centrifugation with Lymphocyte Separation Medium 1077

(Promocell, C-44010) and Leucosep tubes (Greiner Bio-One, 227290) according to the supplier's protocols.

For cytokine analysis, cells were incubated with the respective treatments for 6 h at 37°C; afterwards supernatants were collected and stored until analysis at -80°C. The concentration of the cytokines was determined using Human IL-10 ELISA Set (Diaclone, Besançon, France) and Human TNF-α ELISA Set (Diaclone, Besançon, France), the experimental procedure was carried out according to the supplier's protocols. Raw data was normalized to the cytokine concentration of the inflamed lipopolysaccharide controls.

Preparation of the EV-containing hydrogels

Hydrogels were prepared using a simple method derived from the German pharmacopoeia (DAB). Briefly, hydroxyethyl cellulose (1% or 2.5% respectively) was mixed with glycerol (20%) until an even mixture was achieved. Then, ultrapure water (MilliQ) was added, and the mixture was allowed to swell for 16h at 4°C. The gel was then sterilized in an autoclave for 20 min at 121°C at a pressure of 200 kPa. Afterward, any evaporated water was re-added. The gels were then mixed with the MVs or microparticles ($1x10^{11}$ particles/ml for *L. plantarum* and $1x10^{12}$ particles/ml for *L. casei*) and mixed in a 1:1-ratio with the pre-prepared hydrogel. Phosphatebuffered saline mixed with hydrogel was used as control.

In vivo experiments

Animal experiments were performed with 8-weeks old male mice on C57BL76 background and approved by the local authorities (20/2021 LAV Saarland). Mice were anesthetized for 30 min with 100 mg/kg BW ketamine and 5 mg/lg BW xylazine and a skin flap of 10 x 3 mm approximately 0.5 cm distal to the tail base. Thereby and by holding in single cages, removal of the hydrogel was avoided. A template built from scalpels ensured consistency in length, width and depth of the wounds. Wounds were covered with 50 µl hydrogel containing extracellular vesicles obtained from *Lactobacillus plantarum* (MV loaded hydrogel) or microparticles coated with these vesicles (BP loaded hydrogel). Hydrogel without supplementation served as control treatment. The hydrogel entered the open wound during the wake-up phase avoiding displacement or removal by movement. Licking was avoided by the location of the wound and single holding of the animals. During the first three days, borgal (2 mg/ml) was given in drinking water as prophylactic antibiotic medication. Mice received 0.1 mg/kg buprenorphine *i.p.* twice a day and 1 mg/kg in drinking water overnight for analgesia. Wounds and tail sides were photographed daily and measured for wound length, wound width, and tail width using a sliding caliper. Mice were sacrificed after complete wound closure (28 to 30 days) and probed for tail tissue. The wound was fixed for 48 hours in RotiFix® (Roth, Germany) and embedded in paraffin. Tissue was decalcified, 5 µm sections were subjected to

hematoxylin–eosin (HE) and Ladewig trichrome staining following established protocols, and images were taken using the Axioscan Z1 slide scanner (Zeiss, Germany). Measurements and cell counts in the wound area were performed and analyzed using Zen blue and AxioVision 6.4.1 software (Zeiss).

Immunohistological staining of mouse tissue sections

Sections were deparaffinized, rehydrated, and subjected to antigen retrieval in TRIS/EDTA buffer (pH 9). After blocking in PBS supplemented with 1.5% normal serum, slices were incubated with rabbit anti-mouse CD68 (macrophages), CD3 (lymphocytes) or MPO (neutrophils), washed in PBS, followed by biotin-goat-anti-rabbit and streptavidin-HRP. Antibody binding was visualized by AEC (3-Amino-9-Ethylcarbazole) HRP reaction, and nuclei were counterstained with hemalaun. At least, five quadrants per wound field were counted in a blind manner.

Statistical analyses

Statistics were calculated and graphs were created using the software GraphPad Prism. Plotted values represent the mean value of all performed replicates, error bars indicate the standard deviation. Used statistical tests are indicated in the figure legend. A p-value below 0.05 was regarded as significant.

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Supplementary Information

A cell-free, biomimetic hydrogel based on probiotic membrane vesicles ameliorates wound healing

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Table S1. Detected unique proteins from both Lactobacillus strains with their most important functions as depicted in UniProt.

Function	Protein	Cellular localisation
***L. casei* pH 5**		
metabolism (glycolysis)	L-lactate dehydrogenase	cytoplasm

heat-shock protein	Hsp20/alpha crystallin family protein	cytoplasm
Sugar transfer and metabolism	Phosphocarrier protein HPr	cytoplasm
stress response	Universal stress protein	cytoplasm
nucleotide metabolism	Orotate phosphoribosyltransferase	unknown
amino acid metabolism	DUF1831 domain-containing protein	unknown
***L. casei* pH 6.5**		
cell cycle and cell shape	Cell cycle protein GpsB	cytoplasm
hydrolase	Putative cell wall-associated hydrolase	cell wall
ABC transporter	MetQ/NlpA family ABC transporter substrate-binding protein	membrane (lipid anchor)
stress resistance against oxidative stress	Thioredoxin reductase	cytoplasm
translation	50S ribosomal protein L15	large ribosomal subunit
unknown function	5-bromo-4-chloroindolyl phosphate hydrolysis family protein	membrane
regulation of transcription	Transcription elongation factor GreA	unknown
***L. casei* pH 8**		
nucleotide metabolism	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	cytoplasm
nucleotide metabolism	Proline-tRNA ligase	cytoplasm
metabolism	Iron-containing alcohol dehydrogenase	cytoplasm
anti-viral defense (bacteriophage exclusion)	Probable ATP-binding protein BrxC	unknown
nucleotide metabolism	Inosine-5'-monophosphate dehydrogenase	unknown
unknown function	Phage tail family protein	membrane
peptide transport	ABC transporter ATP-binding protein	membrane
nucleotide metabolism	Alanine--tRNA ligase	cytoplasm

nucleotide metabolism	Ribonuclease J	cytoplasm
cell wall synthesis	Phospho-N-acetylmuramoylpentapeptide-transferase	membrane
***L. casei* anaerobic**		
translation	50S ribosomal protein L17	large ribosomal subunit
translation	50S ribosomal protein L20	large ribosomal subunit
hydrolase activity	Nudix hydrolase domain-containing protein	unknown
cell wall remodelling cell cycle and cell shape	UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	cytoplasm
carbohydrate metabolism	3-hexulose-6-phosphate isomerase	unknown
negative regulation of transcription, DNA-templated	Global transcriptional regulator Spx	cytoplasm
cell wall synthesis	LD-carboxypeptidase	unknown
ribosome metabolism	ABC transporter ATP-binding protein	membrane
cell redox homeostasis	NAD(P)/FAD-dependent oxidoreductase	unknown
***L. plantarum* pH 5**		
ion uptake	High-affinity zinc uptake system membrane protein ZnuB	membrane
cell wall catabolic enzyme	Glycosyl hydrolase family 25	cell wall
ABC transporter	plnH ABC transporter accessory protein	membrane
***L. plantarum* pH 6.5**		
carbon source acquisition	Cell surface protein CscB family	unknown
Ca(2+) transporter	P-type Ca(2+) transporter	membrane
Succinate-semialdehyde dehydrogenase	Succinate-semialdehyde dehydrogenase [NADP(+)] GabD	mitochondrion

cell adhesion / metal ion transport	ABC transporter substrate-binding protein	membrane
antibiotic resistance	Aminoglycoside 3'-phosphotransferase	unknowm

***L. plantarum* pH 8**		
metabolism, CLA production [225]	Transcriptional regulator, LysR family	unknown
metabolism	Glucose-6-phosphate 1-dehydrogenase	unknown
cell redox homoeostasis	FAD-dependent pyridine nucleotidedisulphide oxidoreductase	unknown
glutamine biosynthesis, metabolism	Glutamine synthetase	cytoplasm
glutamine biosynthesis, metabolism	3-oxoacyl-(Acyl-carrier-protein) reductase FabG1	cytoplasm membrane cell wall
metabolism	aminopeptidase c	
translation	50S ribosomal protein	large ribosomal subunit
protein biosynthesis	Methionine-tRNA ligase	cytoplasm
protein biosynthesis	Serine-tRNA ligase 2	cytoplasm
general stress response	general stress response GLS24 family	unknown
metabolism	Glucose-6-phosphate 1-dehydrogenase	cytoplasm

***L. plantarum* anaerobic**		
ion transport	Iron(3+)-hydroxamate-binding protein	cytoplasm
ion transport	Fe(3+) dicitrate-binding periplasmic protein	unknown
translation	50S ribosomal protein	large ribosomal subunit
function unknown	Extracellular protein DUF1093	extracellular
resistance to concurring microorganisms, anti-inflammatory effects	Extracellular transglycosylase, membrane-bound	membrane

structural constituent of cell wall	S-layer protein	cell wall
candidate for probiotic activity, antibiotic and anti-inflammatory effects	Cell surface hydrolase, DUF915 family	cell wall
antibiotic resistance	Beta-lactamase	cell wall

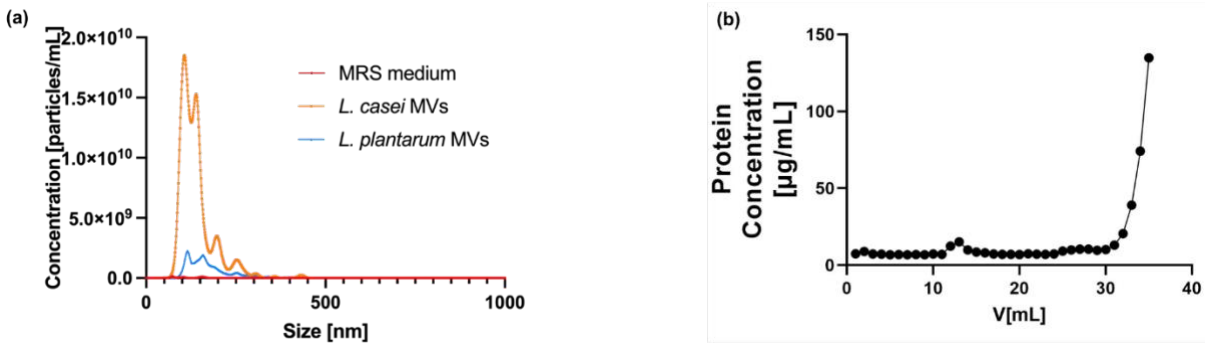


Figure S1. Characterisation of MVs by nanoparticle tracking analysis and size exclusion chromatography. (a) Representative spectra of MVs and medium control using nanoparticle tracking analysis. The average particle concentration in each sample was $c_{MRS\ medium}$ = 6.1E+09 ± 1.5E+09, $c_{L.\ casei}$ = 1.2E+12 ± 9.1E+10, and $c_{L.\ plantarum}$ = 1.9E+11 ± 9.9E+09. (b) representative elution profile of size exclusion purification of MVs measured by the protein concentration of individual fractions.

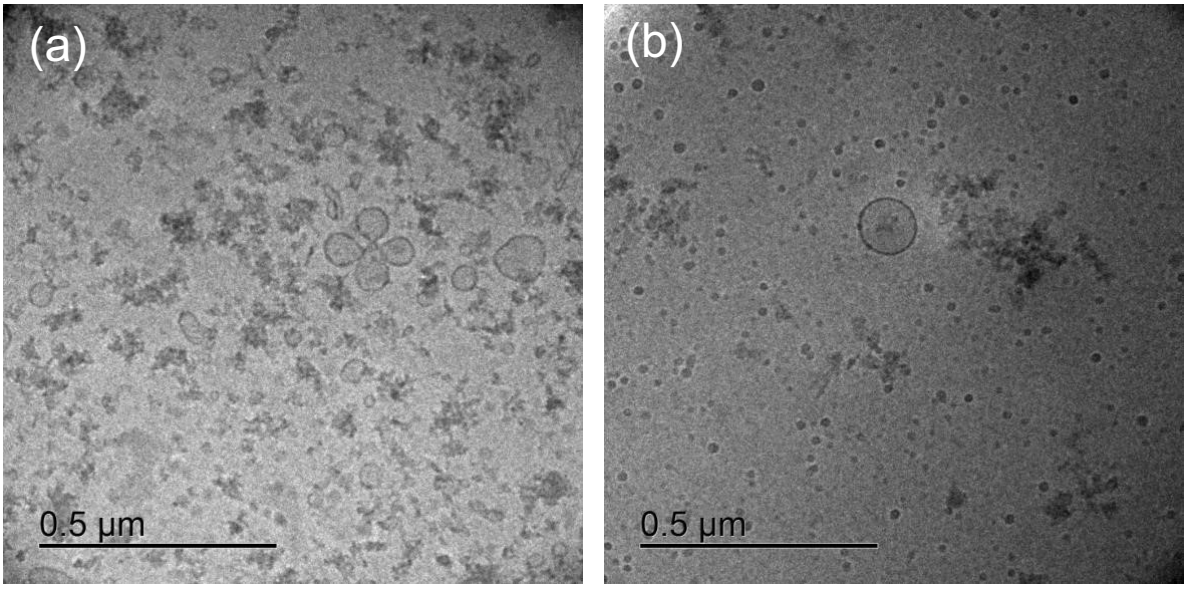


Figure S2. Representative cryo-transmission electron microscopy images of (a) *L. casei* and (b) *L. plantarum* MVs from pH 6.5 standard cultures, and prior to purification. Arrows indicate the MVs (round to off-round shaped vesicles in a typical size range between 50 and 200 nm. *L. casei* images typically show a higher abundance of MVs compared to *L. plantarum* when cultured under the same conditions.

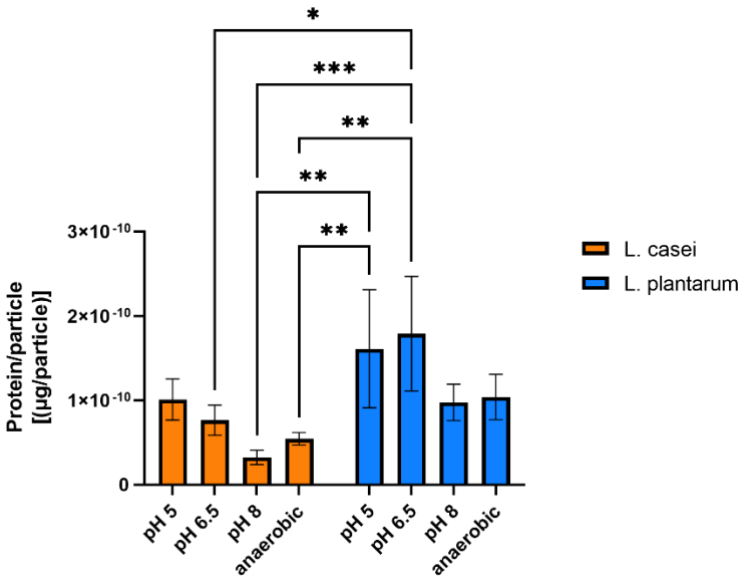


Figure S3. Average protein content per MV for the different culture conditions. The protein quantification of biomimetics was done following bicinchoninic acid assay. The protein content of bulk MVs was normalized to the particle concentration determined by nanoparticle tracking analysis. Data were analyzed by two-way ANOVA (factor one: protein/particle and factor two: culture conditions) to allow enhanced comparability of data from Fig. 3b. *p<0.05, **p<0.01, *p<0.001.

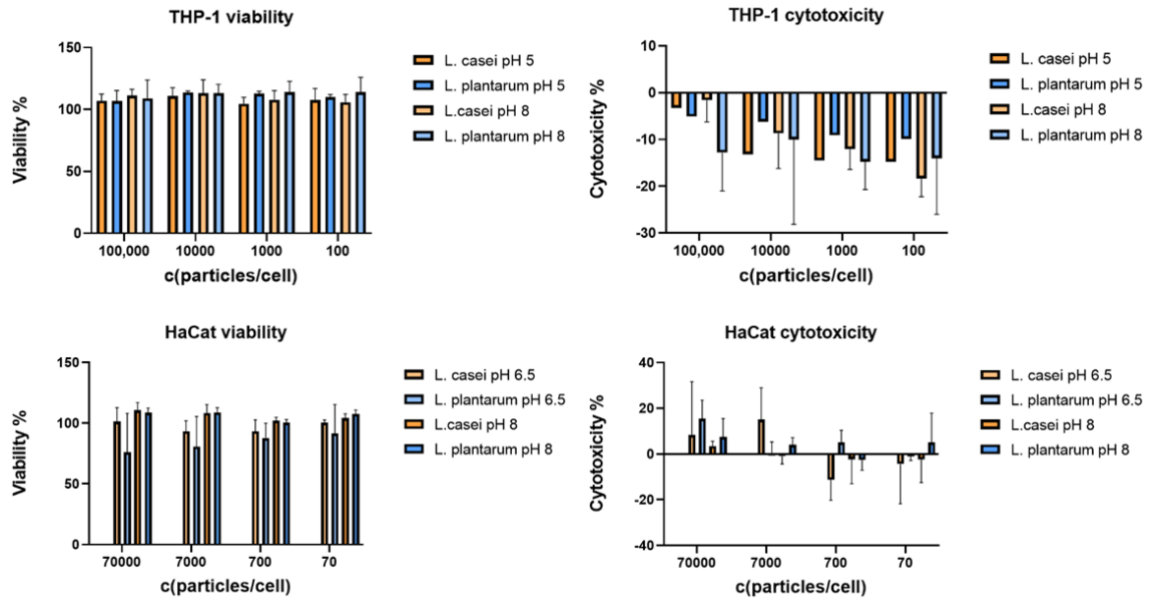


Figure S4. Biocompatibility assessments of bacteriomimetic particles. PMAdifferentiated THP-1 cells (a,b) and HaCaT cells (c,d) were treated with bacteriomimetic particles and analyzed for viability using the PrestoBlue assay (a,c) and cytotoxicity measured as increase in lactate dehydrogenase release (b, d). Data are shown as mean + SD of three independent experiments. No significant cytotoxic effects or changes in the viability were observed.

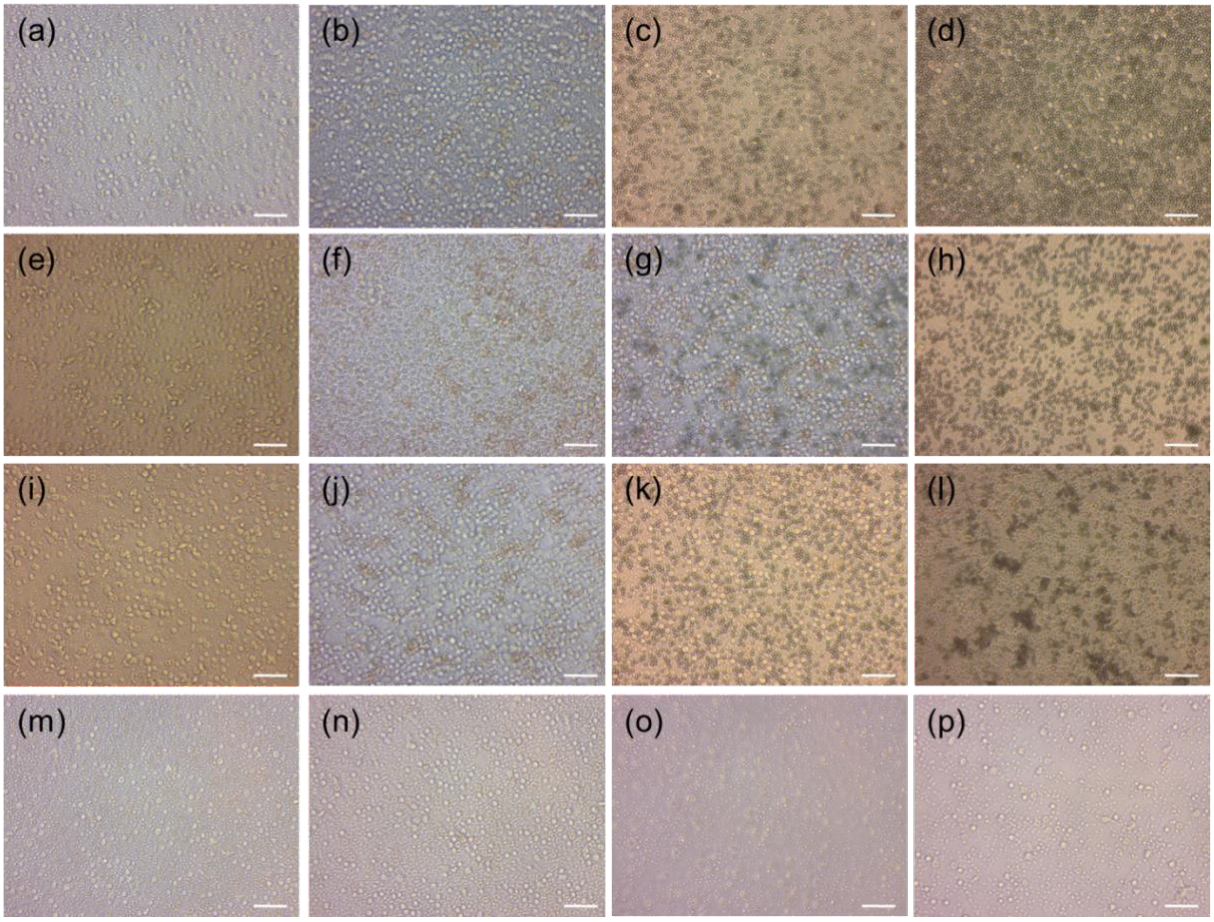


Figure S5. Representative images of peripheral blood mononuclear cells treated with MV or BP respectively, after t=4h. a. *L. casei* pH 5 MV b. *L. plantarum* pH 5 MV c. *L. casei* pH 5 MP d. L. plantarum pH 5 MP e. L. casei pH 6.5 MV f. *L. plantarum* pH 6.5 MV g. *L. casei* pH 6.5 MP h. *L. plantarum* pH 6.5 MP i. *L. casei* pH 8 MV j. *L. plantarum* pH 8 MV k. *L. casei* pH 8 MP l. *L. plantarum* pH 8 MP m. *L. casei* anaerobic MV n. *L. plantarum* anaerobic MV o. *untreated control* p. LPS only control. Error bars indicate 20 μm. BP, bacteriomimetic microparticles; MV, microvesicles.

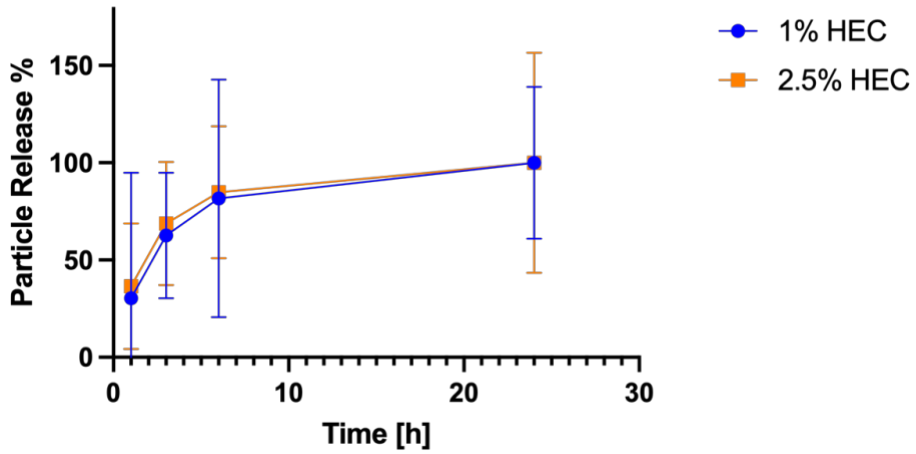
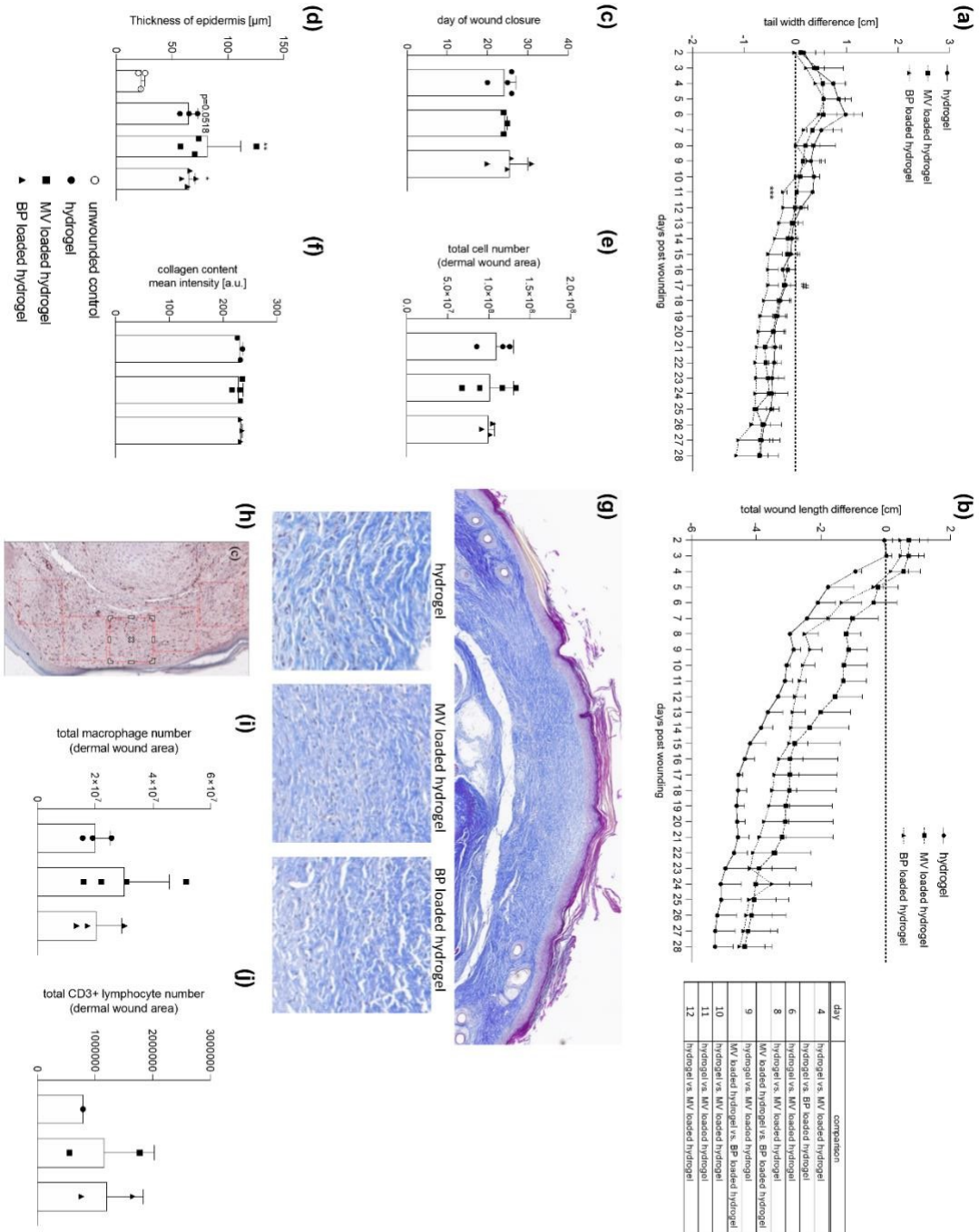


Figure S6. Cumulative release of model MVs. Microvesicles were stained with CellMaskOrange and embedded in 1% and 2.5% hydroxyethylcellulose-10000 hydrogel (HEC). CellMask Orange shows fluorescence only when incorporated into phospholipid membranes. Hydrogels were covered with a 300 µL layer of phosphate-buffered saline, and at each timepoint, 100µl of buffer were replaced with fresh phosphate-buffered saline. The removed buffer samples were then measured using fluorescence assisted nanoparticle tracking analysis, which only measures the stained vesicles, excluding any other particles in the suspension. Data are shown as mean +/- SD of three independent experiments.



day	comparison	p-value
4	hydrogel vs. MV loaded hydrogel	0.0144
	hydrogel vs. BP loaded hydrogel	0.0467
6	hydrogel vs. MV loaded hydrogel	0.0262
8	hydrogel vs. MV loaded hydrogel	0.0069
	MV loaded hydrogel vs. BP loaded hydrogel	0.0147
9	hydrogel vs. MV loaded hydrogel	0.0112
	MV loaded hydrogel vs. BP loaded hydrogel	0.0347
10	hydrogel vs. MV loaded hydrogel	0.0301
11	hydrogel vs. MV loaded hydrogel	0.0218
12	hydrogel vs. MV loaded hydrogel	0.0401

Figure S7. Histological analyses of the skin sections after closure of the wounds. Fullthickness wounds of 10 x 3 mm size were set in the tails and covered with either hydrogel, MV loaded hydrogel or BP loaded hydrogel (MVs obtained from *L. plantarum* cultured at pH 6.5). Tail width (a), wound length (b), and wound closure time were macroscopically measured each day. Hematoxylin-eosin, Ladewig trichrome (exemplary images shown in g) and immunohistochemical stainings were analyzed for thickness of epidermis (c). total number of cells (e), collagen content (f), total macrophage number (i, exemplary images for immunohistochemistry shown in h), and total $CD3^+$ lymphocyte number (j). Quantitative data are shown as mean + SD with data points for each animal. Statistical differences were analysed by One-way ANOVA and Tukey's post-hoc test. Asterisks indicated significant differences to the unwounded control. *p<0.05, **p<0.01.

7.3. Stimulation of Probiotic Bacteria Induces Release of Membrane Vesicles with Augmented Anti-inflammatory Activity

Original article

Stimulation of probiotic bacteria induces release of membrane vesicles with augmented antiinflammatory activity

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KEYWORDS

Autoimmune dispositions; biomimetics; bacteriomimetics; anti-inflammatory therapy; extracellular vesicles

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Stimulation of Probiotic Bacteria Induces Release of Membrane Vesicles with Augmented Anti-inflammatory Activity

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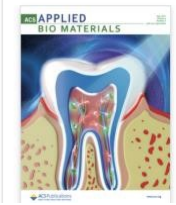

ABSTRACT

During infection, inflammation is an important contributor to tissue regeneration and healing but it may also negatively affect these processes should chronic overstimulation take place. Similar issues arise in chronic inflammatory gastrointestinal diseases such as inflammatory bowel diseases or celiac disease, which show increasing incidences worldwide. For these dispositions, probiotic microorganisms, including lactobacilli are studied as an adjuvant therapy to counterbalance gut dysbiosis. However, not all that are affected can benefit from the probiotic treatment, as immunosuppressed or hospitalized patients can suffer from bacteremia or sepsis when living microorganisms are administered. A promising alternative is the treatment with bacteria-derived membrane vesicles that confer similar beneficial effects as the progenitor strains themselves. Membrane vesicles from lactobacilli have shown antiinflammatory therapeutic effects but it remains unclear whether stimulation of probiotics induces vesicles that are more efficient.

Here, the influence of culture conditions on the anti-inflammatory characteristics of *Lactobacillus* membrane vesicles was investigated. We reveal that the culture conditions of two Lactobacillus strains, namely *L. casei* and *L. plantarum*, can be optimized to increase the anti-inflammatory effect of their vesicles. Five different cultivation conditions were tested, including pH manipulation, agitation rate and oxygen supply, and the produced membrane vesicles were characterized physico-chemically regarding size, yield and zeta potential. We furthermore analyzed the anti-inflammatory effect of the purified vesicles in macrophage inflammation models. Compared to standard cultivation conditions, vesicles obtained from *L. casei* cultured at pH 6.5 and agitation induced the strongest interleukin-10 release and tumor necrosis factor alpha reduction. For *L. plantarum*, medium adjusted to pH 5 had the most pronounced effect on the anti-inflammatory activity of their vesicles. Our results reveal that the anti-inflammatory effect of probiotic vesicles may be potentiated by expanding different cultivation conditions for lactobacilli. This study creates an important base for the utilization of probiotic membrane vesicles to treat inflammation.

INTRODUCTION

Inflammation is a hallmark of several pathophysiological dispositions, ranging from acute and chronic infections and cancer to autoimmune-based dispositions, such as those in the

gastrointestinal (GI) tract.[1] For bacterial infections, colonization of pathogens in inflamed areas such as on the skin can severely hinder the physiological regeneration of the affected tissue. In general, inflammation is defined as a stimulating response induced by invading pathogens or endogenous signals such as damaged cells. This signalling usually results in tissue repair or – when the response is unresolved – in different pathologies.[2, 3] The understanding of this mechanism, including the role and context of inflammation under physiological immune responses and pathology is constantly evolving.

Chronic inflammation is thus a major challenge in the treatment of infections, but also autoimmune-based disorders, for example in the gastrointestinal (GI) tract. Inflammatory bowel diseases (IBDs) affect patients with increasing incidences globally[4, 5] and they are the result of dysbiosis in the gut – an imbalance between harmful and protective GI bacteria – caused by either increased or reduced microbial diversity.[6] IBDs include Crohn's disease (CD) and ulcerative colitis (UC), which manifest in a chronic inflammation of the GI tract provoked by an exaggerated immune response to the luminal microflora.[7] Affected patients suffer from symptoms such as diarrhea, fatigue or weight loss and display an increased risk for colon cancer.[8] Conventional treatment of IBDs includes corticosteroids and immunomodulators, such as tumor necrosis factor (TNF)-α blockers, which can cause severe side effects.[9] To increase drug efficacy in the gut and reduce off-site effects, several delivery systems have been investigated in the past, such as hydrogels[10] or nanocarriers. Some of these approaches work well, but because they are often of artificial origin, their biocompatibility needs to be given consideration, especially when inflammation opens the GI tight junctions. Thus, it would be desirable to treat inflammation without inducing immunostimulatory effects.

As an adjuvant therapy form, probiotic microorganisms are used to alleviate the patients' symptoms and have shown promising effects in the case of inflammatory disorders, such as arthritis or ulcerative colitis.[11, 12] By administering certain probiotic strains – for example lactobacilli, bifidobacteria or lactococci – the epithelial barrier function can be enhanced, GI homeostasis can be restored and favorable immunoregulatory effects were achieved.[13] Unfortunately, probiotic treatment with living microorganisms can put immunosuppressed or hospitalized patients at risk of bacteremia or sepsis,[11] because they lack a functioning immune system and an efficient microbial clearance[14]. Therefore, a safer alternative for the administration of probiotics is needed. A promising solution is the treatment with bacteriaderived membrane vesicles (MVs) that most likely confer the same health benefits as the progenitor strains themselves.[15, 16]

Membrane vesicles belong to the group of extracellular vesicles (EVs), which are spherical particles shed from the cell surface of all three domains of life, namely eukaryotes, bacteria and archaea.[17] They are released into the extracellular milieu and carry parental cell-derived

molecular cargo, such as enzymes or nucleic acids, and are involved in cell-cellcommunication.[18, 19] Bacteria-derived MVs show a high heterogeneity in their characteristics, range in size from 20 to 400 nm and are employed in intercellular signaling,[17, 20] biofilm formation, vaccine development[21] or even as biogenic carrier for secondary metabolites.[22] In natural scenarios, MV biogenesis can be triggered by the genetic background of the releasing cell or phage-derived endolysins.[20, 23] Under artificial conditions, such as the laboratory, the MV release can be modulated by altering growth conditions, such as media composition or oxygen presence [17]. The genus of lactobacilli, a gram-positive, non-pathogenic and mostly anaerobic group of probiotic microorganisms, has shown therapeutic effects on inflammatory diseases such as IBDs[24] and is known to release membrane vesicles. However, the effect of *Lactobacillus*-derived membrane vesicles on inflammatory processes has not been fully investigated yet. Recently, it was demonstrated that *L. plantarum*-derived MVs can be used to regulate neuronal function and anxiety,[25] as well as to alleviate the symptoms of atopic dermatitis.[26] For *L. casei*, characterization studies were carried out, revealing a wide range of cargo, such as immunomodulatory factors or quorum sensing signals, and heterogeneous sized subpopulations of MVs.[27]

As there is only little evidence of the effects of MVs derived from lactobacilli in general, even less is known about how culture conditions are affecting the production and characteristics of these vesicles. In our work, we addressed this need by studying the influence of culture conditions on the characteristics of *Lactobacillus casei*- and *Lactobacillus plantarum*derived membrane vesicles. Different stimuli, including pH, agitation and depletion of oxygen were applied and their effect on physicochemical properties and anti-inflammatory activity against immune cells were assessed. This work aims to clarify whether cultivation conditions influence MV production and MV characteristics in general and if GI mimicking conditions trigger the release of higher particle yields with optimized anti-inflammatory effects. Our results form an important base for engineering *Lactobacillus*-derived MVs as a suitable alternative to the administration of living probiotic microorganisms and their potential use for antiinflammatory therapies.

EXPERIMENTAL SECTION Microbial culture

L. casei (strain DSMZ 20011) and *L. plantarum* (strain NCIMB 8826) were selected as MV producing probiotic strains. As listed in **Table** 2, different cultivation conditions were applied and will be referred to as “reference”, “agitation” / ”static”, “pH 5”, “pH 8” or “anaerobic” condition. As reference condition, the strains were cultivated as indicated from the manufacturer, meaning *L. casei* was incubated at 37 °C without agitation and *L. plantarum* was incubated at 30 °C with shaking at 180 rpm. The agitation / static condition consisted of growing *L. casei* at 180 rpm and *L. plantarum* as a static culture. When applying the two different pH conditions to mimic different areas of the intestine, the pH of the used MRS broth was lowered or raised from 6.5 to a value of 5 or 8 by either adding hydrochloric acid or sodium hydroxide, respectively. In order to perform the anaerobic condition, culture flasks with a bromobutyl rubber plug (GL 45, DURAN, DWK Life Sciences, Germany) were used and anaerobic conditions were attained by adding 0.5% (w/v) of the reducing agent sodium thioglycolate (VWR International, Germany) to the medium. Bacteria were grown for 48 hours in 100 mL of MRS medium (Carl Roth, Germany) in 100 mL culture flasks.

Table 2: Characteristics of different cultivation conditions.

Condition	Microbial Strain	Temperature [°C]	Agitation [rpm]	Medium
Reference	*L. casei* *L. plantarum*	37 30	0 180	MRS (pH 6.5)
Agitation **Static**	*L. casei* *L. plantarum*	37 30	180 0	MRS (pH 6.5)
pH 5	*L. casei* *L. plantarum*	37 30	0 180	MRS (pH 5)
pH 8	*L. casei* *L. plantarum*	37 30	0 180	MRS (pH 8)
Anaerobic	*L. casei* *L. plantarum*	37 30	0 180	MRS (pH 6.5), supplemented with 0.5% (w/v) sodium thioglycolate

MV isolation and purification

MVs were harvested by centrifuging the bacterial suspension at 9,500 × *g* for 10 min at 4 °C to remove the intact lactobacilli. Afterwards, the supernatant was sterile filtered through Stericup Quick Release 0.45 µm (Merck, Germany) to remove residual bacteria. To obtain a vesicle-rich pellet, 70 mL of the supernatant was transferred into ultracentrifuge tubes (Beckman Coulter, Germany) and centrifuged at 100,000 × g for 2 h at 4 °C (rotor SW 45Ti, Optima L-90k, Beckmann Coulter, Germany). The resulting supernatant was discarded and the pellet was resuspended in 400 µL filtered PBS (Gibco, Thermo Fisher Scientific, USA). For further purification from soluble protein impurities in the sample, size exclusion chromatography (SEC) was applied, using a 50 mL Sepharose CL-2B (GE Life Science, UK) column. Eluted fractions of 1 mL were collected in 1.5 mL microtubes (Axygen, Corning, Germany) and stored at 4 °C for a maximum of two days until further characterization.

Bicinchoninic Acid Assay (BCA)

To verify the efficiency of SEC purification and to quantify the protein content of the collected fractions, BCA assays were carried out using the QuantiPro BCA Assay Kit (Sigma Aldrich, Germany). All steps were carried out following the manufacturer's manual and protein concentration in the fractions was quantified by comparison to a BSA standard curve.

Particle size, concentration and size distribution

The hydrodynamic diameter of the MVs and the concentration and size distribution of the particle suspension were assessed by Nanoparticle Tracking Analysis (NTA LM-10, Malvern, UK). To maintain equal conditions, the obtained MV fractions were diluted up to 1,000-fold with sterile PBS in a way that 20-120 particles per frame were measured. The viscosity was set to 1.050, corresponding to PBS, and a sample of 300 µL volume was introduced into the measuring chamber. Subsequently, high-sensitivity videos of 30 s length were recorded in triple at a camera level of 15. Particle concentration and diameters were calculated by NanoSight 3.3 software with a detection threshold set to five. Average particle sizes are displayed as mode values.

Dynamic light scattering to determine zeta potential

Zeta potential was determined by diluting the desired fraction 1:10 with filtered PBS. Approximately 800 µL of the sample were transferred into a folded capillary zeta cell (Malvern Panalytical, UK). PBS was selected as the dispersant. Equilibration time was adjusted to 120

s and the measurement duration was set to “Automatic” with a minimum of 10 and a maximum of 100 runs. Measurements were carried out in triple using Zetasizer Nano ZSP (Malvern Panalytical, UK). Smoluchowski algorithm and a value of 1.50 were applied as the $F_{(ka)}$ method.

Electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed after MV harvest and concentration via ultracentrifugation. Briefly, 3 µl of the MV solution were placed onto a holey carbon TEM grid (type S147-4, Plano Wetzlar, Germany), plotted for 2 seconds and plunged into liquid ethane at T = 108 K using a Gatan Cryoplunge 3 (Pleasanton, CA, United States). Samples were transferred under liquid nitrogen to a Gatan model 914 cryo-TEM holder operating at T = 100 K and investigated under low-dose conditions at 200 kV accelerating voltage (JEM-2100 LaB6 HR, JEOL, Akishima, Tokio, Japan). Bright field TEM images were acquired using a Gatan Orius SC1000 CCD camera (1024 pixel × 1024 pixel at binning 2, exposure time 4 seconds).

Treatment of THP-1 cells & testing of anti-inflammatory effects

THP-1 monocytic cells (DSMZ ACC 16) cells were seeded at a density of 100,000 cells in 200 µL/well of RPMI medium (1640, Gibco, Thermo Fisher Scientific, USA) supplemented with 10% FCS (Gibco, Thermo Fisher Scientific, USA). THP-1 cells were differentiated by addition of 7.5 ng/mL phorbol 12-myristate-13-acetate (PMA, Sigma Aldrich, Germany) and incubation at 37 °C in a humidified 5% CO_2 ambience for 48 h. Treatment solution was a dilution of 1 part purified MVs in 2 parts of PBS, and a 1 in 100 lipopolysaccharide (LPS, from *E. coli* O111:B4, γ-irradiated, Merck, Germany) dilution (stock solution: 1 mg/mL) in RPMI medium. Cytokine levels measured were normalized to the protein content of the reference culture of each strain to allow comparability of all groups. After 48 h medium was aspirated from each well and replaced with 100 µL of RPMI medium or LPS dilution and 100 µL of MV solution. The cells were incubated for 6 h at 37 °C in a humidified 5% CO_2 ambience. After incubation, the supernatants were stored at -80 °C until further analysis. Enzyme-linked Immunosorbent Assay (ELISA) testings were performed using human interleukin (IL)-10 and TNF-α ELISA sets (Biomol, Germany) and following the manufacturer’s protocol.

Statistical analysis of data

All data is displayed in mean (x) ± standard deviation (SD) with *n* indicating the number of independent experiments. The minimum of independent experiments was set as triplicates. To

compare the different cultivation conditions, statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. Significance levels were determined by comparison to the reference condition and significant *p*-values were stated as * for $p < 0.05$, ** for $p < 0.01$, **** for $p < 0.0001$ or the exact *p* value.

RESULTS AND DISCUSSION Different cultivation conditions affect MV protein content and concentration, but do not influence MV size

In this work, we studied the influence of different cultivation conditions on the characteristics of lactobacilli-derived membrane vesicles (**Figure S1**). When culturing lactobacilli under different conditions (**Table 1**), we did not observe major differences in their growth curves with the exception of pH 5 (**Figures S2 and S3**). The slightly acidic pH induced a reduction in the maximum optical density reached of 25% and 27% compared to the reference for *L. casei* and *L. plantarum*, respectively. For the different pH conditions, pH of the cultures was measured over the course of their growth (**Figure S6**). *L. casei* cultures all declined in pH to approximately 4.5 during the first 24 h, while the pH was more stable in *L. plantarum* cultures.

For standardization and comparability of the different conditions, we generally used 70 mL of bacterial supernatant for MV isolation. The protein content of the eluted MV fractions was determined by BCA assay (**Figure S4**). The reference condition showed an average MV protein concentration 160.1 ± 19.6 µg/mL in the highest fraction for *L. casei*, whereas *L. plantarum* produced less than a third of protein compared to *L. casei*, namely 53.0 ± 8.9 µg/mL (**Figure 1a and b**). When applying other cultivation conditions on *L. casei*, final protein concentrations ranged from 23.0 ± 3.6 µg/mL under shaking conditions to 141.9 ± 31.9 µg/mL of protein at pH 8 (**Figure 1a**). All but the pH 8 condition generated significantly lower results than the reference *L. casei* culture. In the case of *L. plantarum*, we found that the altered cultivation conditions produced the lowest protein concentration at pH 5 with 13.0 ± 7.7 µg/mL and the highest protein content under static conditions with a total protein concentration of 102.8 ± 12.3 µg/mL (**Figure 17b**), which is about twice the reference. Same as for *L. casei*, all conditions except for the pH 8 cultivation generated significantly different results compared to the reference.

By doing analysis of the MV suspension via NTA, we determined the hydrodynamic diameter of the MVs and the particle concentration of the suspension (**Figure S5**). In general, the diameters obtained under different culture conditions and produced by the two bacterial strains, did not cause significantly different results when compared to the reference condition, showing average diameters of 116 ± 5 nm for *L. casei* and 116 ± 9 nm for *L. plantarum* (**Figure 17c and d**). Only a small difference in particle size was observed when growing *L. plantarum* in medium adjusted to pH 5 (**Figure 17d**), creating a 17% lower but significant result. We nevertheless reckon this difference not to be biologically relevant.

Subsequently, we compared the particle concentration of the fractions. The MVs produced by *L. casei* under reference conditions showed particle concentrations of $2.1 \times 10^{12} \pm 2.6 \times 10^{11}$ per milliliter, whereas the highest yield for *L. plantarum* was at $3.2 \times 10^{11} \pm 1.1 \times$

10^{11} particles/mL (**Figure 17e and f**). Concerning the altered cultivation conditions, the highest particle concentration of $4.4 \times 10^{12} \pm 3.6 \times 10^{11}$ particles/mL was achieved when cultivating *L. casei* in medium adjusted to pH 8, whereas the lowest concentration was obtained at pH 5 with $3.3 \times 10^{11} \pm 3.0 \times 10^{10}$ particles/mL (**Figure** 17**e**). Compared to the reference, this equates a two-fold increase or 6.5-fold decrease, respectively. Significantly different results compared to the reference were obtained when growing *L. casei* under agitation conditions or in medium adjusted to pH 5 or 8. In contrast, *L. plantarum* cultures yielded the highest particle concentration of $1.7 \times 10^{12} \pm 4.9 \times 10^{11}$ particles/mL under static conditions and the lowest when using medium adjusted to p 5, which was at $7.2 \times 10^{10} \pm 4.0 \times 10^{10}$ particles/mL. Compared to the reference, this equals a 5.3-fold increase in MV yield or a 4.5-fold loss of MVs, respectively. For *L. plantarum*, significantly higher results were only obtained at static and anaerobic conditions. In addition, the obtained values for protein and particle concentration were subsequently normalized to the final optical density of the each culture condition (**Figure S8**) with very similar trend.

For *L. casei*, which is commonly grown under static cultures, shaking while cultivation did not only lead to low protein amounts, but also seemed to decrease its vesicle production. Interestingly, cultivating *L. casei* at pH 8 increased vesicle production up to two-fold compared to the reference. As this strain already showed preference for this cultivation scenario beforehand by producing a high amount of protein in general and therefore indicating an increased metabolism, vesicle production may also be more likely. This may be due to an increased number of bacteria which consequently increases the number of released vesicles. In the case of *L. plantarum*, a highly significant difference in particle concentration was only observed at static conditions, indicating lower tolerance to agitation and therefore MV biogenesis may be triggered by less shear stress or MV stability suffers under shear stress. Seemingly, as mentioned before, increased bacterial growth due to improved environmental conditions lead to higher bacterial cell numbers and therefore more vesicles. As rod-shaped bacteria are more sensitive to mechanical stress than spherical shaped cocci,[28] this could be a possible explanation for the lower particle amounts in all conditions at shaking rates applied to *L. plantarum*. The observed low particle concentrations at pH 5 for both strains may be due to a mechanisms that *Lactobacillus* strains use to cope with acid stress. To maintain their internal pH, the bacteria tend to increase their membrane's rigidity and compactness,[29] therefore vesicle shedding or membrane disruption with subsequent MV release may be less likely.

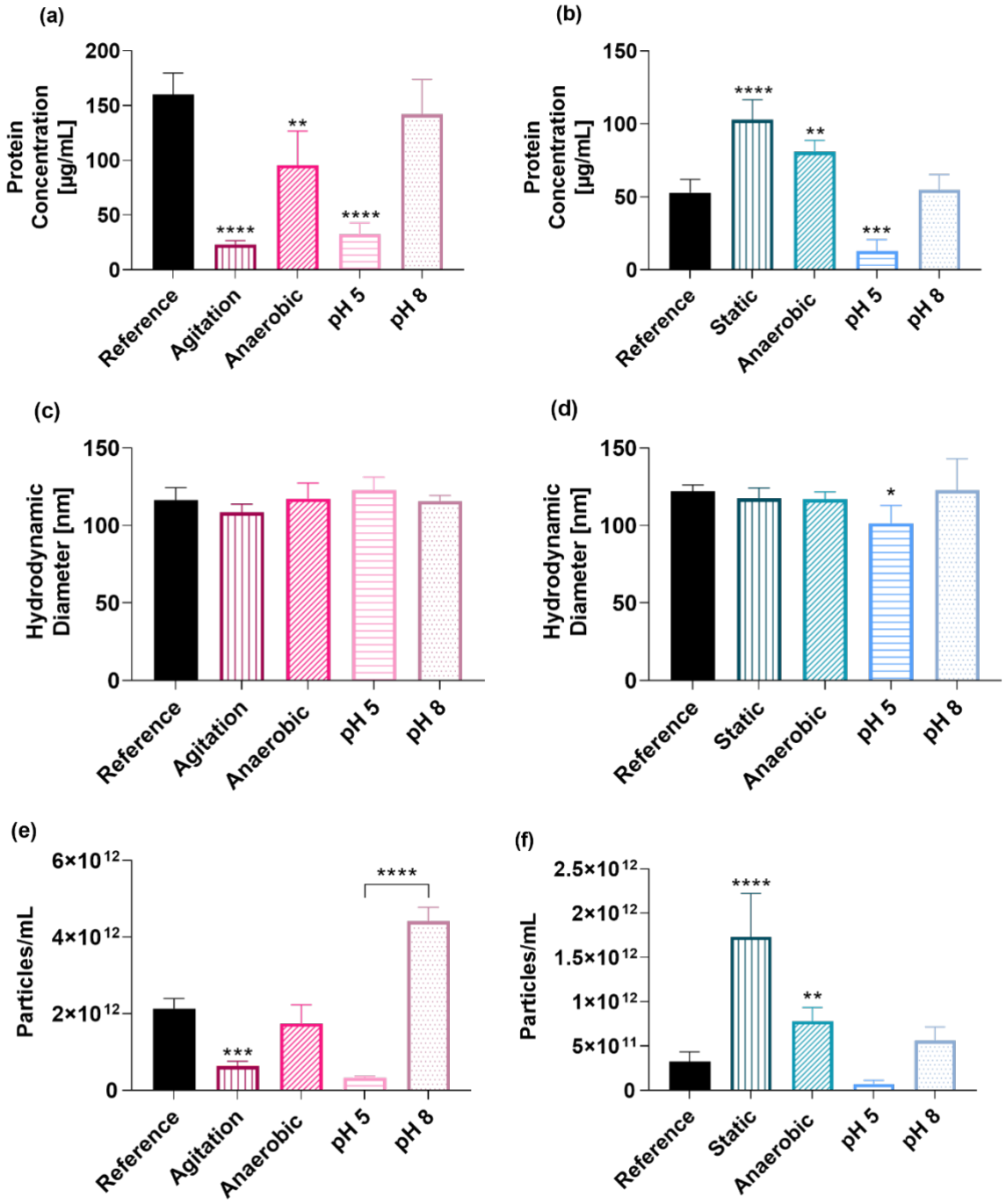

*Figure 17: Analysis of lactobacilli derived membrane vesicle-rich eluted fractions after size exclusion chromatography (SEC). Comparison of protein quantification via bicinchoninic acid (BCA) assay of SEC eluted fraction with highest protein content for (a) L. casei and (b) L. plantarum. Mean particle sizes for MV-rich SEC eluted fraction after nanoparticle tracking analysis (NTA) for (c) L. casei and (d) L. plantarum. Average particle concentration obtained by NTA for (e) L. casei and (f) L. plantarum. Shown are mean ± SD, with n = 3-6 and ***p = 0.0009 / 0.0001 (protein content / particle concentration), **p = 0.0031 / 0.0096 (protein content / particle concentration) and *p = 0.0180 (ANOVA followed by Dunnett's multiple comparison test).*

Altered cultivation conditions affect the surface charge and morphology of MVs

To further characterize the purified membrane vesicles, we determined their surface charge by measuring zeta potential *via* dynamic light scattering. Particles derived from *L. casei* under the reference condition showed zeta potentials of -6.3 ± 0.7 mV (**Figure 2a**). When cultured under the altered conditions, significantly different results were obtained when grown at agitation or pH 8 conditions. In these cases, the zeta potential was at approximately -13 mV, hence more than twice as high compared to the reference. In contrast, zeta potential measured under reference conditions of *L. plantarum* was at -3.3 ± 0.2 mV (**Figure 2b**) and only the pH 5 condition showed higher values of -5.6 ± 2.0 mV, which was approximately 70% higher than the reference. When comparing the measured zeta potential with the protein amount per particle, we observed that *L. casei* showed a higher zeta potential in combination with a lower protein amount per particle and vice versa (**Figure 2c**). This effect was less pronounced for *L. plantarum* which only showed such pattern at static conditions (**Figure 2d**). Here, in most cases high protein amounts per particle occurred concomitantly with a high zeta potential. These results indicate that zeta potential is affected by cultivation conditions and most likely changes in altered environments. In the case of *L. casei*, a clear assumption can be made that a low protein content per particle leads to a highly negative zeta potential, whereas high protein amounts produce lower zeta potentials. For *L. plantarum*, the static condition showed a very similar pattern to *L. casei*, as a low protein content per particle also led to a more negative zeta potential. All other conditions applied on *L. plantarum*, except for the reference, showed a clear correlation between protein content and zeta potential, as an increasing protein amount led to a rise in zeta potential. Seemingly, both *Lactobacillus* strains behave differently in respect to surface charge of the vesicles and therefore altered lipid and protein compositions may be observed. Future proteomic and lipidomic evaluations will be needed to better understand this phenomenon.

To gain insights into the morphology of the MVs produced under different cultivation conditions, we selected the culture conditions with the largest difference in particle concentration and compared to the reference using cryo-TEM imaging. In all cases, the vesicles appeared to be closed membrane structures, ranging in size from 50 to 200 nm (**Figure 2e-j**). When comparing the reference and pH 8 condition of *L. casei*, it became obvious that in both cases heterogeneous populations of particles were present (**Figure 2e and f**). The MVs showed different sizes and morphologies. Sizes ranged from about 50 nm to 200 nm diameter. Various different shapes could be observed, including spherical, elliptical and also nongeometrical ones. In several cases, there were also smaller MVs inside of bigger particles (**Figure 2e**) and some vesicles exhibited a higher granularity than others (**Figure 2f**). In contrast, vesicles generated by *L. plantarum* under different cultivation conditions, revealed

similarly looking cryo-TEM images (**Figure 2g-j**). All conditions showed uniformly shaped spherical MVs of approximately 100 nm in diameter and only a few smaller elliptical or spherical ones of about 50 nm in size (**Figure 2h and j**). In literature, mean diameters for *L. casei* MVs of approximately 140 nm were found (Dean et al. 2019), which matches our findings here. In 2017, *L. plantarum*-derived MVs were characterized and over 80% of the isolated MVs showed sizes ranging from 31 nm to 200 with a peak of high particle abundancy at around 101 nm.[30] Very recently, our research group published cryo-TEM images of MVs from probiotic strains,[31] demonstrating the heterogeneous nature of MVs concerning their size, morphology and shape that was also observed in this work.

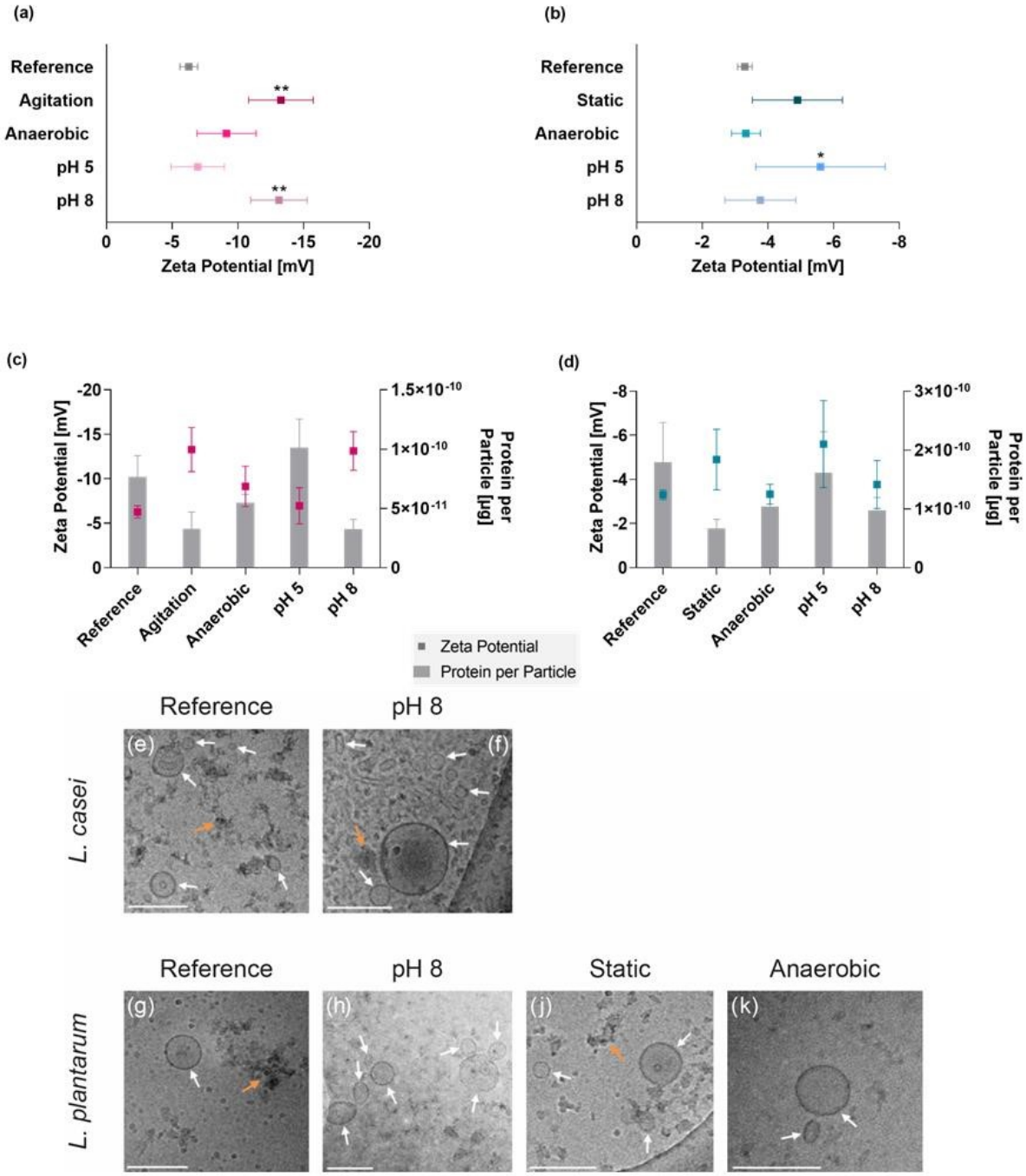

*Figure 18: Zeta potential and cryo-TEM images of lactobacilli-derived membrane vesicles. Comparison of zeta potential (mV) measured by dynamic light scattering for (a) L. casei and (b) L. plantarum and (c,d) protein content per particle. Shown are mean ± SD, with n = 3-6 and **p = 0.0013 (L. casei – agitation), **p = 0.0041 (L. casei – pH 8), *p = 0.0352 (L. plantarum – pH 5) (ANOVA followed by Dunnett's multiple comparison test). Cryo-TEM images of MVs produced by L. casei under (e) reference and (f) pH 8 conditions and L. plantarum under (g) reference, (h) static, (j) pH 8 and (k) anaerobic conditions. White arrows indicate presence of MVs, orange arrows indicate protein impurities; scale bars are 200 nm in all images.*

Lactobacillus-derived MVs trigger anti-inflammatory immune responses in dTHP-1 cells

To find suitable cultivation conditions that lead to MVs with a potential anti-inflammatory effect, we treated differentiated THP-1 cells with a mixture of either medium and MVs or medium combined with LPS and MVs and measured the release of different cytokines. Six hours after MV treatment, cytokine release was determined by ELISA. Initially, Interleukin-10 (IL-10) levels were determined for both bacterial strains. The obtained results of the controls showed moderate IL-10 levels of around 40 pg/mL, with or without LPS co-treatment (**Figure 19a and b**). For *L. casei*, the agitation condition showed the most potent results: the MVs boosted the IL-10 levels from around 40 pg/mL (control) to either 636.3 ± 125.6 pg/mL in inflamed dTHP-1 cells or 1,727.1 ± 796.2 pg/mL in cells without LPS treatment (**Figure 19a**). For pH 5 stimulation, MVs induced levels up to 1,113.3 ± 672.8 pg/mL, which represented a nearly 28-fold increase of IL-10 levels. In contrast, MVs generated by *L. plantarum* showed the highest IL-10 levels when cultivated at pH 5. Here, IL-10 levels increased from 40 pg/mL (control) to 487.4 ± 143.1 pg/mL with LPS and 721.0 ± 173.8 pg/mL with MVs alone (**Figure 19b**). Conclusively, *L. casei*-derived MVs obtained in agitation conditions showed most potent results in inflamed THP-1 cells compared to *L. plantarum* MVs derived from cultures at pH 5. To the best of our knowledge, no data has been published yet about the influence of cultivation conditions on the anti-inflammatory effect of MVs derived from probiotic strains by measuring IL-10 levels. In non-obese diabetic (NOD) dendritic cells treated with living probiotics *L. casei,* Maniraroa et al. observed an induced IL-10 release of about 800 pg/mL.[32] Upon injection of *L. casei* stimulated dendritic cells into NOD mice IL-10 serum levels of around 400 pg/mL were still measured after 28 h. Different studies showed that ingestion of *L. casei* led to elevated IL-10 levels of around 30 pg/mL for *Helicobacter pylori* infected mice.[33] Mice treated with *L. casei* for 7 days before being infected with *Salmonella enterica* serovar Typhimurium nearly tripled their IL-10 release from around 65 pg/mL to 187 pg/mL.[34] Here, we achieved IL-10 values of up to 1,700 pg/mL when treating THP-1 cells with cell-free *L. casei* MVs derived at agitation conditions (**Figure 19a**), indicating a potential enhancement of IL-10 release which needs verification in more complex models.

Furthermore, we determined the release of the pro-inflammatory marker TNF-α. The control of inflamed dTHP-1 cells showed signals of approximately 188 pg/mL while co-treated with LPS, whereas the negative control was at around 47 pg/mL (**Figure 19c and d**). MVs generated by *L. casei* under agitation, anaerobic and pH 5 conditions inhibited the TNF-α release nearly entirely with or without LPS co-treatment (**Figure 19c**). The reference condition was the only cultivation setting which had greater negative impacts on the TNF-α release by THP-1 cells and increased the cytokine level nearly two-fold up to 360 pg/mL. In the case of

L. plantarum, the anaerobic and pH 5 condition set the TNF-α levels nearly to zero (**Figure 19d**). Same as for *L. casei*, cells only treated with MVs alone did not release any detectable signals of TNF-α. The obtained results indicated that the *Lactobacillus*-derived MVs intervene in the signal cascades produced by the THP-1 cells, therefore influencing cytokine release. Monocytic cells, such as THP-1 cells, can be differentiated by stimulation with PMA and transferred into resting state (M0 macrophages).[35] M0 macrophages can then be polarized toward two different phenotypes: potent antimicrobial effector cells (M1) or alternatively activated macrophages (M2).[36] M1 macrophages release pro-inflammatory cytokines, such as TNF-α, whereas M2 macrophages produce anti-inflammatory mediators such as IL-10.[37] LPS acts as the main ligand of toll-like receptor 4 (TLR-4) of dTHP-1 cells and initiates a cascade of different signals by extracellular signaling to the nucleus, resulting in a switch to the M1 phenotype and pro-inflammatory cytokine production, which ultimately leads to the clearing of the infection.[37, 38] As LPS and the *Lactobacillus*-derived MVs are added to the THP-1 cells at the same time, high IL-10 levels coupled with low TNF-α signal may be concomitantly induced. The vesicles may block the TLR-4 receptor or interact with the TLR-4 pathway, so that LPS cannot interact and does not lead to an M1 switch combined with a high TNF-α release. In literature, *L. casei* Shirota strains have already shown their effect to attenuate the activation of the TLR-4 signaling cascade and other *Lactobacillus* strains even reduced the expression of the TLR-4 itself, therefore blocking TNF-α expression.[39] These beneficial effects of upregulation of IL-10 and downregulation of TNF-α may also be conferred to *Lactobacillus*derived vesicles.

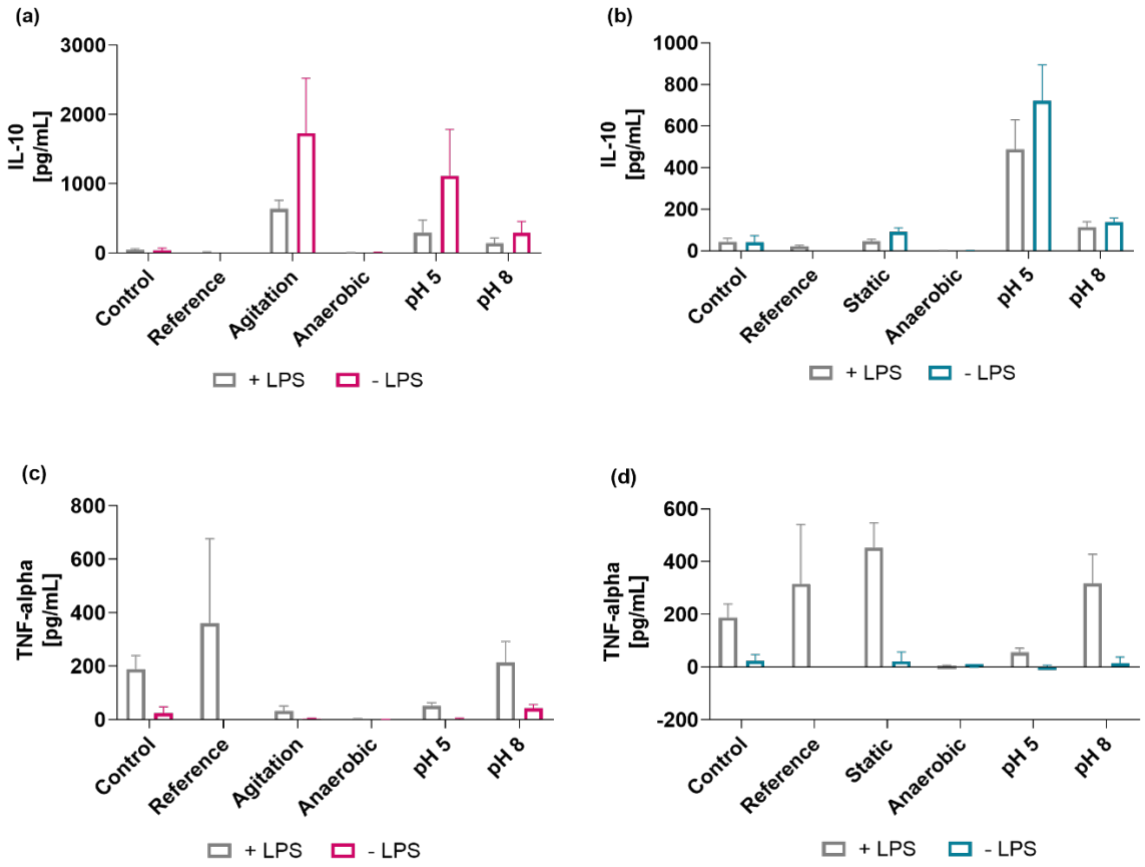

Figure 19: IL-10 and TNF-α concentration released by dTHP-1 cells before (control) and upon treatment with MVs generated by L. casei and L. plantarum under different cultivation conditions. Cytokine levels were determined by ELISA. IL-10 levels of (a) L. casei, (b) L. plantarum are shown. TNF-α production induced by (c) L. casei-derived MVs and by (d) L. plantarum-derived MVs. Shown are mean ± SD from n = 3 independent experiments.

Optimal culture conditions for *L. casei* and *L. plantarum* lead to favorable MV characteristics

Inherent anti-inflammatory effects have been shown for cell-derived vesicles from different sources.[40] When comparing all characteristics of *L. casei*-derived MVs, we did not observe a clear pattern in the distribution of the determined vesicle characteristics (**Figure 20a and b**). For the reference condition, a high protein content, moderate particle concentrations and zeta potentials and nearly no anti-inflammatory effect were determined. A similar distribution pattern as the reference with lower values of all characteristics was observed when growing *L. casei* under anaerobic conditions, indicating the reference conditions' resemblance – being reduced input of oxygen resulting from non-agitation. All other conditions showed different patterns, presenting high zeta potential coupled with high particle concentrations and protein contents (pH 8 condition) or low protein content and particle concentrations accompanied by high zeta potentials (agitation condition). We concluded that a high amount of

protein does not automatically lead to a low zeta potential when cultivating *L. casei*, same as a high protein content does not indicate high particle concentrations. Interestingly, a high particle yield also does not correlate with a potent anti-inflammatory effect. As previously discussed, the biological effect of the *Lactobacillus*-derived MVs can be due to either beneficial protein on the vesicle surface or cargoes. This has to be further analyzed in ensuing experiments. For *L. plantarum,* the reference condition showed moderate protein amounts and zeta potentials and low particle concentrations and IL-10 signals (**Figure 20c**). The static condition produced high values in all characteristics, except for IL-10, suggesting that high protein amounts and particle concentrations do not correlate with a potent anti-inflammatory effect (**Figure 20d**). Again, the lowest particle concentration obtained at pH 5 induced a high IL-10 signal. Interestingly, for *L. casei* a higher oxygen uptake into the medium and most likely higher shear forces lead to the production of fewer but IL-10 generating vesicles, whereas *L. plantarum* produces more abundant vesicles when not agitated. This is in accordance with the observation that both strains showed a common low vesicle concentration coupled with a potent anti-inflammatory effect. It should be highlighted that all ELISA data were normalized to the protein concentration of the control reference conditions to allow comparability of samples. In conclusion, the two *Lactobacillus* strains in this study produced different MVs under the same cultivation conditions, which consequently forbids any generalization regarding MV characteristics or biological activity based on culture conditions.

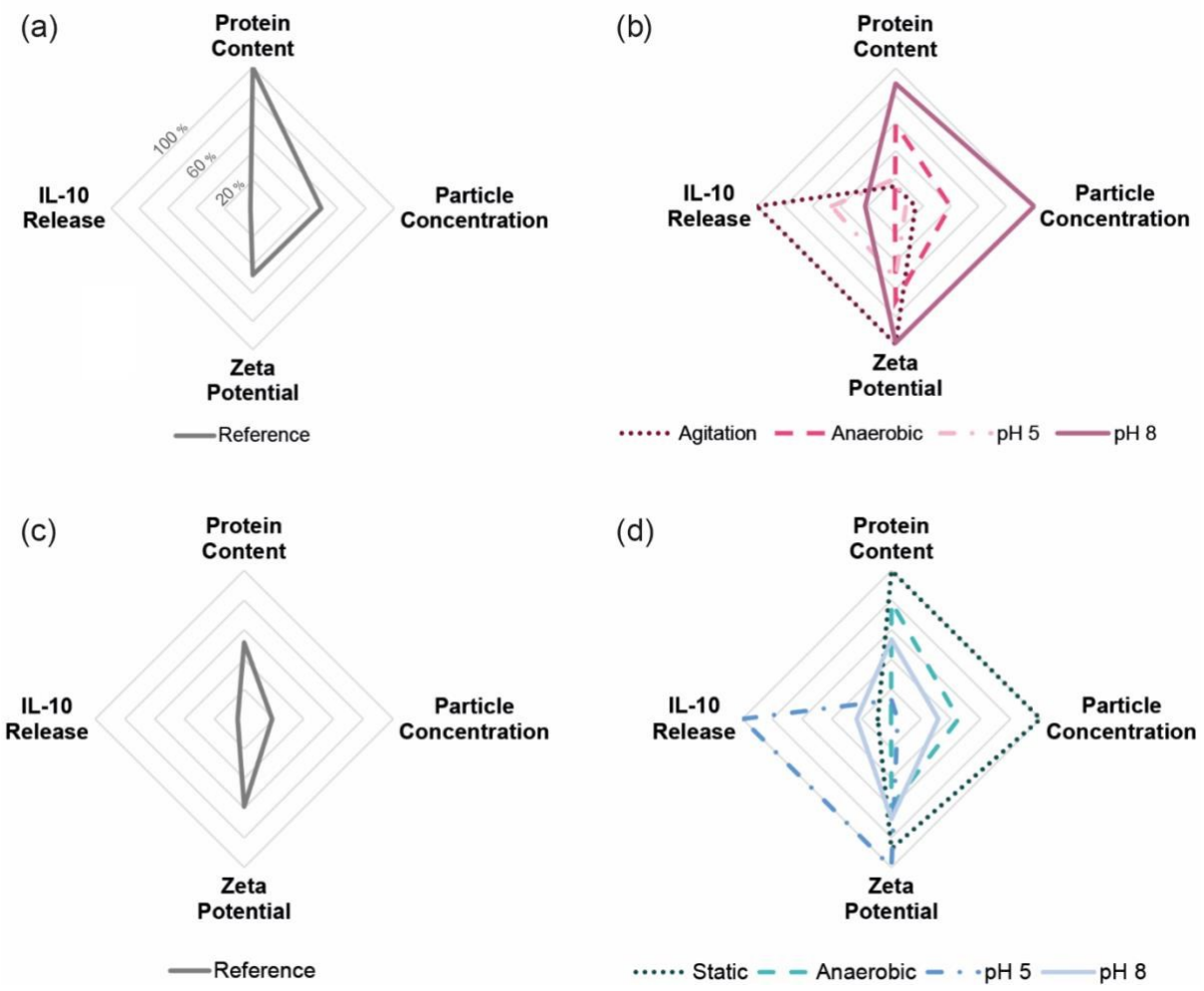

Figure 20: Overall comparison of vesicle characteristics determined in different cultivation scenarios applied on L. casei and L. plantarum. (a) L. casei reference compared to (b) stimulation conditions and (c) L. plantarum reference in comparison to (d) stimulation conditions. Characteristics were normalized to the highest value of each category.

CONCLUSION

In this work, we studied the impact of different cultivation conditions on the diverse characteristics of membrane vesicles produced by two *Lactobacillus* strains. We showed that bacterial strains adapt to their environment and confer altered released vesicles. Surprisingly, cultivation conditions leading to a high MV yield did not cause a desired anti-inflammatory biological effect. However, for each strain, one cultivation condition was identified that conferred a lower MV yield but coupled with a potent anti-inflammatory effect, being agitation and pH 5 for *L. casei* and *L. plantarum*, respectively. Surprisingly, the optimal conditions for each strain were substantially different which forms the basis for further optimization. Furthermore, the described results serve as the starting point for the advanced analysis of the MV release mechanisms of Gram-negative bacteria, their potential probiotic cargo and behavior in complex inflammation models.

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Author Contributions

L.M. conducted all experiments on vesicle isolation and characterization and wrote the manuscript; T.K. conceived the study, helped setting up protocols and provided cells for cytokine testing; M.K. acquired cryo-electron microscopy images; L.M. wrote the manuscript together with G.F.; G.F. supervised the study and wrote the overall project. All authors have given approval to the final version of the manuscript.

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SUPPORTING INFORMATION

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Figure S7. Additional cryo-transmission electron microscopy images

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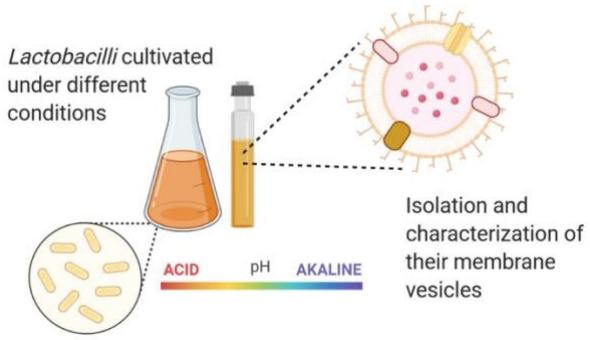

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Supporting information

Stimulation of probiotic bacteria induces release of membrane vesicles with augmented antiinflammatory activity

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Supporting Figures

Figure S1. Schematic overview of the isolation and characterization of probiotic membrane vesicles

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Figure S3. Growth curves of *L. plantarum*, cultivated under static, anaerobic, pH 5 and pH 8 condition

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Figure S5. Typical size distribution profile of isolated MVs produced by L. casei and L. plantarum under reference conditions

Figure S6. Measurement of the pH-value of the bacterial cultures over the course of their growth

Figure S7. Additional cryo-transmission electron microscopy images

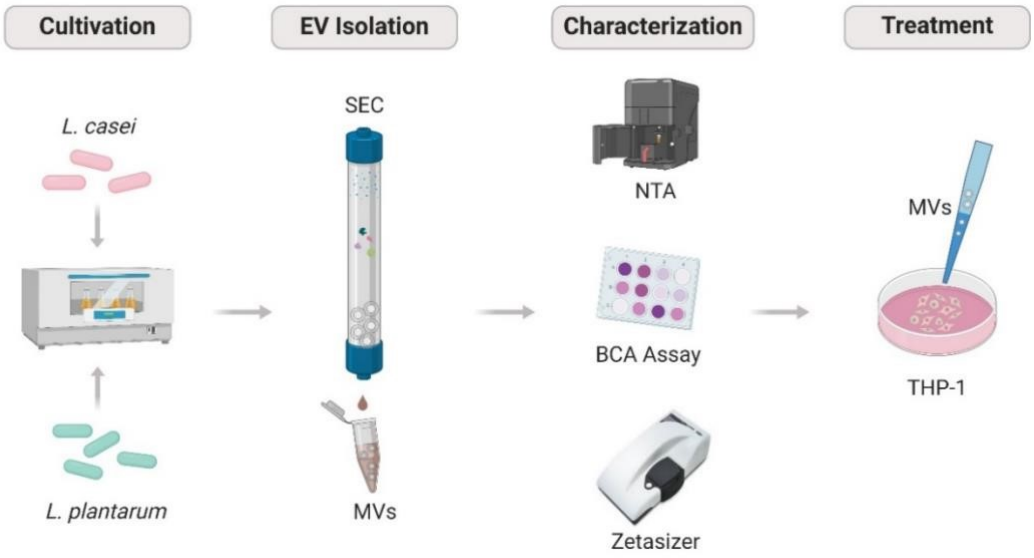

Figure S1. Schematic overview of the isolation and characterization of probiotic membrane vesicles. Different cultivation conditions of L. casei and L. plantarum were carried out to determine their influence on MV production. MVs derived under different conditions were isolated by means of SEC and characterized with different analysis methods, including nanoparticle tracking analysis, bicinchonic acid protein kit and Zetasizer analysis. The vesicles' anti-inflamatory activity was assessed on macrophage like cells treated by ELISA. [Own representation via BioRender]

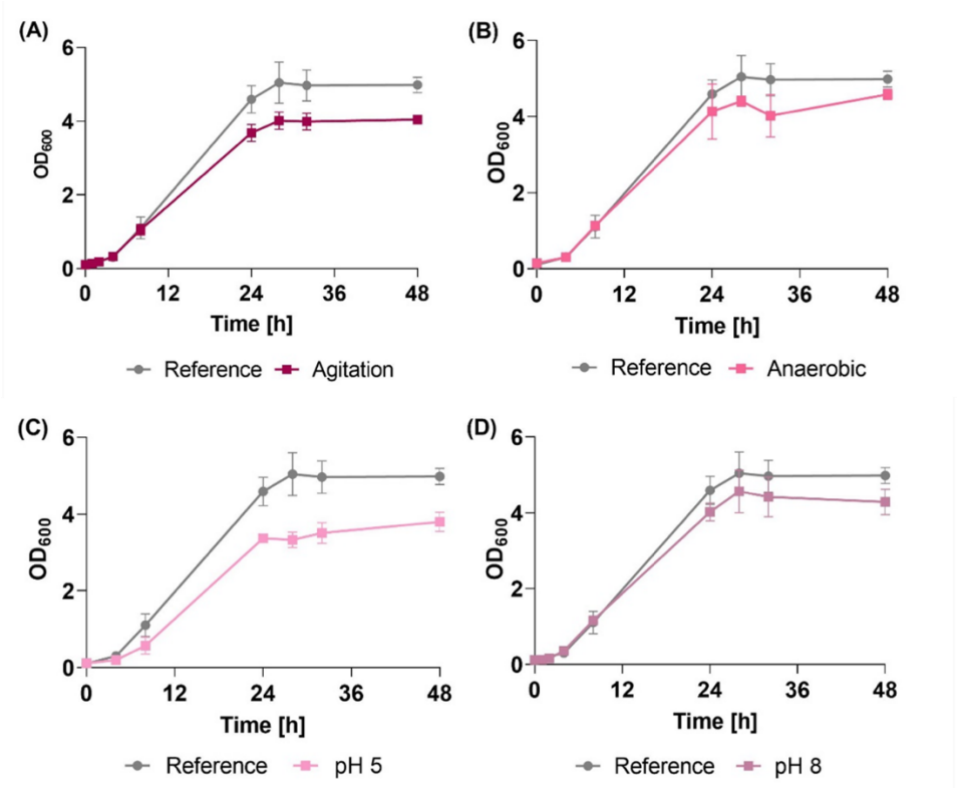

Figure S2. Comparison of growth curves of L. casei, cultivated under (A) agitation, (B) anaerobic, (C) pH 5 and (D) pH 8 condition. Grown at 37 °C and 0 rpm in MRS medium (reference), 180 rpm (agitation), anaerobic flasks (anaerobic) or MRS medium adjusted to pH 5 or 8. OD measured at 600 nm. Shown are mean values ± SD from independent triplicates.

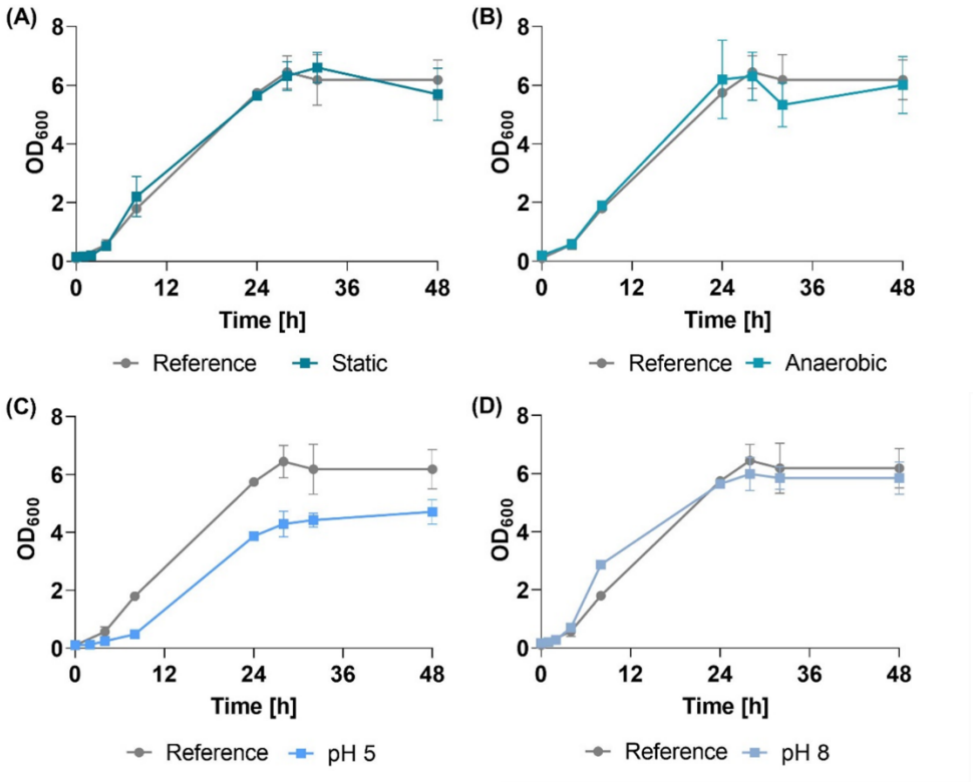

Figure S3. Growth curves of L. plantarum, cultivated under (A) static, (B) anaerobic, (C) pH 5 and (D) pH 8 condition. Grown at 30 °C and 180 rpm in MRS medium (reference), 0 rpm in MRS medium (static), anaerobic flasks (anaerobic) or MRS medium adjusted to pH 5 or 8. OD measured at 600 nm. Shown are mean values ± SD from independent triplicates.

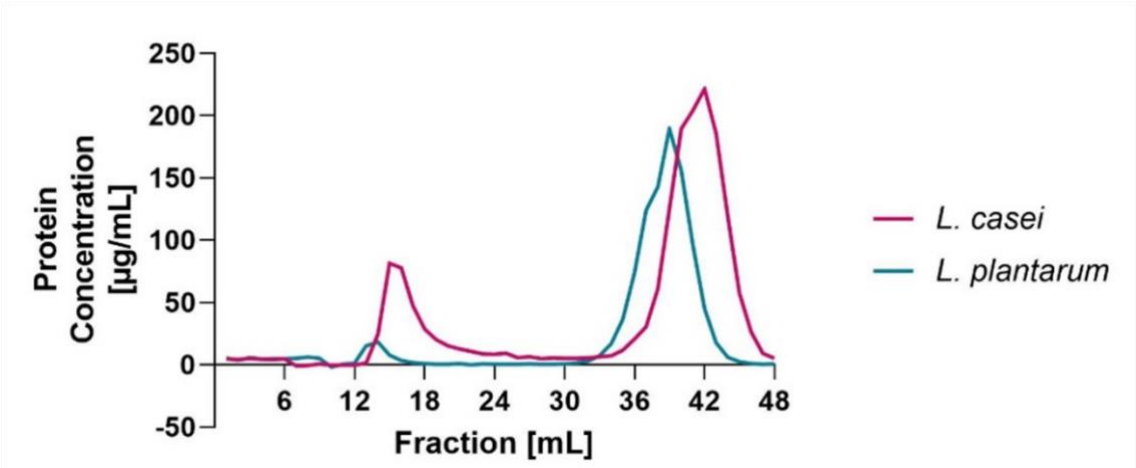

Figure S4. SEC column characterization for eluted fractions of L. casei and L. plantarum. Protein content was determined by BCA assay

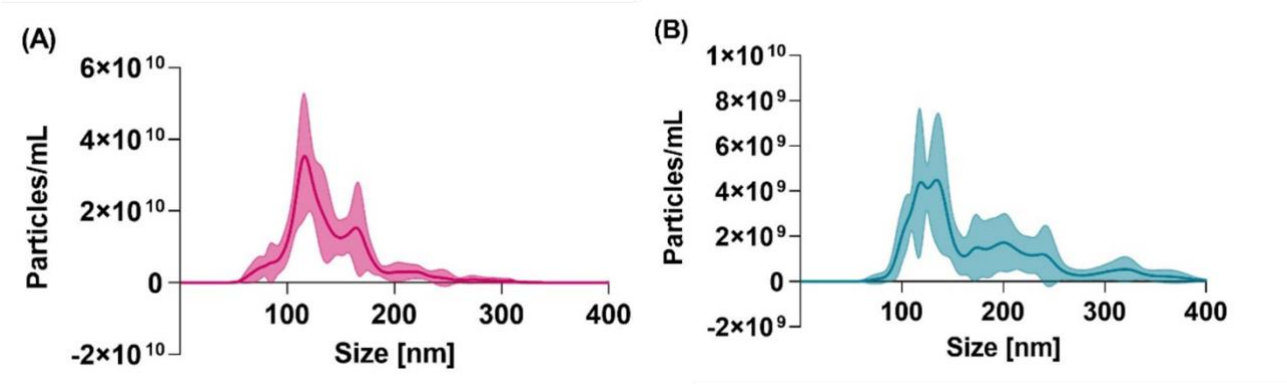

Figure S5. Typical size distribution profile of isolated MVs produced by (A) L. casei and (B) L. plantarum under reference conditions. Particle sizes and concentrations were obtained by NTA measurement. Shown are mean values ± SD from n ≥ 3 independent experiments; SD is demonstrated as colored area below and above mean values.

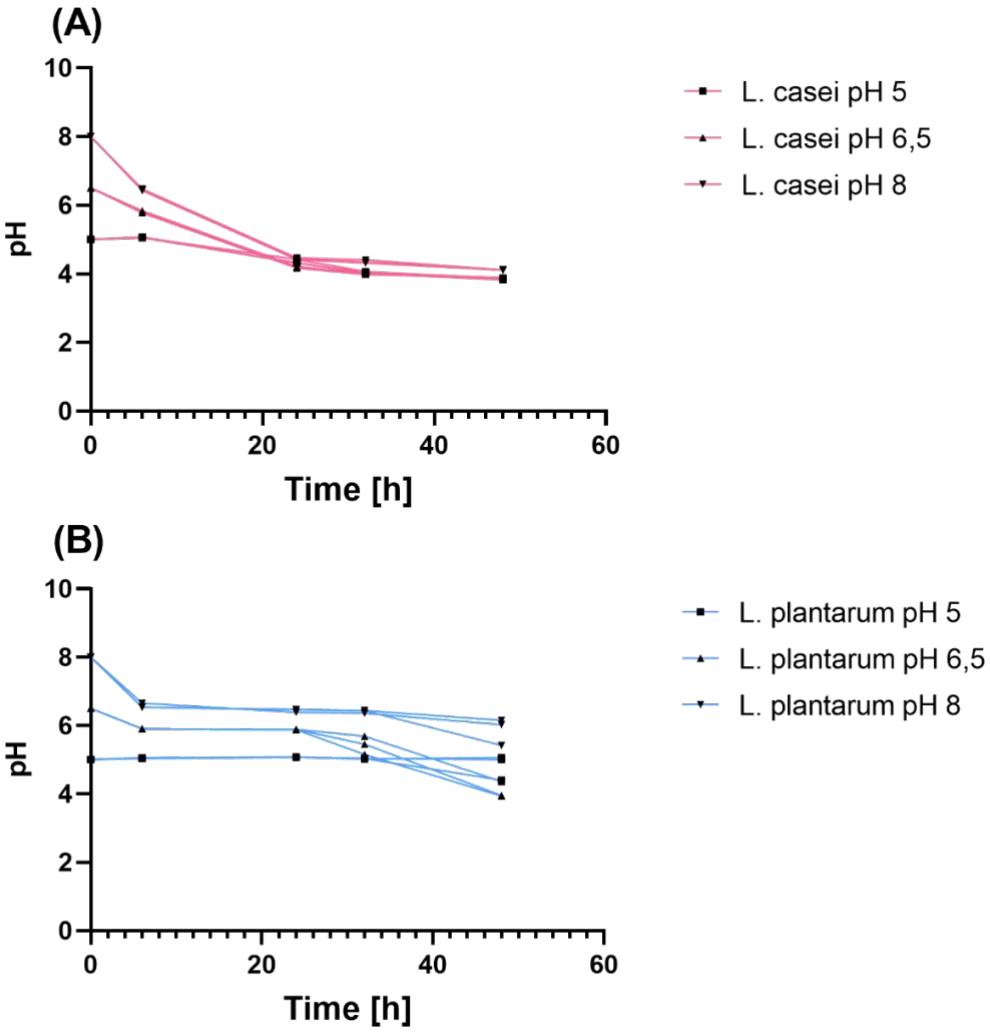

Figure S6. Measurement of the pH-value of the bacterial cultures over the course of their growth.

For the different pH-conditions, the pH-change of the cultures of **(A)** *L. casei* and **(B)** *L. plantarum* was monitored. Individual values are represented by each data point. The experiment was performed in n=3.

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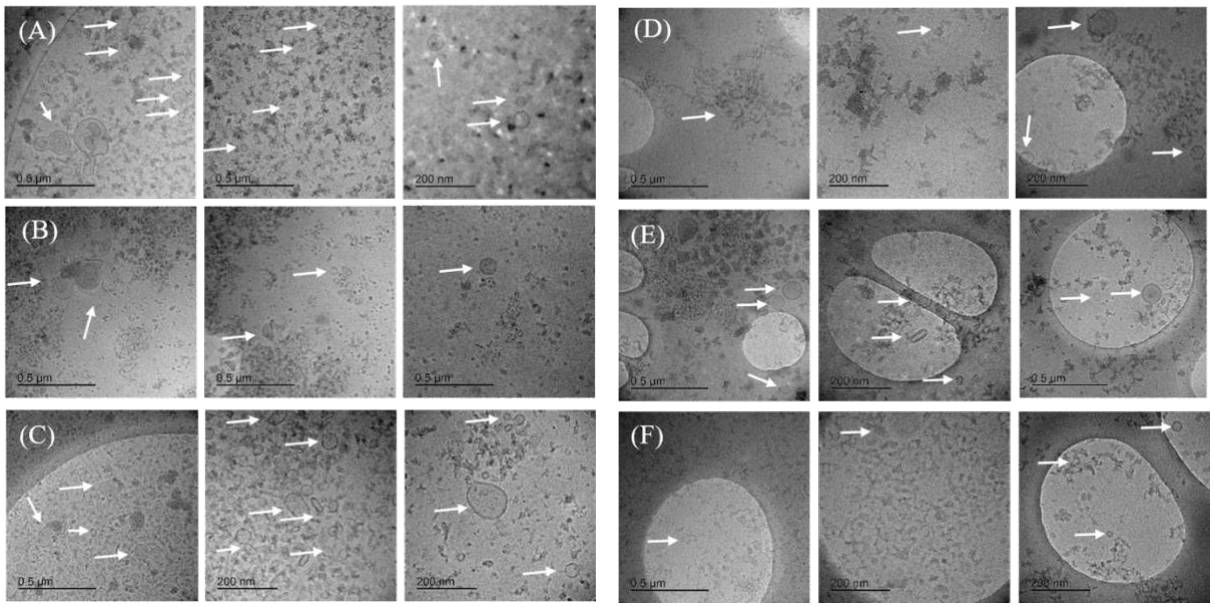

Figure S7. Additional cryo-transmission electron microscopy images of *Lactobacillus* MVs. (A) *L. casei* reference condition **(B)** *L. plantarum* reference condition **(C)** *L. casei* grown at pH 8 **(D)** *L. plantarum* static growth condition **(E)** *L. plantarum* grown at pH 8 **(F)** *L. plantarum* anaerobic growth condition.

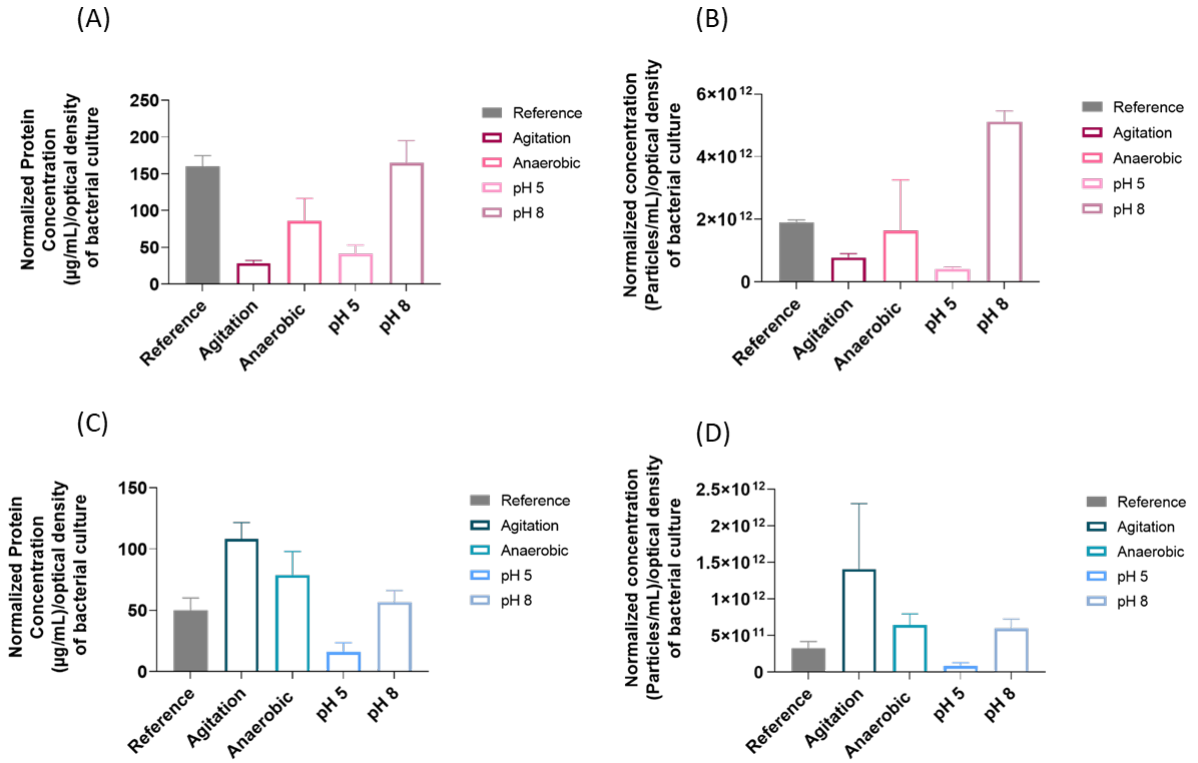

Figure S8. Normalized protein and particle concentrations. Values were normalized to the optical density of the cultures. (A) and (B) protein and particle concentration of L. casei MVs, (C) and (D) protein and article concentration of L. plantarum MVs. Shown are mean values ± SD from independent triplicates.

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